COPYRIGHT AND USE OF THIS THESIS

This thesis must be used in accordance with the provisions of the Copyright Act 1968.

Reproduction of material protected by copyright may be an infringement of copyright and copyright owners may be entitled to take legal action against persons who infringe their copyright.

Section 51 (2) of the Copyright Act permits an authorized officer of a university library or archives to provide a copy (by communication or otherwise) of an unpublished thesis kept in the library or archives, to a person who satisfies the authorized officer that he or she requires the reproduction for the purposes of research or study.

The Copyright Act grants the creator of a work a number of moral rights, specifically the right of attribution, the right against false attribution and the right of integrity.

You may infringe the author’s moral rights if you:

- fail to acknowledge the author of this thesis if you quote sections from the work
- attribute this thesis to another author
- subject this thesis to derogatory treatment which may prejudice the author’s reputation

For further information contact the University’s Copyright Service.

sydney.edu.au/copyright
THE INVOLVEMENT OF α9α10-nICOTINIC ACETYLCHOLINE RECEPTORS IN PAIN STATES

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

By

Sarasa Mohammadi

Faculty of Medicine
The University of Sydney
2015
“Knowing is not enough; we must apply. Willing is not enough; we must do.”

-Goethe
ABSTRACT

The α9 subunit of the nicotinic acetylcholine receptor (α9-nAChR) has recently attracted the interest of pain researchers, after the discovery of highly efficacious α9α10-nAChR-inhibiting analgesics. The α9α10-nAChR has since been pursued as a novel analgesic target. However, all evidence to implicate this receptor subunit in nociception and analgesia has been indirect, through pharmacological studies, and no anatomical site of action has been confirmed. A more direct approach to studying the role of the α9α10-nAChR in pain was therefore undertaken, through behavioural testing of mice with germ line deletion of the α9-nAChR subunit.

Comparable pain responses were seen in α9-nAChR knockout (KO) mice and their wild-type (WT) counterparts in a number of pain modalities both in the naive state, and after the induction of neuropathic and inflammatory pain. Mechanical allodynia, thermal hyperalgesia and cold allodynia were unaffected by α9-nAChR subunit deletion. Conversely, the development and maintenance of mechanical hyperalgesia was altered in α9-nAChR KO mice, wherein the magnitude and duration of mechanical hyperalgesia were attenuated. Furthermore, sham-operated WT mice exhibited gradual repeated-testing induced hyperalgesia due to the noxious nature of the testing. The KO mice were resistant to this repeated-testing effect. Overall, the contribution of α9α10-nAChRs to pain perception appears to be limited to the modality of mechanical hyperalgesia, suggesting that specific pharmacological inhibitors of the receptor would have limited clinical applications.

A proposed mechanism of action of α9α10-nAChR-inhibiting analgesics is through peripheral immune cells at injury sites, and such compounds have been shown to reduce immune cell infiltration into injured nerves. Histological comparison of the injury sites of WT and α9-nAChR-KO mice revealed no involvement of the receptor in the severity or maintenance of pro-nociceptive
pathology. This suggests that the mechanism of action of known α9α10-nAChR-inhibiting analgesics is independent of their α9α10-nAChR-inhibiting properties.

While no side effects of α9-nAChR-inhibiting analgesics have been reported, further behavioural phenotyping of α9-nAChR KO mice revealed a susceptibility of this genotype to stress-induced dysregulation of behavioural and physiological affective responses, which is likely hypothalamic-pituitary-adrenal (HPA)-axis mediated. These findings have negative implications for the clinical application of pharmacological inhibitors of α9α10-nAChRs, wherein such compounds may have undesirable side effects impacting HPA-axis function.

The results have expanded on our understanding of the role of α9α10-nAChRs in pain. α9α10-nAChRs were found to have a limited role in the development and maintenance of chronic pain, indicating that the clinical application for specific α9α10-nAChR-inhibiting analgesics would be narrow. Furthermore, the potential for unforeseen negative side effects was uncovered.
ACKNOWLEDGEMENTS

My sincerest thanks to my supervisor, Prof Mac Christie, for your guidance that has ensured my growth as a scientist and as an individual. Your encouragement of autonomy and independent thought are qualities that I hope I have achieved and which I value above all technical skills that I have acquired over the years. The breadth and depth of your knowledge that seems so effortlessly acquired, retained and recited has and will continue to be an inspiration to me. Thank you for setting the example of uncompromising scientific integrity.

I am very grateful to the past members of the Christie lab that started me on my practical journey into behavioural neuroscience and immunohistochemistry, Christine Mozar, Dr Ian Napier, Dr Billy Chieng and Dr Yael Azriel. To those who have ensured the progress of my research; Dr Petra Van Nieuwenhuijzen, thanks for the uplifting chats and brilliant ideas; Dr Jonathon Arnold and Alana Anderson thank you for your time and use of equipment and resources; and most of all, thank you Thomas Burton, for your patience, support, ideas and help with all of the work I’ve done in the Animal Behavioural Facility.

To all of the staff at Lab Animal Services, your care and maintenance of the animals is invaluable. Thank you! Thank you Dr Yan Ping Du for maintaining our animal colonies, and all the hard work you do for our lab.

Thanks to Dr Richard Clark for the provision of the conotoxins, and to the Lewis (UQ), Adams (RMIT) and Alewood (UQ) labs, for the enjoyable annual meetings. To the Balleine lab, with whom our lab crossed paths for a large part of my PhD, and the current members of the Christie and Bagley labs, it has been great working alongside such hard working and intelligent people.

To my parents, Thomas, and all those friends and family who I have not seen enough of lately, thank you for keeping my life happy and healthy outside of the lab!
LIST OF TABLES

Chapter 1
Table 1.1. Analgesic α-conotoxins with proposed dual mechanisms of action

Chapter 5
Table 5.1 Comparison of CCI and PNL pain models on the Incapacitance test

Chapter 7
Table 7.1. Timing schedule of stress paradigm
Table 7.2 Summary of behavioural and physiological phenotype of WT and KO mice after acute and persistent stress
LIST OF FIGURES

Chapter 1
Figure 1.1. Cone snail anatomy
Figure 1.2. Mechanism of action of MVIIA (Ziconotide)
Figure 1.3. Acetylcholine synthesis, release and removal
Figure 1.4. Phylogenetic tree (A) and composition rules (B) of muscle- and neuronal-type nAChRs
Figure 1.5 Structure of nicotinic acetylcholine receptors
Figure 1.6. Developmental changes in expression of α9 and α10 nAChRs in the rat cochlea
Figure 1.7 Medial olivocochlear (MOC) projections from brainstem to outer hair cells (OHCs) of the cochlea
Figure 1.8 Proposed sites of action of analgesic α-conotoxins such as Vc1.1
Figure 1.9. Major components of the HPA axis
Figure 1.10. Glucocorticoid receptor (GR)-regulated gene expression
Figure 1.11. Schematic representation of the interactions between the HPA-axis and the immune system.

Chapter 2
Figure 2.1. Chronic neuropathic pain models of the sciatic nerve in the mouse
Figure 2.2. Hot and cold pain threshold tests
Figure 2.3. Non-noxious mechanical threshold tests
Figure 2.4. Noxious mechanical threshold test
Figure 2.5. Routes of drug administration in the mouse
Figure 2.6 The IntelliCage system
Figure 2.7. IntelliCage “intelligent” corners
Figure 2.8. The elevated plus maze (EPM)
Figure 2.9. The forced swim test (FST)
Figure 2.10. Mouse restraint
Chapter 3
Figure 3.1. Baseline pain thresholds for mechanical allodynia, mechanical hyperalgesia and thermal hyperalgesia
Figure 3.2. Hot and cold thermal hypersensitivity in chronic neuropathic and inflammatory pain models
Figure 3.3. Mechanical hyperalgesia in chronic neuropathic and inflammatory pain models
Figure 3.4. Mechanical hyperalgesia in chronic neuropathic and inflammatory pain

Chapter 4
Figure 4.1. H & E staining of naïve and cuffed sciatic nerves.
Figure 4.2. Oedema in sciatic nerves of WT and α9-nAChR KO mice.
Figure 4.3. Representative lymphocyte (CD3ε+) staining in injured and naive WT sciatic nerves.
Figure 4.4. Representative staining of ChAT-immunoreactive cells in injured and sham sciatic nerves
Figure 4.5. Immune cell infiltration of sciatic nerves.

Chapter 5
Figure 5.1. Timeline of conotoxin testing
Figure 5.2. Dose response relationships of GABA_B receptor ligands
Figure 5.3. Conotoxin testing in C57Bl/6j mice; Vc1.1 and Rg1A
Figure 5.4. Vc1.1 testing in 129Sv/Ev WT and α9-nAChR KO mice.
Figure 5.5. cVc1.1 testing in 129Sv/Ev WT and α9-nAChR KO mice; mechanical allodynia.
Figure 5.6. cVc1.1 testing in 129Sv/Ev WT and α9-nAChR KO mice; mechanical hyperalgesia.

Chapter 6
Figure 6.1. General activity measures over the course of IntelliCage testing.
Figure 6.2. Average activity measures per module.
Figure 6.3. Circadian patterns of exploratory activity for WT and KO mice during the Free Adaptation module of IntelliCage testing.

Figure 6.4. Sucrose preference developed in both WT and α9-nAChR KO mice.

Figure 6.5. Anhedonia developed in KO mice.

Chapter 7
Figure 7.1. Time spent immobile during the forced swim test was altered in persistently stressed mice

Figure 7.2. Locomotor activity on the elevated plus maze did not differ between WT and α9-nAChR KO mice

Figure 7.3. Bouts of arm crosses were elevated in persistently stressed WT mice, but not KO mice

Figure 7.4. Anxiety-like behaviour is increased in persistently stressed α9-nAChR KO mice

Figure 7.5. The physiological stress response of corticosterone release was dysregulated in α9-nAChR KO mice

Chapter 8
Figure 8.1. Interplay between external stimuli and neuroendocrine centres.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-BGTx</td>
<td>α-Bungarotoxin</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChRs</td>
<td>acetylcholine receptor</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ARC</td>
<td>Animal Resources Centre</td>
</tr>
<tr>
<td>ASIC</td>
<td>acid sensing ion channel</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BMRI</td>
<td>Brain and Mind Research Institute</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C57</td>
<td>C57Bl/6j</td>
</tr>
<tr>
<td>CCI</td>
<td>chronic constriction injury</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund's adjuvant</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin releasing hormone</td>
</tr>
<tr>
<td>CRH-R1</td>
<td>CRH receptor type 1</td>
</tr>
<tr>
<td>CRH-R2</td>
<td>CRH receptor type 2</td>
</tr>
<tr>
<td>cVc1.1</td>
<td>cyclic Vc1.1</td>
</tr>
<tr>
<td>DEG/ENaC</td>
<td>degenerin/epithelial Na⁺ channel</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>DS</td>
<td>drinking sessions</td>
</tr>
<tr>
<td>DSM</td>
<td>diagnostic and statistical manual of mental disorders</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalograph</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPM</td>
<td>elevated plus maze</td>
</tr>
<tr>
<td>FA</td>
<td>free adaptation</td>
</tr>
</tbody>
</table>
FST  
  forced swim test

GABA  
  gamma-aminobutyric acid

GPCR  
  G-protein coupled receptor

GR  
  glucocorticoid receptor

H & E  
  haematoxylin and eosin

HPA  
  hypothalamic-pituitary-adrenal

i.m.  
  intra-muscular

i.p.  
  intra-peritoneal

IR  
  immunoreactive

i.u.  
  international units

i.v.  
  intravenous

IL  
  interleukin

Imp  
  Impulsivity

IVC  
  Individually Ventilated Cages

K$_{2p}$  
  two-pore-domain $K^+$ channel

KO  
  knockout

LC-NE  
  locus coeruleus-norepinephrine

LHS  
  left hand side

mAChR  
  muscarinic acetylcholine receptor

MAO  
  monoamine oxidase

Mgprs  
  Mas-related GPCRs

MMP  
  matrix metaloproteases

MOC  
  medial olivocochlear

MR  
  mineralocorticoid receptor

nAChR  
  nicotinic acetylcholine receptor

NHMRC  
  National Health and Medical Research Council

NK1  
  neurokinin-1

NPA  
  Nosepoke adaptation

NSAIDs  
  non-steroidal anti-inflammatory drugs

OC  
  olivocochlear

OHC  
  outer hair cell

PAM  
  pressure application measurement

PBS  
  phosphate buffered saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNL</td>
<td>partial nerve ligation</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>RFID</td>
<td>radio-frequency identification</td>
</tr>
<tr>
<td>RHS</td>
<td>right hand side</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>s.c.</td>
<td>sub-cutaneous</td>
</tr>
<tr>
<td>SA</td>
<td>sucrose adaptation</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SES</td>
<td>socio economic status</td>
</tr>
<tr>
<td>SK2</td>
<td>small conductance Ca(^{2+})-dependent K(^{+}) channel</td>
</tr>
<tr>
<td>SNL</td>
<td>spinal nerve ligation</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin reuptake inhibitors</td>
</tr>
<tr>
<td>TCA</td>
<td>tri-cyclic antidepressants</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage gated calcium channel</td>
</tr>
<tr>
<td>VGSC</td>
<td>voltage gated sodium channel</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
# Table of Contents

ABSTRACT ........................................................................................................................................ III

ACKNOWLEDGEMENTS .................................................................................................................. V

LIST OF TABLES ............................................................................................................................. VI

LIST OF FIGURES .......................................................................................................................... VII

LIST OF ABBREVIATIONS .............................................................................................................. X

## Chapter 1

GENERAL INTRODUCTION .............................................................................................................. 1

1.1 THE PAIN BURDEN AND RISK FACTORS .................................................................................. 2

1.1.1 THE ECONOMIC BURDEN OF CHRONIC PAIN ...................................................................... 2

1.1.2 EPIDEMIOLOGY, DEMOGRAPHICS & RISK FACTORS OF CHRONIC PAIN ......................... 2

1.1.3 CHRONIC PAIN AS A DISEASE ENTITY .................................................................................. 4

1.2 CURRENT ANALGESICS .............................................................................................................. 5

1.2.1 MILD TO MODERATE PAIN: NSAIDs .................................................................................... 5

1.2.2 Severe or persistent pain: Opiates ............................................................................................ 6

1.2.3 ANTICONVULSANTS, ANTIDEPRESSANTS & SYNERGISTIC ANALGESICS ......................... 6

1.3 NOVEL MOLECULAR TARGETS OF ANALGESICS ..................................................................... 9

1.3.1 SIGNAL TRANSDUCTION MOLECULES .................................................................................. 9

1.3.2 ION CHANNELS ...................................................................................................................... 10

1.3.2.1 SODIUM CHANNELS .......................................................................................................... 10

1.3.2.2 CALCIUM CHANNELS ......................................................................................................... 10

1.4 CONOTOXINS FOR THE TREATMENT OF PAIN .......................................................................... 12

1.4.1 MU CONOTOXINS .................................................................................................................. 14

1.4.1.1 KIIIA .................................................................................................................................. 14

1.4.2 OMEGA CONOTOXINS .......................................................................................................... 14

1.4.2.1 MVIIA ............................................................................................................................... 15

1.4.2.2 CVII, CVIE AND CVIF ..................................................................................................... 18

1.4.3 ALPHA CONOTOXINS .......................................................................................................... 18

1.4.3.1 Vc1.1 AND RGIA .............................................................................................................. 19

1.4.3.2 PeIA AND AuIB .................................................................................................................. 20

1.5 ACETYLCHOLINE RECEPTORS & PAIN ...................................................................................... 22

1.5.1 NICOTINIC ACETYLCHOLINE RECEPTORS .......................................................................... 24

1.5.2 NICOTINIC ACETYLCHOLINE RECEPTORS IMPLICATED IN PAIN ........................................ 26
1.5.3 α9 Subunit of Nicotinic Acetylcholine Receptors ............................................. 27
1.5.3.1 Discovery of α9- and α10-nAChR Subunits .................................................. 27
1.5.3.2 Function of α9α10-nAChRs in the Cochlea .................................................... 30
1.5.4 α9α10-nAChR Tissue Distribution ..................................................................... 32
1.5.4.1 Sensory epithelia .............................................................................................. 32
1.5.4.2 Bone ................................................................................................................ 32
1.5.4.3 Breast and Lung Epithelia ............................................................................... 32
1.5.4.4 Keratinocytes .................................................................................................. 32
1.5.4.5 Immune Cells ................................................................................................... 33
1.5.4.6 Neuroendocrine Cells ..................................................................................... 33
1.5.4.7 Neuronal Cells ................................................................................................ 34
1.6 α9α10-nAChRs in Pain .......................................................................................... 35
1.6.1 α9α10-nAChR Inhibition Without Analgesia ....................................................... 35
1.6.2 Pain-Relevant Anatomical Sites of α9α10-nAChR Expression ......................... 35
1.6.3 Peripheral Afferent Nerves as α-Conotoxin Targets ......................................... 38
1.6.3.1 Peripheral Afferent Nerve nAChR Expression ................................................. 38
1.6.3.2 ACh Source at Peripheral Afferent Nerve nAChRs: Central Terminals ........ 38
1.6.3.3 ACh Source at Peripheral Afferent Nerve nAChRs: Peripheral Terminals .... 39
1.7 Validity of Animal Models of Human Pain States ............................................... 41
1.7.1 Endpoints Beyond Evoked, Reflexive Withdrawals ......................................... 41
1.7.2 CCI Model of Neuropathic Pain; Cuff versus Catgut .................................... 42
1.7.3 PNL Model of Neuropathic Pain ...................................................................... 44
1.8 The HPA-Axis in Pain, Immune Function & Mental Health ............................... 45
1.8.1 The HPA-Axis Stress Response ......................................................................... 46
1.8.2 The HPA Axis in Stress & Affective Disorders .................................................. 49
1.8.3 The HPA-Axis in Chronic Pain Disorders .......................................................... 50
1.8.4 Overlapping Targets for Pain, Mood & Inflammation ..................................... 52
1.8.4.1 CRH Receptors ............................................................................................... 52
1.8.4.2 Substance P Neuropeptide Receptors ............................................................. 53
1.8.4.3 Cytokines ....................................................................................................... 53
1.9 Validity of Animal Models of Affective State ...................................................... 56
1.10 Project Goals & Significance ............................................................................ 58

Chapter 2

General Materials & Methods ................................................................................. 60
2.1 Animals ............................................................................................................... 61

xiv
2.5.5.2 Oedema ........................................... 85

2.6 DRUG ADMINISTRATION ......................................................... 86
2.6.1 Scruffing ................................................................. 86
2.6.2 Sub-cutaneous injections ........................................... 87
2.6.3 Intra-peritoneal injections ........................................... 87
2.6.4 Intra-muscular injections ............................................. 88

2.7 IntelliCage ................................................................. 90
2.7.1 The IntelliCage system .................................................. 90
   2.7.1.1 Cage composition and software .............................. 90
   2.7.1.2 Intelligent corner composition ............................... 93
   2.7.1.3 RFID transponder implantation ............................... 95
2.7.2 Experimental design .................................................. 95
   2.7.2.1 General activity & circadian rhythms ...................... 97
   2.7.2.2 Socio-environmental effects of group housing .......... 97
   2.7.2.3 Sucrose preference .............................................. 97
   2.7.2.4 Learning tasks: Impulsivity & Aversion .................. 98
2.7.3 IntelliCage for greater bench-to-bedside translation .......... 98

2.8 STRESS & AFFECTIVE DISORDERS ........................................ 100
2.8.1 Elevated plus maze ................................................... 100
2.8.2 Forced swim test ....................................................... 102
2.8.3 Restraint stress ......................................................... 104
2.8.4 Physiological stress response ...................................... 106
   2.8.4.1 Blood collection ............................................... 106
   2.8.4.2 Corticosterone measurement .................................. 106

Chapter 3

Pain phenotype of \( \alpha^9 \)-nAChR KO mice ........................................ 107

3.1 INTRODUCTION .............................................................. 108

3.2 METHODS ................................................................. 110
   3.2.1 Pain testing paradigms ........................................... 111
   3.2.2 Expression of data & statistical analyses ................... 111

3.3 RESULTS ................................................................. 112
   3.3.1 Acute nociception is unaffected by deletion of the \( \alpha^9 \)-nAChR subunit 112
   3.3.2 Chronic hot and cold hypersensitivity is normal in \( \alpha^9 \)-nAChR KO mice 112
   3.3.3 Chronic mechanical allodynia develops normally in \( \alpha^9 \)-nAChR KO animals, but mechanical hyperalgesia shows a unique phenotype 114
3.3.4 Repeated testing-induced hypersensitivity does not develop normally in α9-nAChR KO animals ................................................................. 116
3.3.5 Further evidence for abnormal responses of α9-nAChR KO animals to repeated nociceptive testing ................................................................. 116

3.4 Discussion ............................................................................................. 119
3.4.1 Disadvantages of pharmacological probes ........................................... 119
3.4.2 Disadvantages of gene knockout models ............................................ 121
3.4.3 α9-nAChR KO mice show dissociation between mechanical allodynia & hyperalgesia ......................................................................................... 121
3.4.4 Anatomical site of action of α9α10-nAChR-inhibiting analgesics .......... 122
3.4.5 Potential neuroendocrine involvement of α9α10-nAChR in pain .......... 123
3.4.6 Potential side effects of α9α10-nAChR-inhibiting analgesics ............... 124
3.4.7 Unusual properties of α9α10-nAChRs ................................................. 125
3.4.8 Conclusions ......................................................................................... 125

Chapter 4

Immunohistochemical comparison of WT and KO injured sciatic nerves ...127

4.1 Introduction .......................................................................................... 128
4.2 Methods ............................................................................................... 131
4.2.1 Timing of nerve collection ................................................................... 132
4.2.2 Regions of interest (ROIs) for quantification ...................................... 132
4.3 Results ................................................................................................... 133
4.3.1 Nerve morphology ............................................................................. 133
4.4.2 Oedema ............................................................................................ 135
4.3.3 Immune cell infiltration of sciatic nerves ........................................... 137
4.3.3.1 Lymphocyte infiltration ................................................................. 137
4.3.3.2 ChAT immunoreactive cell infiltration ....................................... 140
4.4 Discussion ............................................................................................ 142
4.4.1 The α9α10-nAChR is not necessary for inflammatory response in injured sciatic nerves ................................................................. 142
4.4.2 α9α10-nAChR deletion in mice may be subject to functional compensation .............................................................................................................. 143
4.4.3 Oedema & immune reactions are not pain model-dependent ............ 143
4.4.4 Conclusions ......................................................................................... 145
CHAPTER 5

α-Conotoxins in α9-nAChR KO mice ................................................................. 146

5.1 Introduction .............................................................................................. 147

5.2 Methods .................................................................................................... 149

  5.2.1 GABA<sub>B</sub> receptor-ligands .............................................................. 150
  5.2.2 Toxin preparation .................................................................................. 150

  5.2.2.1 Saline solvent ................................................................................... 150
  5.2.2.2 Conotoxin doses .............................................................................. 150
  5.2.2.3 BSA ................................................................................................. 151

  5.2.3 Pain testing paradigms .......................................................................... 151

  5.2.3.1 Conotoxin acute administration ...................................................... 151
  5.2.3.2 Conotoxin repeated administration ................................................. 151

  5.2.4 Expression of data & statistical analyses .............................................. 152

5.3 Results ....................................................................................................... 154

  5.3.1 Baclofen analgesia is attenuated by SCH50911 ..................................... 154
  5.3.2 α-Conotoxins in vivo in C57BL/6J mice ................................................. 156
  5.3.3 VC1.1 in vivo in 129Sv/Ev mice ............................................................... 159
  5.3.4 cVC1.1 in vivo in 129Sv/Ev mice ............................................................. 161

5.4 Discussion ................................................................................................. 164

  5.4.1 Discontinuation of ACV1 clinical trials ............................................... 164
  5.4.2 GABA<sub>B</sub> receptor-dependent VC1.1 analgesia ............................... 165
  5.4.3 Peptide drug stability ........................................................................... 165

  5.4.4 Binding site(s) of α-conotoxins on α9α10-nAChRs & species differences in affinity .......................................................... 166
  5.4.5 Conclusions ......................................................................................... 168

CHAPTER 6

Behavioural phenotyping of α9-nAChR KO mice in the IntelliCage .......... 169

6.1 Introduction .............................................................................................. 170

6.2 Methods .................................................................................................... 173

6.3 Results ....................................................................................................... 174

  6.3.1 The IntelliCage monitoring system measures gross activity levels:

  Visits, Nosepokes & Licks ........................................................................... 174

  6.3.2 Module switches in the IntelliCage have minimal effect on behaviour 176
6.3.3 Circadian patterns are altered in α9-nAChR KO mice but are sensitive to socio-environmental factors ................................................................. 178
6.3.4 Sucrose preference develops in WT & KO mice in the IntelliCage home-
cage ........................................................................................................ 181
6.3.5 Anhedonia develops in KO mice after cognitive challenge .............. 183
6.4 Discussion .......................................................................................... 186
6.4.1 KO mice showed variable increases in general activity compared to WTs
...................................................................................................................... 186
6.4.2 KO mice showed an altered circadian pattern of activity ............... 187
6.4.3 KO mice showed normal hedonic preferences, but were more susceptible
to stress-induced anhedonia ........................................................................ 188
6.4.4 Future experiments: Improvements & new tasks ................................ 190
6.4.5 Conclusions ..................................................................................... 191

Chapter 7

Stress, Anxiety & the α9α10-nAChR ...................................................... 193
7.1 Introduction ....................................................................................... 194
7.2 Methods ............................................................................................ 196
7.2.1 Housing considerations .................................................................... 196
7.2.2 Temporal considerations of stressors and behavioural tests ........... 197
7.2.3 Stress paradigm ................................................................................ 198
7.2.4 Expression of data & statistical analyses ........................................ 198
7.3 Results ............................................................................................... 199
7.3.1 Behavioural stress responses ........................................................... 199
7.3.1.1 Stress-induced arousal behaviour is decreased in α9-nAChR KO mice...... 199
7.3.1.2 Anxiety-like behaviour is increased in α9-nAChR KO mice ............... 203
7.3.2 Physiological stress response .......................................................... 205
7.3.2.1 The corticosterone stress-response is dysregulated in α9-nAChR KO mice. 205
7.4 Discussion .......................................................................................... 207
7.4.1 Decreased stress-induced arousal .................................................... 207
7.4.2 Increased anxiety-like behaviour ....................................................... 208
7.4.3 Dysregulation of physiological stress response .............................. 209
7.4.4 HPA-axis dysregulation vs. pain phenotype in α9-nAChR KO mice .... 211
7.4.5 Conclusions ..................................................................................... 212
CHAPTER 8

GENERAL DISCUSSION ........................................................................................................213

8.1 ADVANTAGES OF α-CONOTOXIN ANALGESICS .........................................................214
8.2 α9α10-nAChR–INHIBITING ANALGESICS .................................................................215
8.3 THE ANATOMICAL SITES OF ACTION OF α9α10-nAChRs IN ANALGESIA .............217
  8.3.1 α9α10-nAChRs ON IMMUNE CELLS ......................................................................218
  8.3.2 α9α10-nAChRs ON NEURONAL CELLS .................................................................219
  8.3.3 PERIPHERAL ACETYLCHOLINE SOURCE .............................................................220
  8.3.4 α9α10-nAChR EXPRESSION IN INJURED TISSUE ..............................................220
8.4 POTENTIAL RISKS OF α9α10-nAChR INHIBITORS ..................................................221
8.5 AFFECTIVE DISORDERS & α9α10-nAChRs .................................................................223
  8.5.1 DYSREGULATION OF AROUSAL STATES IN α9-nAChR KO MICE ......................223
  8.5.2 BEHAVIOURAL RESPONSES OF α9-nAChR KO MICE AFTER STRESS ............224
  8.5.3 POTENTIAL PHYSIOLOGICAL CHANGES IN α9-nAChR KO MICE AFTER STRESS 226
  8.5.4 IMPLICATIONS OF STRESS DYSREGULATION FOR PAIN STATES IN α9-nAChR KO MICE ..................................................................................................................227
  8.5.5 BROADER IMPACT OF α9-nAChR SUBUNIT DELETION OR INHIBITION ............229
8.6 FUTURE EXPERIMENTS .................................................................................................232
  8.6.1 THE α9α10-nAChR IN NOCICEPTION VS. PAIN ...............................................232
  8.6.2 CHRONIC PAIN AS A STRESSOR IN MICE .........................................................232
  8.6.3 AFFECTIVE DISORDERS IN α-CONOTOXIN-TREATED MICE ..............................233
8.7 CONCLUSIONS ............................................................................................................234

REFERENCES ....................................................................................................................236

APPENDICES ....................................................................................................................275
  APPENDIX A ....................................................................................................................276
  INTELLICAGE EXPERIMENTAL MODULES .................................................................276
  APPENDIX B ....................................................................................................................279
  INTELLICAGE EXPERIMENTAL OPTION NOTES .......................................................279
  APPENDIX C ....................................................................................................................283
  INTELLICAGE MODULE SWITCH STATISTICAL ANALYSIS OUTCOMES ..................283
  APPENDIX D ....................................................................................................................289
  PUBLICATIONS AND PROCEEDINGS ............................................................................289
CHAPTER 1

GENERAL INTRODUCTION
1.1 THE PAIN BURDEN AND RISK FACTORS

Chronic pain is a highly prevalent condition with far reaching negative implications. At the individual level, chronic pain significantly reduces quality of life and may ultimately lead to disability and the need for specialised care. At an economic level, the impact of pain is two-fold. Firstly, the direct costs of pain management are significant for patients, carers and the health care system. Secondly, the indirect costs of chronic pain that result from reduced productivity and lost wages is equally significant (Gaskin and Richard, 2012).

1.1.1 THE ECONOMIC BURDEN OF CHRONIC PAIN

Estimating the economic impact of chronic pain is difficult, as chronic pain often begins as a secondary consequence of other illnesses, and as such, is not always a diagnosed condition. While estimates vary, all studies agree that the burden is large. A recent evaluation of the economic cost of pain in the United States adult population conservatively estimated the total impact at US$560 to US$635 billion per year (Gaskin and Richard, 2012). This far exceeded the economic burdens of the most costly diagnosed diseases (e.g. cardiovascular disease, cancer) by at least double. The authors found that chronic pain affected at least 21% of the adult working population. This pain-afflicted adult-aged working population had lower wages, worked fewer hours and paid significantly more for health care services, compared to the non-pain population. Of the approximately $600 billion economic cost of chronic pain, the incremental costs (out of pocket costs paid by patients, Medicare costs, health insurance, workers compensation etc.) accounted for $261 to $300 billion, while indirect costs (reduced productivity, sick leave/days of work missed etc.) made up the remaining $299 billion to $334 billion.

1.1.2 EPIDEMIOLOGY, DEMOGRAPHICS & RISK FACTORS OF CHRONIC PAIN

The estimated prevalence of chronic pain is relatively uniform worldwide at 1 in 5 adults. Cross national studies report prevalence at 22% (Gureje et al., 2001),
while national studies report approximately 15% in the USA (Gaskin and Richard, 2012), 17% and 18% in New Zealand and Australia respectively (Blyth et al., 2001; Dominick et al., 2012) and 19-20% in Europe (Breivik et al., 2006; van Hecke et al., 2013).

The propensity of an individual to develop chronic pain may be, to an extent, modifiable so far as lifestyle, employment status and occupational factors are concerned (Smith et al., 2007). Although prevention is always better than cure, there are many non-modifiable factors that affect the likelihood of developing chronic pain that are beyond an individual’s control. The main socio-demographic factors associated with chronic pain are female gender, older age, lower socio economic status (SES), geographical and cultural background and a history of abuse or interpersonal violence (van Hecke et al., 2013). Chronic pain is more prevalent among individuals with other chronic health conditions, known as multi-morbidity (Butchart et al., 2009). A better understanding of the competing demands and interactions of multiple chronic health conditions is required for improving both quality of care and the health outcomes of multi-morbid patients (Butchart et al., 2009). Furthermore, patients with severe chronic pain have an increased risk of 10-year mortality, independent of the cause of the pain or socio-demographic factors. This increased mortality is believed to be due to the high intensity of the pain, and/or the resulting disability (Torrance et al., 2010).

Both the epidemiological study of chronic pain, as well as its clinical management, require careful consideration of multi-morbidity, where conditions exist both as discrete measurable entities, as well as contributing to a cumulative allostatic load (Dominick et al., 2012). Allostatic load refers to the costs incurred to the body as a result of adaptation to stressors, in order to regain homeostasis (McEwen, 2001), on which chronic pain is thought to bear significantly. Both physiological and emotional stressors affect allostatic load (Chrousos and Gold, 1992) and multi-morbid patients with both physical and mental health conditions, alongside chronic pain, have a much poorer quality of life (Dominick et al., 2012). This is likely due to the bidirectional temporal relationship between
mental health and chronic pain, and the overlapping multiple system involvement (Gatchel, 2004; van Hecke et al., 2013).

1.1.3 CHRONIC PAIN AS A DISEASE ENTITY

Chronic pain is generally considered a symptom secondary to causative diseases, and therefore treatment approaches focus on the pathology causing the pain. However, the effects of persistent or chronic pain on physiology (e.g. receptor expression, neural plasticity) and psychology (e.g. emotional, cognitive and social impact) suggest that chronic pain should be considered as a distinct disease entity (Siddall and Cousins, 2004). The estimates of the prevalence of chronic pain are likely conservative due to the assumption of equivalence of chronic pain with discrete painful conditions such as arthritis, chronic back pain, and migraine.

It is apparent that chronic pain is a major social and economic burden. Patients with chronic pain give lower ratings for the quality of pain care compared to their overall healthcare, and are less satisfied with overall care than patients suffering from chronic diseases without chronic pain (Butchart et al., 2009). Better pain management treatments are overdue.
1.2 CURRENT ANALGESICS

Modern medicine has given us many treatments to promote health, increase longevity and increase the quality of life. One of the main factors that affect subjective assessments of quality of life is pain. The primary approach of modern medicine to increase the quality of life of the ill is to alleviate pain. This is often effective for acute pain that is relatively mild or moderate, and often of an inflammatory origin. Pain that is neuropathic, that is, originating from damage to the somatosensory nervous system (Jensen et al., 2011), pain that has become chronic, and pain that is severe in intensity is much harder to treat (Martin and Eisenach, 2001). Efforts are ongoing to find medical treatments that alleviate these types of pain.

1.2.1 MILD TO MODERATE PAIN: NSAIDs

For mild to moderate pain, non-opioid analgesics, such as COX-inhibitors, are the primary means of treatment. However, these are inadequate in treating many neuropathic and chronic pain conditions and suffer from both ceiling-effects, and unfavourable side-effect profiles (Katz and Barkin, 2008).

Drugs such as paracetamol and non-steroidal anti-inflammatory drugs (NSAIDs e.g. aspirin, diclofenac and ibuprofen) act by inhibiting the cyclooxygenase (COX) family of enzymes. Although the mechanism of action of paracetamol is incompletely understood, its predominant mechanism of action is involves a COX-2-selective pathway (Graham and Scott, 2005). NSAIDs are COX inhibitors that ultimately reduce the production of prostaglandins and thromboxanes (Campos et al., 1999). In addition to non-compliance due to the necessity for multiple dosing, NSAIDs are associated with cumulative gastrointestinal and cardiovascular risks, and are unsuitable for those with compromised renal and hepatic functions (Barkin, 2009). Much of the adverse gastric and renal side effects and antithrombic activity were attributed, perhaps falsely (Fukai et al., 2011), to inhibition of the constitutively active COX-1 isozyme, thus COX-2 selective agents were developed. Inhibition of the inducible COX-2 isoform
proved to have fewer gastrointestinal side effects, however the risks are not abolished, and the cardiovascular risks still remain (Campos et al., 1999; Katz and Barkin, 2008).

1.2.2 Severe or Persistent Pain: Opiates

The treatment of more severe or persistent pain relies heavily on opioid analgesics, primarily targeting the μ-opioid receptors. Opioids are prone to tolerance, requiring increased dosing over time, which in turn increases the likelihood of the development of adverse side effects such as suppression of gastrointestinal motility, nausea and vomiting, sedation and cognitive impairment and at high doses, respiratory depression. Other major concerns with opioid analgesics are the risks of physical dependence and addiction, leading to abuse and misuse (Barkin, 2009; Walwyn et al., 2010). Stringent regulation of prescription and dispensing of opioid analgesics may cause them to be prescribed at suboptimal doses or under-prescribed (Katz and Barkin, 2008).

1.2.3 Anticonvulsants, Antidepressants & Synergistic Analgesics

Other treatments for neuropathic pain include anti-depressants, anticonvulsants and muscle relaxants, which may be used alone or in combination with non-opioid and opioid analgesics (Barkin, 2009; Lui and Ng, 2011). Antidepressants modulate synaptic transmission at the spinal cord level to enhance the endogenous inhibitory pain pathways, and may dampen conduction by blocking voltage-dependent Na\textsuperscript{+} channels (Zuliani et al., 2010). Anticonvulsants dampen neuronal hyperexcitability by inhibiting excitatory (glutamatergic) neurotransmission (Shimoyama et al., 2000), enhancing inhibitory (GABA-ergic) neurotransmission, or by blocking voltage-dependent ion (Na\textsuperscript{+} and Ca\textsuperscript{2+}) channels (Swerdlow, 1984). Pregabalin and gabapentin were developed as anticonvulsants, but are now the first line treatment for some neuropathic pain conditions (Levendoglu et al., 2004; Wiffen et al., 2013). Pregabalin and gabapentin bind to the \( \alpha2\delta \) subunit of voltage-gated calcium channels (Ca\textsubscript{\( \alpha2\delta \)}) which are expressed at particularly high density at spinal dorsal horn synapses (Bennett, 2011). Their analgesic properties are believed to be through the decrease of Ca\textsubscript{\( \alpha2\delta \)}-mediated neurotransmitter release at central
nervous system (CNS) synapses. In particular, they are thought to act at synapses that undergo plastic changes associated with chronic neuropathic or inflammatory pain (central sensitisation) (Tuchman et al., 2010). The unique mechanism of action of gabapentin and pregabalin warrant their categorisation as a distinct class of analgesics.

Although only modest improvements in pain relief are gained from current adjuvant analgesics, and despite being associated with their own respective side effects, combination therapy has the benefit of allowing for reduced doses of each drug, minimising the occurrence and intensity of adverse effects, particularly those associated with opioid treatments (Bennett, 2011; Gilron et al., 2005). Successful combination therapies also include single drugs/molecules that have more than one mechanism of action. Analgesics with intrinsic combination opioid and monoamine reuptake-inhibiting mechanisms, including tramadol and tapentadol, have multiple benefits. These compounds offer greater analgesia and a broader therapeutic window, as their analgesic mechanisms act synergistically, while the side effect mechanisms remain distinct. Tapentadol, in particular offers this synergism at a constant ratio, due to its single molecule binding to two targets- µ-opioid receptors and noradrenaline transporters- and having no active metabolites (Raffa et al., 2012; Tzschentke et al., 2014). Such dual-mechanisms reduce opioid-typical side effects, and may decrease dependence and abuse potential. Combination gabapentin and morphine treatment has also been associated with lower doses of the respective drugs needed, and reduction of adverse side effects when compared to single drug therapies (Gilron and Weaver, 2005).

Although progress has been made in analgesic research, inadequate response to the currently available analgesics in a large proportion of pain sufferers constitutes a substantiated unmet need in patients with neuropathic pain (Finnerup et al., 2015). Clinically, only half of patients are able to attain partial pain relief and the maintenance of this relief often requires the addition of multiple therapeutic agents (Backonja, 2012). Thus, there remains a pressing
need for the discovery and development of novel non-opioid analgesics that are effective in alleviating severe and persistent pain.
1.3 Novel molecular targets of analgesics

As the physiological processes behind pain transmission and maintenance continue to be better understood, the development of analgesics is able to proceed as a more focused and directed pursuit.

1.3.1 Signal transduction molecules

The transduction of an external stimulus into a painful subjective experience depends on the functioning of a multitude of molecular markers that characterise different neuronal subtypes.

Hot and cold stimuli activate specific transient receptor potential (TRP) proteins (e.g. TRPV1 and TRPM8 respectively) that transduce the stimuli into electrical signals (Bautista et al., 2007; Caterina et al., 1997; Lapointe and Altier, 2011).

Acid sensing ion channels (ASICs) are voltage-independent, depolarising cationic channels. They are chemo-electrical transducers important for somatic and visceral nociception. ASIC channels are proton gated and can respond to very small reductions in pH (Deval et al., 2008; Yagi et al., 2006). ASICs are thus believed to play a primary role in the pain-response to tissue injury, where protons are released by damaged cells, as well as other conditions of acidosis (Deval et al., 2010).

In chronic pain conditions, mechanical hypersensitivity (alldynia and hyperalgesia) is a major clinical problem. Mechanosensation is perhaps the most complicated and the least well characterised sense at the molecular level. Different pressures and textures are perceived by a variety of mechanosensitive neuronal subtypes that translate mechanical stimuli both quantitatively and qualitatively (Basbaum et al., 2009). Knowledge of the molecules that comprise the mechanotransduction machinery is still in its infancy. Candidate mechanotransducers include Piezo2 which are responsible for non-painful, low threshold mechanosensation (Ranade et al., 2014), degenerin/epithelial Na\(^{2+}\)
channel (DEG/ENaC) superfamily, including ASIC channels (Chen and Wong, 2013; Price et al., 2000), certain TRP proteins (Liedtke et al., 2000), and two-pore-domain K+ channel (K2P) subunits such as TREK-1, TREK-2 and TRAAK (Noel et al., 2009; Pereira et al., 2014). G-protein coupled receptors (GPCRs) such as the Mas-related GPCRs (Mgprs) also play a role in the modulation of mechanical nociception (Rau et al., 2009). However, none of these candidate proteins completely explain the mechanisms of mechanotransduction or chronic mechanosensitivity and further research is therefore needed.

A more detailed understanding of the function of signal transduction molecules in health and pathology will undoubtedly offer medical science new targets for analgesics. This will allow for highly targeted and specialised analgesic care.

1.3.2 Ion Channels

Ion channels- in particular, sodium (Cummins et al., 2007; Wang et al., 2011) and calcium (Schmidtko et al., 2010) channels are the key propagators and modulators of nociceptive inputs, and are currently the targets of pain research.

1.3.2.1 Sodium Channels

Voltage-gated sodium channels (VGSCs) are the primary mediators of action potential initiation and propagation of in excitable cells. There are nine known mammalian subtypes (Na\textsubscript{v}1.1- Na\textsubscript{v}1.9) which each have distinct distribution and biophysical properties, with Na\textsubscript{v}1.3, Na\textsubscript{v}1.7, Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9 believed to be involved in pain transmission (Knapp et al., 2012).

1.3.2.2 Calcium Channels

Voltage-gated calcium channels (VGCCs) are vital in coupling electrical signalling at the plasma membrane to physiological responses within the cell. In pain transmission, neurotransmitter release is modulated by these receptors. Multiple calcium channel types have been identified, which can be distinguished according to pharmacological and electrophysiological characteristics. Six have
thus far been identified, named L-, T-, R-, P/Q- and N-type. Those most relevant to pain transmission are the T-, P/Q and N-types, with the N-type channel being the most suitable therapeutic target, as inhibitors of this type show fewer side-effects and neuroprotective properties (Schroeder and Lewis, 2006).
1.4 Conotoxins for the Treatment of Pain

The first analgesic drug to be approved for clinical use in decades with a novel molecular target is the synthetic version of a naturally occurring conotoxin.

Conotoxins are peptides isolated from the venom of marine cone snails (Figure 1.1). Cone snails have evolved extremely effective venomous apparatuses for both hunting and self-defence. Each snail produces venom that is composed of hundreds of peptides, or conotoxins, each of which is highly selective for specific cellular targets (Dutertre et al., 2014; Livett et al., 2004). When isolated, the specific function of each peptide may be exploited. Conotoxin peptides can be classed according to their respective target ion channel or receptor. Numerous conotoxin classes act on pain targets, and can therefore be used to dissect the roles of specific ion channels and receptors that contribute to pain, as well as holding potential as novel therapeutics (Lewis et al., 2012). Those classes of interest as potential analgesics include μ- and μO-conotoxins, which target voltage-gated sodium channels, ω-conotoxins, which target voltage-gated calcium channels, and α-conotoxins, which target nAChRs (Lewis et al., 2000; Zhang et al., 2007).

One of the great appeals of using natural venoms as starting points in the search for new drugs and drug leads is that their products have inherent biological activity, selectivity and high potency. While this high selectivity potentially minimises the likelihood of off-target side effects, the protein targets involved in nociception are often ubiquitous, both centrally and peripherally, and are therefore prone to on-target adverse events. Nevertheless, heterogeneity in the subunit composition of certain ion channels, such as N-type Ca$^{2+}$ channels (Altier et al., 2007; Andrade et al., 2010), brings about functional heterogeneity, which is recognised by certain conotoxins.
Figure 1.1. Cone snail anatomy. Cone snails have sophisticated envenomation strategies for which the hollow harpoon-like radula is used to inject venom into their predators or prey. Photograph from http://ocean.nationalgeographic.com/ocean/photos/dangerous-sea-creatures/
The specificity of conotoxins allow structure-activity relationship (SAR) evaluation that facilitate the engineering of the most optimal peptide structures by elucidating the important residues that contribute to the pharmacophore’s structure and target-interactions (Schroeder and Lewis, 2006). By guiding the rational development of specific channel inhibitors, conotoxins allow for the design of more effective drugs for clinical use and may overcome many side effects (Cummins et al., 2007; Mould et al., 2004).

1.4.1 Mu conotoxins

Mu (μ) conotoxins are characterised by their affinity for voltage-gated Na\(^{2+}\) channels.

1.4.1.1 KIIIA

The remarkable selectivity of the first μ-conotoxin to be isolated, GIIA from Conus geographus, for the muscle-specific VGSC subtype Na\(_{1.4}\) (Cruz et al., 1985) suggested that other μ-conotoxins would display similar selectivity for other subtypes. Of interest were conotoxins that were selective for neuronal VGSCs that are primarily involved in pain signalling.

KIIIA is a μ-conotoxin from Conus kinoshitai that has nanomolar affinity for Na\(_{1.7}\), a VGSC subtype associated with congenital pain disorders in humans with gain- and loss-of-function mutations (Cox et al., 2006; Dib-Hajj et al., 2005; Faber et al., 2012). KIIIA has analgesic properties in animal models of inflammatory pain (Zhang et al., 2007). Properties such as its small size, high affinity and unique VGSC channel selectivity means that the structural framework of KIIIA is being used to develop new analgesic drugs that inhibit Na\(_{1.7}\) sodium channels.

1.4.2 Omega conotoxins

Omega (ω) conotoxins are characterised by their affinity with voltage-gated calcium channels.
1.4.2.1 MVIIA

The first conotoxin to reach the market for pain treatment was an ω-conotoxin, MVIIA (generic name ziconotide, commercial name Prialt ®), from Conus magus. MVIIA is an ideal example of therapy that was rationally designed based on an understanding of basic pain mechanisms (Backonja, 2012). It achieves its effects through inhibition of N-type VGCCs on the central terminals of primary afferent neurons in the dorsal horn of the spinal cord (Schmidtko et al., 2010). This inhibition prevents the release of pro-nociceptive neurochemicals such as glutamate, calcitonin gene-related peptide (CGRP) and substance P, from being released, and thus prevents the pain signal from ascending into the central nervous system (CNS) (Figure 1.2).

MVIIA is available as an intrathecal infusion for the treatment of severe chronic pain in individuals who are intolerant of or whose pain is refractory to conventional treatments. MVIIA overcomes some of the major adverse events that limit opiate dosage; it does not cause respiratory depression (Dean et al., 1999) and tolerance and dependence have not been reported (Schmidtko et al., 2010) meaning it does not have abuse potential. Furthermore, MVIIA does not reduce gastrointestinal motility; an effect of opiates which often extends the duration of postoperative hospitalisation (Mathur, 2000).

Despite cases of MVIIA providing complete pain relief and increased quality of life, the therapeutic window of the drug is very small. MVIIA’s efficacy is unpredictable and variable, with only a small subset of patients finding treatment beneficial, while severe adverse side effects are common (Schmidtko et al., 2010). Of particular concern are neurological side effects such as confusion, agitation, auditory and visual hallucinations and unresponsiveness (Penn and Paice, 2000).

Intrathecal routes of drug administration have the benefits of equipotency at lower doses when compared to systemic administration, as well as stability of concentration owing to continuous drug delivery (Schmidtko et al., 2010). However, intrathecal delivery is reserved as one of the last choices for
pharmacological pain management due to the highly invasive nature and the high risk of infection, particularly meningitis. Thus, although MVIIA provides immeasurable benefits for some individuals, the small therapeutic window in which it is active limits its use.
Figure 1.2. Mechanism of action of MVIIA (Ziconotide). A] Pain signals are transmitted from the periphery via primary afferent nerves to the CNS. The afferent sensory neurons terminate in the dorsal horn of the spinal cord; the first point of synaptic processing before the pain signal ascends via the spinothalamic tract to the brain. B] At the central synapse of primary afferent nerves, N-type VGCCs regulate signalling. Membrane depolarisation, as caused by an action potential, opens the N-type VGCCs, causing Ca$^{2+}$ influx, which triggers neurotransmitter (e.g. glutamate) release into the synaptic cleft. Ziconotide inhibits pain transmission at the level of the spinal cord by binding to the N-type VGCCs, blocking Ca$^{2+}$ permeability and preventing neurotransmission. Image taken from Schmidtko et al. (2010).
1.4.2.2 CVID, CVIE AND CVIF

The cone snail species *Conus catus* has yielded a number of ω-conotoxins that show analgesic properties. One that has reached human clinical trials is CVID, which has even greater selectivity for the N-type VGCCs over P/Q-type than MVIIA (Lewis et al., 2000), possibly by targeting a pharmacologically distinct subset of N-type VGCCs (Adams et al., 2003). CVID has much lower toxicity than MVIIA and may therefore be administered through non-spinal, parenteral routes (e.g. i.v.), allowing a greater range of patients to be treated (Scott et al., 2002). This conotoxin has proven most effective when administered synergistically with analgesics that have differing mechanisms of action (e.g. morphine (Kolosov et al., 2011) and dexmedetomidine (Blake et al., 2005)), possibly giving rise to new mechanisms of analgesia. As with all combination therapies, smaller respective doses of each drug ensures decreased risks of toxicity and adverse events.

The newest ω-conotoxins to be isolated from *Conus catus* that are potent, selective and reversible inhibitors of the N-type VGCCs are CVIE and CVIF. These peptides have been shown to have channel interactions that are unique in their dependence on membrane potential, channel isoform and channel activation state. CVIE and CVIF show analgesic activity in rats, and these peptides, and their R10K analogues, are valuable for the rational development of N-type VGCC-specific inhibitors (Berecki et al., 2010), giving promise of better therapeutic indices.

1.4.3 ALPHA CONOTOXINS

Alpha (α) conotoxins are characterised by their affinity for nicotinic acetylcholine receptors. A nicotinic mechanism of pain is known to exist (see Section 1.5.2) but due to the high degree of complexity of nAChR assembly and stoichiometry (see Section 1.5.1), the precise subunits of nAChRs involved are unknown. The native affinity of specific conotoxins for different nAChR subunits is allowing gradual elucidation of the matter.
1.4.3.1 Vc1.1 and RgIA

Vc1.1 is another conotoxin that has reached clinical trials for treatment of neuropathic pain. However, Phase 2A trials were discontinued when a large species-difference in potency was discovered at the proposed site of action (Metabolic, 2007).

Vc1.1 is an α-conotoxin that has shown a great deal of promise in pre-clinical testing in rats, having unmatched analgesic efficacy (Clark et al., 2010), as well as being long-acting, with no tolerance or documented side effects. Additional pre-clinical experiments indicate that it may also facilitate functional recovery from nerve injury (Livett et al., and issued 10 October 2002; Livett et al., and issued 29 September 2005; Satkunanathan et al., 2005). Investigations into the mechanism of action of Vc1.1 have proven less straightforward.

Vc1.1 is a member of the α-class of conotoxins, which are ligands at nicotinic acetylcholine receptors (nAChRs). Early publications suggested that Vc1.1 interacted with nAChRs containing the α3 subunit with either β2 or β4, however the affinity at these subunits was too weak to account for its analgesic effects (Clark et al., 2006). The peptide has since been identified as a highly potent antagonist of the α9α10-nAChR, and inhibition of this receptor was said to be the mechanism of Vc1.1 analgesia (Vincler et al., 2006). During the early phases of human clinical trials, it was discovered that the potency of Vc1.1 at the human α9α10-nAChR was approximately 100-fold less than at the rat α9α10-nAChR, and continuation of human clinical trials was deemed cost-prohibitive (Azam and McIntosh, 2012; McIntosh et al., 2009). Despite confident assertions that the analgesic effects of Vc1.1 was due to α9α10-nAChR antagonism (Vincler et al., 2006), this receptor had never been implicated in pain before, and had mostly been associated with audition and development in the olivocochlear system (Murthy et al., 2009). The role of the α9α10-nAChR in pain perception has since come into doubt (see Section 1.6). An alternative mechanism has subsequently been proposed, involving a unique GABABR-dependent pathway that results in N-type VGCC inhibition (Callaghan and Adams, 2010; Callaghan et al., 2008). Another α-conotoxin that likely acts through a similar mechanism as Vc1.1 is
RgIA (Callaghan et al., 2008; Ellison et al., 2006). RgIA is the most potent inhibitor of the α9α10-nAChR currently known, and also inhibits N-type VGCC currents via the GABA₉R. RgIA has proven to be an effective analgesic, and repeated dosing of both RgIA and Vc1.1 proffers restorative properties in rat pain models (Mannelli et al., 2014; Vincler et al., 2006).

1.4.3.2 PeIA and AuIB

PeIA from Conus and AuIB from Conus aulicus are two other α-conotoxins that likely share the GABA₉R-dependent mechanism of analgesia, alongside their respective nAChR-subunit targets. PeIA is selective for α9α10-nAChR over α7, as well as a potent inhibitor α3β2-receptors and chimeric α6/α3β2β3 receptors (McIntosh et al., 2005). AuIB is selective for α3β4-nAChRs (Luo et al., 1998). AuIB has been shown to have analgesic effects in rodent models of chronic neuropathic pain, thus adding more support to the GABA₉R-dependent mechanism of analgesia proposed for Vc1.1 and RgIA (Klimis et al., 2011; Napier et al., 2012). The inhibition of their respective nicotinic subunits may also contribute to their analgesia, however the diversity of nAChR-subtypes inhibited by this group of α-conotoxins suggests that the inhibition of VGCCs via GABA₉Rs is likely to be the primary mechanism of analgesia.

While the action of ω-conotoxins is well characterised as being through direct binding of VGCCs, the inhibition of Ca²⁺ channels by Vc1.1 requires significantly more investigation. Increasingly more α-conotoxins with this unique GABA₉R-dependent VGCC inhibiting mechanism are being identified that show promise as novel analgesics (Table 1.1). The relevance of nAChR inhibition for α-conotoxin analgesia is unknown, and is an interesting domain to be investigated.
Table 1.1. Analgesic α-conotoxins with proposed dual mechanisms of action.

<table>
<thead>
<tr>
<th>Conotoxin</th>
<th>Sequence</th>
<th>Target</th>
<th>Analgesic?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vc1.1</td>
<td>GCCSDPRNCYDHPEIC*</td>
<td>α9α10 N-type VGCC via GABA&lt;sub&gt;B&lt;/sub&gt;R</td>
<td>Yes</td>
</tr>
<tr>
<td>RgIA</td>
<td>GCCSDPRCRYR---CR</td>
<td>α9α10 N-type VGCC via GABA&lt;sub&gt;B&lt;/sub&gt;R</td>
<td>Yes</td>
</tr>
<tr>
<td>PeIA</td>
<td>GCCSHPACSVPNHELC*</td>
<td>α9α10, α3β2 N-type VGCC via GABA&lt;sub&gt;B&lt;/sub&gt;R</td>
<td>Not Tested</td>
</tr>
<tr>
<td>AuIB</td>
<td>GCCSYPPCFATNPD- C*</td>
<td>α3β4 N-type VGCC via GABA&lt;sub&gt;B&lt;/sub&gt;R</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Amidated C-terminus. Lines linking the cystine (C) residues in the conotoxin sequences represent disulphide bonds that contribute to structural stability.

Images from http://www.conchology.be/?t=1
1.5 Acetylcholine receptors & pain

Acetylcholine (ACh) is well known as an important neurotransmitter, but is also a local mediator that is evolutionarily conserved across not only species and taxonomic class, but across all domains of life. ACh, and ACh synthesizing activity, is present in animals (vertebrate and invertebrate), plants, fungi and bacteria, wherein it regulates physiological functions (Horiuchi et al., 2003). Although ACh was initially identified as a neurotransmitter, and was indeed the first neurotransmitter ever identified (Dale and Loewi, 1965; Fishman, 1972), the function of non-neuronal ACh is being increasingly well characterised.

ACh synthesis is catalysed in the cytoplasm by choline acetyltransferase (ChAT) enzymes, from precursors choline and acetyl CoA. ACh is stored in vesicles until it is released into the synaptic cleft. Acetylcholinesterase (AChE) hydrolises ACh, liberating choline, which is transported back into the nerve terminal for resynthesis into ACh (Figure 1.3).

Acetylcholine is the endogenous ligand for ACh receptors (AChRs) that are either metabotropic or ionotropic. The two subtypes are named according to the pharmacological agents that characterise their activity. Metabotropic AChRs are exclusively activated by muscarine from the Amanita muscaria mushroom, thus dubbed muscarinic AChRs (mAChRs), while ionotropic AChR are sensitive to nicotine, from the tobacco plant, and known as nicotinic AChRs (nAChRs).

Rapidly activated ionotropic nAChRs regulate diverse physiological processes such as maintaining metabolic tone, controlling inflammatory processes and their widely studied influence over inhibitory and excitatory transmission in the nervous system (Albuquerque et al., 2009).
Figure 1.3. Acetylcholine synthesis, release and removal. AChE, acetylcholinesterase; ChAT, Choline acetyltransferase; CoA, coenzyme A.
1.5.1 Nicotinic acetylcholine receptors

Mammalian nAChRs are classed as muscle- or neuronal-type. The muscle-type receptors are uniform pentamers with fixed subunit stoichiometry, consisting of two α1, one β1, one δ and either a γ((α1)2β1δγ) or an ε((α1)2β1δε) subunit, depending on developmental maturity. The neuronal-type nAChRs are more diverse in their composition, forming either homo- or hetero-pentamers made up of α (α2-α10) and/or β (β2-β4) subunits (Figure 1.4). The ligand binding site is at the interface of two subunits, with a principal (+) and complementary (-) component. α and β subunits are defined by the presence or absence, respectively, of two adjacent cystines in the putative ACh binding domain (Cordero-Erausquin et al., 2000) (Figure 1.5). α7 subunits natively express as functional homomers as well as heteromers with other subunits (α8 in chick retina, α5, β2, β4 in recombinant receptors (Millar and Gotti, 2009)). α8 and α9 subunits have also been shown to form homomers in heterologous expression systems (Elgoyhen et al., 1994; Gotti et al., 1994). Not only does the subunit composition of the neuronal nAChR confer function, but so to does the stoichiometry, the characterisation of which is still in its infancy. This incredible diversity in neuronal nAChR subtypes, despite the relative lack of understanding of them, suggests an evolutionary refinement of function, and offers highly promising target sites for therapeutic agents. Indeed, in parallel with the increasing understanding of nAChR pharmacology and their role in physiology and pathology, there has been growing interest in nAChRs as potential drug targets, for the treatment of psychiatric, neurological and peripheral disorders (Dutton and Craik, 2001; Hurst et al., 2013; Livett et al., 2006; Millar and Gotti, 2009).
Figure 1.4. Phylogenetic tree (A) and composition rules (B) of muscle- and neuronal-type nAChRs. The subunits are classified as muscle- or neuronal-type. They form homo- or hetero-pentameric receptors, with only select alpha subunits, 7, 8 and 9, capable of assembling as homo-pentamers. Localisations of the ACh binding sites at the subunit interfaces are shown (yellow; principal site at the base and complementary site at the point). Image adapted from Cordeiro-Erausquin et al (2000).

Figure 1.5. Structure of nicotinic acetylcholine receptors. A| nAChR subunits have four hydrophobic transmembrane domains (M1-M4), extracellular N- and C-termini, and a large intracellular loop. B| Schematic representation of the quaternary structure of the nAChR pentamer, with acetylcholine binding sites and the axial cation-conducting channel. C| Representation of the ligand binding site. Amino acid residues (circles) that participate in ligand binding are shown on loops A, B and C of the principal component, and loops D, E, and F on the complementary component of the binding site. Elements of these images are taken from Changeux & Edelstein (2001), Gotti et al (2006), Clementi (2004), Karlin (2002) and Taly et al (2009).
Modern drug discovery has identified numerous ligands, particularly natural products, for use as molecular probes of nAChRs, yet few nAChR ligands have reached late stage development and only one has been approved since the 1950’s: varenicline, an α4β2 nAChR partial agonist, prescribed for smoking cessation. The main factor that has limited the success of nAChR ligands is their narrow therapeutic range i.e. inadequate clinical efficacy and/or high incidence of adverse events (Arneric et al., 2007; Hurst et al., 2013).

Since cholinergic communication and regulation is so ubiquitous in the mammalian system and the complexity of nAChRs so great, therapeutic nAChR ligands continue to pose a great challenge.

1.5.2 Nicotinic Acetylcholine Receptors Implicated in Pain

It has long been known that nicotinic compounds alleviate pain. Nicotine, in the form of tobacco, brought back by Colonialists from the New World, has been known to alleviate pain since the 16th century (Hurst et al., 2013). In more recent times, the identification of epibatidine, from the poison arrow frog (Spande et al., 1992) and the development of a more selective synthetic nAChR agonist, ABT-594 (Bannon et al., 1998), spurred the quest for nicotinic analgesics. However, progress has been limited. The classical nicotinic compounds, nicotine and epibatidine, have small therapeutic windows and prohibitive side effect liabilities, due to their lack of selectivity. For example epibatidine causes hypothermia and hypertension, as well as sensitisation that can turn nonlethal doses into lethal ones (Umana et al., 2013); nicotine causes nausea and dizziness and tolerance to the analgesic effects has been shown to develop in rats (Cepeda-Benito et al., 1998). Thus clinical use of nicotinic-analgesics has not yet been realised.

The primary nAChR subtypes in the nervous system are α4β2 (most abundant in CNS), α3β4 (most abundant in PNS) and α7 (Jain, 2004). The α4β2 nAChR has been a leading target of analgesic research, with agonists of the α4β2 receptor, such as epibatidine, nicotine and ABT-594, all having analgesic efficacy
comparable to or greater than that of opiates (Umana et al., 2013) in animal models of pain. However, agonism of \( \alpha 4\beta 2 \) nACHRs (primarily spinal) has been shown to be necessary but not sufficient to produce nicotinic analgesia (Gao et al., 2010). Additional subunits such as \( \alpha 3, \alpha 5, \alpha 6 \) and \( \beta 3 \) are believed to comprise part of the nicotinic analgesic effect (Lang et al., 2003; Takeda et al., 2003; Vincler and Eisenach, 2004; Wieskopf et al., 2015).

### 1.5.3 \( \alpha 9 \) Subunit of Nicotinic Acetylcholine Receptors

In the last decade, a novel nACHR subunit has been suggested to be involved in pain. The \( \alpha 9 \) subunit of nACHRs is now being pursued as a novel target for nicotinic analgesics, however the evidence for the validity of this target is conflicted.

#### 1.5.3.1 Discovery of \( \alpha 9 \)- and \( \alpha 10 \)-nACHR Subunits

The \( \alpha 9 \)-nACHR was first described in the context of vestibular and auditory physiology. Elgoyhen et al (1994) identified a novel gene from a rat genomic library, and classified the protein as a nACHR based on the primary structure. This novel nACHR had a unique and unusual pharmacological profile when expressed in *Xenopus laevis* oocyte recombinant expression systems, which coincided with hair cell cholinergic properties (Chen et al., 1996; Guth and Norris, 1996). The \( \alpha 9 \)-nACHR was described as mixed nicotinic-muscarinic, wherein ACh activated the receptor, but nicotine, the classical nicotinic agonist, caused inhibition. Additionally, agonists of both nicotinic (DMPP) and muscarinic (0X0-M) AChRs activated the receptor. The \( \alpha 9 \)-nACHR was shown to be expressed in the hair cells of the cochlea, and was credited as the main nACHR in the cochlea.

Despite the coincidence of recombinant \( \alpha 9 \)-nACHRs and cochlea hair cell pharmacology, \( \alpha 9 \) homomers were difficult to express and produced unusually small ACh-evoked currents, which suggested that additional subunit(s) might be required for full function. The discovery of the \( \alpha 10 \) subunit fits this description.
The rat (Elgoyhen et al., 2001) and human (Lustig et al., 2001; Sgard et al., 2002) α10-nAChRs were cloned and characterised, and shown to co-assemble with α9-nAChRs, in similar tissue distribution patterns (Elgoyhen et al., 2001; Sgard et al., 2002). Co-expression of α9- and α10-nAChR subunits in oocytes revealed that the α10 subunit confers functionality to the α9-nAChR. The α9α10-nAChR pharmacological profile was largely unchanged, but ~100-fold larger ACh-evoked currents and biophysical properties (current-voltage relationship, desensitisation kinetics, response to extracellular Ca²⁺ concentrations) that matched those of the hair cells, strongly supporting the theory that the α9α10 heteromer is the native receptor in the cochlea (Elgoyhen et al., 2001; Sgard et al., 2002). α9- and α10-nAChR subunits differ slightly in their developmental and spatial expression (Katz et al., 2004; Morley and Simmons, 2002; Simmons and Morley, 2011) (see Figure 1.6), but KO mouse studies have shown that the α10 subunit is required for biologically relevant cochlear hair cell nAChRs to form (Vetter et al., 2007). The putative stoichiometric conformation of α9α10-nAChRs is (α9)₂(α10)₃, where two α9- and three α10-subunits make up the receptor (Plazas et al., 2005).

The α10 subunit fails to reconstitute a functional receptor alone, or in combination with any other known nAChRs, except for α9 (Elgoyhen et al., 2001; Sgard et al., 2002). It is thus clearly established that co-expression of α9 and α10 nAChR subunits is required to form functional channels and, more importantly, deletion of the α9 nAChR subunit effectively knocks out functional α9α10 nAChRs.
Figure 1.6. Developmental changes in expression of α9 and α10 nAChRs in the rat cochlea. During early development (E18) olivocochlear innervation of the organ of Corti initiate α9 expression. In intermediate development (P1-P10), as efferent olivocochlear innervation reaches the outer hair cells, α9 and α10 expression is differentially regulated in IHCs and OHCs. In the mature cochlea (P31) IHC downregulate both α9 and α10 subunits, however α9 receptors remain at detectable levels. GER, greater epithelial ridge; IHC, inner hair cell; OHC, outer hair cell. Image taken from Morley & Simmons (2002).
1.5.3.2 **Function of α9α10-nAChRs in the Cochlea**

The α9α10-nAChR is necessary for normal functioning of the cochlea, primarily through its role in normal synapse formation between the olivocochlear (OC) efferent nerves and the hair cells of the cochlea (Murthy *et al.*, 2009) (Figure 1.7). The absence of either the α9- or α10-subunit (Vetter *et al.*, 2007), via germline deletion, has been shown to cause disruptions in hair cell physiology and cochlear function. The hair cells are functionally deafferented, meaning that the innervation of the hair cells by OC terminals is abnormal or absent. However, despite these physical and physiological abnormalities, α9-nAChR KO mice exhibit very little deficit in hearing behaviourally. Tone detection and intensity discrimination are normal and no abnormal age-related hearing loss has been reported (May *et al.*, 2002). Increased functionality of the α9-nAChR, on the other hand, shows a significant benefit in protecting against acoustic injury. Overexpression of the receptor (Maison *et al.*, 2002) or increased sensitivity due to point mutation (Taranda *et al.*, 2009) results in less hearing loss from exposures that cause either temporary or permanent threshold shifts, without changing pre-exposure cochlear sensitivity to low- or moderate-level sound. The α9α10-nAChR has been suggested as a potential target for the treatment of tinnitus that is associated with hearing loss, through a mechanism that alleviates tinnitus when hearing impairment is reduced (Vetter *et al.*, 1999).

Since the α10 subunit is considered necessary for biologically relevant nAChRs to form (Vetter *et al.*, 2007), and that the α9α10-nAChR is considered the native receptor (Elgoyhen *et al.*, 2001), α9 subunit-containing nAChRs will herein be denoted as α9α10-nAChRs.
Figure 1.7. Medial olicocochlear (MOC) projections from brainstem to outer hair cells (OHCs) of the cochlea. A| MOC neurons project from the superior olivary complex of the brainstem to the cochlea. B| MOC projections make direct synaptic contacts at the base of the OHCs. C| MOC terminals release ACh, which binds OHC α9α10-nAChRs, causing Ca2+ influx and activation of Ca2+-dependent SK2 K+ channels. Image taken from Taranda et al (2009).
1.5.4 α9α10-nAChR TISSUE DISTRIBUTION

Although the α9α10-nAChR has been best characterised in the cochlea, tissue distribution extends to other sensory, immune and endocrine tissues.

1.5.4.1 SENSORY EPITHELIA

In addition to the hair cells of the cochlea, α9α10-nAChRs are expressed in the sensory epithelia of cochlear and vestibular labyrinths and labyrinthine bone marrow of adult rats and guinea pigs (Elgoyhen et al., 1994; Hiel et al., 1996; Luo et al., 1998). Other developing sense organs in the rat also show α9α10-nAChR expression including embryonic olfactory epithelium and tongue (Elgoyhen et al., 1994).

1.5.4.2 BONE

Developing rats express α9α10-nAChRs in inner sulcus cells of the cochlea as well as chondrocytes and/or osteoblasts in the cochlear capsule and interscalar laminae, and the vestibular capsule. Both developing and adult rats express high levels of α9-nAChR mRNA in bone marrow (Luo et al., 1998).

1.5.4.3 BREAST AND LUNG EPITHELIA

Tobacco smoking-related cancers have implicated the α9α10-nAChR in tumour development. The α9-nAChR appears to be important for nicotine–induced transformation of breast epithelial cells into precancerous or cancerous cells (Lee et al., 2010). Upregulation of the receptor is associated with lower survival rate, and is mediated by the transcription factor oestrogen receptor (Lee et al., 2011). Nicotine-induced breast cancer cell proliferation is inhibited by α9α10-nAChR downregulation (Chen et al., 2011). The α9α10-nAChR may also play a role in lung cancer development and progression (Chikova and Grando, 2011).

1.5.4.4 KERATINOCYTES

The α9α10-nAChR mediates the activity of adhesion molecules and cytoskeletal proteins in keratinocytes, thus playing a role in the early stages of
epithelialisation and wound healing (Chernyavsky et al., 2007). The receptor has also been implicated in Pemphigus vulgaris, an autoimmune disease of mucocutaneous adhesion, causing blistering of oral and skin epithelial cells (Nguyen et al., 2000). Lack of α9α10-nAChR function due to autoantibodies is believed to cause acantholysis - the separation of keratinocytes from each other.

1.5.4.5 IMMUNE CELLS
There is a growing literature on the presence on α9α10-nAChRs on many populations of immune cells. CD3+ T cells (both CD4+ and CD8+ subsets), as well as CD19+ and CD80+ B lymphocytes all express both α9 and α10 nAChR mRNA (Peng et al., 2004). However the expression of AChRs, both nicotinic neuronal and muscarinic, has been shown to be highly variable between individuals (Sato et al., 1999). The function of the α9α10-nAChR subtype in immune cells in unknown and, as yet, attempts to elicit α9α10-mediated ACh responses have not succeeded in either human B or T-lymphocytes or Jurkat immortalised T cells (Peng et al., 2004). α9α10-nAChRs are not upregulated in activated B-lymphocytes (Koval et al., 2011), but may be involved in nicotine exposure-related inhibition of allergic asthma (Mishra et al., 2010). α9α10-nAChRs are expressed in both developing and adult bone marrow cells (Luo et al., 1998), which may reflect the developmental origins of α9α10-nAChR-expressing immune cells.

1.5.4.6 NEUROENDOCRINE CELLS
It has recently emerged that the α9α10-nAChR has functions in the neuroendocrine system. The α9α10-nAChR appears to play an important role in cholinergic synaptic transmission between splanchnic nerve terminals and chromaffin cells in the adrenal medulla during stress. The receptors are responsible for maintaining tonic inhibitory control on the gap junction coupling between chromaffin cells, and are upregulated in the adrenal medulla under conditions of stress to become the dominant contributor to ACh-induced current (Colomer et al., 2010). α9α10-nAChR expression has been shown in the pituitary
gland, although the functional role of the receptor here is yet to be determined (Elgoyhen et al., 1994).

1.5.4.7 Neuronal cells
The predominant CNS nAChR subtypes are α4β2 and α7, while the α3β4 subtype is most abundant in the peripheral nervous system (Jain, 2004). α9α10-nAChRs are thought to be absent in CNS tissue (Elgoyhen et al., 1994). α9-nAChR mRNA has been inconsistently shown to be present in dorsal root ganglion (DRG) cell bodies but not sensory nerve axons, and no functional cell-surface protein has been identified in this tissue (Callaghan and Adams, 2010; Haberberger et al., 2004; Lips et al., 2002). α9α10-nAChRs are expressed at the sympathetic splanchnic nerve-chromaffin cell synapse in the adrenal gland (Colomer et al., 2010), though whether this expression is pre-synaptic or post-synaptic is unknown.
1.6 α9α10-nAChRs in Pain

The involvement of the α9α10-nAChR in nociception has only been inferred from pharmacological studies. Direct evidence is lacking.

Analgesic conotoxins such as Vc1.1 and RgIA that have very high selectivity for the α9α10-nAChR, have been used as evidence for the involvement of this receptor in pain (see Section 1.4.3). There are however, a number of factors that cast doubt on the validity of this assertion including the lack of analgesic activity of α9α10-nAChR-inhibiting Vc1.1 analogues, the absence of a relevant anatomical site of α9α10-nAChR expression and the unknown source of ACh.

1.6.1 α9α10-nAChR Inhibition Without Analgesia

Pharmacological evidence for the inadequacy of α9α10-nAChR inhibitors as analgesics has been known for years. Nevin et al. (2007) showed that analogues of Vc1.1 that were equipotent at the α9α10-nAChR produced no analgesia in a rat model of neuropathic pain. The analogues, vc1a and [P6O]Vc1.1, were structurally almost identical to Vc1.1 apart from one (P6Ovc1.1) or two (vc1a) post-translational modifications (PTMs), indicating that any differences in biological targets were not due to major changes in the 3D shape of the molecules.

1.6.2 Pain-Relevant Anatomical Sites of α9α10-nAChR Expression

The prevailing explanation for the analgesic actions of α9α10-nAChR-antagonists in the literature is that it occurs through immune cell inhibition (Mannelli et al., 2014; Vincler et al., 2006) (Figure 1.8 B). Proponents of this theory observed that in addition to analgesia, repeated Vc1.1 and/or RgIA administration in rats significantly inhibited the migration of ChAT-immunoreactive cells, ED1-immunoreactive macrophages and CD2-immunoreactive T-cells into CCI injured nerves (Vincler et al., 2006). The high degree of selectivity of both of these conotoxins was inferred to be the mechanism of action of both the inhibition of
immune cell infiltration and the analgesia. The authors suggested that inhibition of α9α10-nAChRs on immune cells in the vicinity of nerve injury reduces the inflammatory milieu, thus reducing the overall algogenic pathology. Peripheral immune cells do express the main components of cholinergic communication, including nAChRs, choline acetyltransferase (ChAT) and ACh (Kawashima and Fujii, 2004; Rinner et al., 1995; Sato et al., 1999), so they could feasibly be targets for nicotinic analgesics. However, the presence of functional, ACh-responsive α9α10-nAChRs on immune cells is yet to be confirmed (see Section 1.5.4.5).

Another potential site of action of systemically acting analgesics is the peripheral sensory nervous system (Figure 1.8 A). Unfortunately, no functional expression of α9α10-nAChRs has been shown on sensory afferent nerve axons, terminals or cell soma. The cell bodies of peripheral sensory nerves, collectively situated in the dorsal root ganglia (DRGs), do indeed express multiple nAChR subtypes (Genzen et al., 2001), though these are predominantly α4 and α7 (Genzen et al., 2001; Haberberger et al., 2004). α9-nAChR RNA expression has been inconsistently found in rat DRG neurons, however no translated functional protein has been detected (Callaghan and Adams, 2010; Haberberger et al., 2004; Lips et al., 2002). Putting this mechanism of action into further doubt is the fact that nAChRs have been shown to be down-regulated in peripheral sensory afferents in neuropathic pain models (Dubé et al., 2005), and in vivo studies show that α-Bungarotoxin (α-BGTx)-sensitive nAChR subtypes (i.e. α7 and α9) are minimally involved in nicotinic analgesia (Damaj et al., 1998).
Figure 1.8. Proposed sites of action of analgesic α-conotoxins such as Vc1.1. A| Neuronal sites on peripheral sensory nerves (green) have been proposed, inhibiting either peripheral or central terminals. Central cholinergic neurons (yellow) are an ACh source. B| Immune cell sites have been proposed. Scissors represent the site of injury along the sensory afferent nerves.
1.6.3 Peripheral afferent nerves as α-conotoxin targets

The expression of nAChRs in peripheral sensory afferents is a relatively new field of study, and the source of ACh at such peripheral nerve nAChRs is yet to be confirmed.

1.6.3.1 Peripheral afferent nerve nAChR expression

Peripheral sensory neurons have not traditionally been a focus of studies of cholinergic signalling, and only recently has the traditional cholinergic machinery been identified in peripheral sensory nerves (Tata et al., 2004). The role of this ACh-signalling in sensory neurons is still not known (Zhang et al., 2015). Although nicotine can evoke sensory nerve responses (Lang et al., 2005) the mechanism of this may not be nAChR-mediated, but rather due to activation of transient receptor potential ankyrin type 1 (TRPA1) receptors (Talavera et al., 2009).

nAChR expression has been shown in the peripheral nerve cell bodies of chicks (α3, α4 (Boyd et al., 1991) and rats (Rau et al., 2005; Zhang et al., 2015). At least three nAChR subtypes (α7, α3β4, α3β4α5) are functionally expressed in the cell bodies of nociceptors that innervate the hairy skin of rats (Rau et al., 2005).

Inflamation increases the current density through α7 and α3β4-nAChRs in rat DRGs (Zhang et al., 2015). As stated in Section 1.5.4.7, no expression of α9α10-nAChR has been found on peripheral afferent axons. Expression of α9-nAChR mRNA, but not protein (Callaghan and Adams, 2010), has been inconsistently reported in DRGs (Haberberger et al., 2004; Lips et al., 2002), and whether the receptors are specifically translated and transported outside of the soma in pain states is not known.

1.6.3.2 ACh source at peripheral afferent nerve nAChRs: Central terminals

Initial theories on the mechanism of action of Vc1.1 favoured neuronal nAChR inhibition. Livett et al. (and issued 10 October 2002) suggested that cholinergic inhibitory interneurons of the spinal dorsal horn, the primary contributor to
dorsal horn cholinergic input (Mesnage et al., 2011), might activate nAChRs expressed presynaptically to the central terminals of primary afferents. Vc1.1 was suggested to inhibit this activation (Figure 1.8). However, conotoxins, like most peptides, are not expected to have significant permeability of the blood-brain (BBB) or blood-spinal cord barrier (BSCB) (Blanchfield et al., 2007), so central nAChR are unlikely targets of peripherally administered conotoxins. The permeability of the BBB and BSCB have been shown to increase significantly after nerve injury, through activation of TRPV1-expressing C-fibres (Beggs et al., 2010), and it is possible that conotoxins such as Vc1.1 may selectively penetrate these barriers in pathological conditions. Spinal cord permeability is also compromised in other disorders (see (Bartanusz et al., 2011) for review) that may benefit from conotoxin treatment. Specific investigation of the permeability of these peptides through BBB and BSCB is required. Intrathecal administration of Vc1.1 produces significant and long-lasting relief of allodynia (Napier et al., 2012), however whether this is via the same mechanism of action as peripherally administered Vc1.1 remains to be determined.

### 1.6.3.3 ACh source at peripheral afferent nerve nAChRs: Peripheral terminals

A peripheral origin of a cholinergic plexus has been suggested, but the evidence is conflicted. Both the absence (Mesnage et al., 2011) and presence (Matsumoto et al., 2007) of ChAT-immunoreactive DRG cells have been reported with the same antibody. The functional role of ACh in peripheral sensory neurons is speculated to be central inhibition of pain (Matsumoto et al., 2007), though more evidence is needed to support this theory. A sub-group of nociceptors (capsaicin-sensitive) do not release ACh centrally (Dussor et al., 2005). Whether other sub-groups of nociceptive fibres do release ACh centrally remains to be determined.

Non-neuronal ACh sources include keratinocytes after cutaneous injury (Grando et al., 1993) as well as immune cells. A cutaneous source would not account for the pain relief attained by Vc1.1 in animal models, which involve nerve injury (CCI, PNL (Clark et al., 2010; Klimis et al., 2011; Nevin et al., 2007;
Satkunanathan et al., 2005; Vincler et al., 2006)), inflammatory (CFA (McCracken, 2005)) and chemogenic (diabetic neuropathy via streptozotocin injection (McCracken, 2005)) pain. Immune cells such as lymphocytes, dendritic cells and macrophages express cholinergic components sufficient to constitute a discrete cholinergic system, synthesising and releasing ACh that has either an autocrine or paracrine effect (Kawashima and Fujii, 2004; Kawashima and Fujii, 2008). Whether or not ACh released from immune cells does activate sensory afferent nerve nAChRs is unknown. It is possible that the main function of such ACh sources is activation of immune cell nAChRs, as nAChRs mRNA has been identified in thymocytes (α3, α5, β4 (Mihovilovic et al., 1998)) and lymphocytes (α2, α5, α6, α7, α10, β2 (Kawashima et al., 2007)). α9α10-nAChR protein has been identified in B- and T-cells, however these receptors were unresponsive to applied ACh (Peng et al., 2004).
1.7 Validity of Animal Models of Human Pain States

Despite ongoing efforts to improve clinical pain treatments and tremendous advances in basic science knowledge of pain physiology, progress in pharmacological analgesic development has been slow (Backonja, 2012). One of the major concerns facing analgesic research is the translation of preclinical successes to clinical outcomes and registration of new drugs. While notable criticisms have been made regarding the apparent lack of predictive power of animal pain models (Mogil et al., 2010b), and a prominent failure commonly cited (i.e. NK1 receptor antagonists (Hill, 2000)), animal models have contributed immeasurably to the understanding of pain pathophysiology and to the development of new drugs (Whiteside et al., 2008).

Mice have long made excellent models for human pain conditions, with behavioural and anatomical correlates proving consistent, providing much enlightenment to the field. Back-translation of virtually all clinically effective compounds have shown efficacy in existing animal models (Mogil et al., 2010a), however the development of new compounds has idled. The construct validity of the pain models used are improving, with highly specific pathophysiological conditions modelled such as post-operative (incision) pain (Brennan et al., 1996) and chemotherapy-induced pain (Nozaki-Taguchi et al., 2001).

1.7.1 Endpoints Beyond Evoked, Reflexive Withdrawals

Most clinical populations report ongoing pain (96%), but only a portion report evoked mechanical (64%) or thermal pain (38%) (Backonja and Stacey, 2004). However, for reasons of practicality, reproducibility and, probably, convention, behavioural pain testing in animals relies almost exclusively (90% of studies) on evoked hypersensitivity to either mechanical or thermal stimuli (Mogil and Crager, 2004). Thus, basic research paradigms are inadequately modelling clinical populations.
A difficulty with using animal models is identifying specific behaviour(s) that sensitively, uniquely and reliably represent the presence and intensity of spontaneous pain (Mogil and Crager, 2004). Most measures of spontaneous behaviour (vocalisation, autotomy/over-grooming, sleep disruption) used in the past do not uniquely reflect pain or its intensity, and at very best complement, but cannot replace, more direct measures of pain and sensitivity (Vierck et al., 2008). Other poor measures of spontaneous pain are hypolocomotion and reduced rearing, or spontaneous licking, lifting, flinching and shaking (Mogil et al., 2010b). Better measures of ongoing spontaneous pain are facial expressions (Langford et al., 2010) and ultrasonic vocalisation (Kurejova et al., 2010), which may see greater utility in the future. Operant measures such as conditioned place preference (Sufka, 1994), as well as quality-of-life measures are gaining favour (Vierck, 2006) and warrant further development.

It is commonly argued that mice, as a prey species, lack overt and consistently observable suffering when experiencing chronic pain and therefore inadequately reflect human chronic pain conditions (Mogil, 2009; Urban et al., 2011). Indeed, reduced quality-of-life and “illness-behaviour” associated with pain, is a predominant complaint in human patients (Breivik et al., 2006; Lame et al., 2005), but difficult to discern in mice.

1.7.2 CCI MODEL OF NEUROPATHIC PAIN; CUFF VERSUS CATGUT

The chronic constriction injury (CCI) model of neuropathic pain was developed in the 1980s (Bennett and Xie, 1988) and has become a widely used model of neuropathic pain. This model does, however, have multiple drawbacks that limit its utility.

A significant shortcoming of the classic CCI procedure is the difficulty in standardising the degree of injury. The large inter- (and often intra-) experimenter variability means that studies that cite the use of this model are not always comparable (Mosconi and Kruger, 1996). Indeed, many investigators that use the term CCI do not constrict the nerve to the extent originally described.
(“just barely constricted”; “retarded...circulation through the superficial epineurial vasculature”; Bennett and Xie (1988)), but place loose ligatures that do not necessarily produce any apparent constriction (Maves et al., 1993). A component of the chromic catgut, either the chromium ions or pyrogallol, is likely producing a chemical/toxic effect (Maves et al., 1993), which in turn induces pain.

The use of chromic catgut is also problematic, in that toxic substances are introduced to the injury site, which complicates the pathology. Chromic salts added to catgut act as a cross-linking agent that increases the tensile strength and resistance to absorption by the body (Edlich et al., 1973). However, chromic catgut alone, without constriction, causes thermal hyperalgesia and alterations in gait and stance, putting into question the aetiology of pain caused by his model (Maves et al., 1993). Since chromic catgut does not induce an immune or inflammatory response beyond other biomaterials (Akinrinmade and Lawal, 2010; Edlich et al., 1973; Herzberg et al., 1994; Stewart et al., 1990; Varma et al., 1981), it has been suggested that it is the acidification around the injured nerve that is responsible for thermal hyperalgesia. Thus, the chemical/toxic effect of the catgut, without the constriction of the nerve, is closer to a model of peripheral neuritis than neuropathic pain (Maves et al., 1995). Inconsistency in the surface area of perineurial sheath removed prior to applying the ligature affects the amount of contact the nerves have with the chromium and pyrogallol, further affects standardisation of the model (Maves et al., 1994). Furthermore, poor knotting, poor knot security, unreliable tensile strength and difficulty in handling (Akinrinmade and Lawal, 2010) of chromic catgut add to the setbacks encountered with this suture material.

The cuff model of CCI used throughout the current experiments was developed (Benbouzid et al., 2008; Mosconi and Kruger, 1996) with specific concern for consistency and standardisation. The cuff model of neuropathic pain possesses far greater face and construct validity than the traditional CCI method, has vastly improved reproducibility, and will hopefully offer greater predictive validity in future drug discovery.
1.7.3 PNL model of neuropathic pain

The partial nerve ligation (PNL) model of neuropathic pain was developed in 1990 by Seltzer et al. (1990), in the hopes of modelling the human causalgiform pain condition. The PNL model is now one of the most commonly employed models of neuropathy (Jaggi et al., 2011). As with the catgut CCI model, variability in the number of nerve fibres ligated across subjects is high, even among experienced experimenters (Challa, 2015). The difficulty in attaining internal consistency is a significant limitation of the PNL model.

Due to the variation in methods used across laboratories such as strain, housing conditions, diet and suture materials, a true comparison of pain models is difficult to obtain from the literature and comparisons of pain models rely on single comprehensive studies (Berge, 2011). Studies comprising of systematic comparisons of neuropathic pain models of peripheral nerve injury have uncovered contrasting features of each model (Dowdall et al., 2005; Kim and Chung, 1997) and these have been suggested to represent different clinical populations (Kim and Chung, 1997). Thus each pain model is useful for the study of different aspects of chronic pain.
1.8. THE HPA-AXIS IN PAIN, IMMUNE FUNCTION & MENTAL HEALTH

There is a complex interplay between the nervous, endocrine and immune systems, which share a "chemical language" with common neurotransmitters, peptide hormones and cytokines used to communicate across systems (Chapman et al., 2008). This trans-system interplay can compound the symptoms of chronic pain, stress, and mental health disorders.

Acetylcholine is one such molecule that permits communication across nervous, endocrine and immune systems. The link between cholinergic mechanisms and mood disorders has been known since the 1940's (Grob et al., 1947), and was formally proposed in 1972 (Janowsky et al., 1972). There is increasing evidence that an imbalance of central ACh causes mood disturbances from depression to mania (e.g. Meyer et al. (2006); Mineur et al. (2013); Saricicek et al. (2012)). However, peripheral nAChRs may also contribute to mood and affective disorders through regulation of the hormonal stress response axis. Although there has not been a great deal of investigation into the role of the α9α10-nAChR in the stress response and affective state, Colomer et al (2010) have shown a dominant role of this subunit in the adrenal gland during stress. Additionally, expression of the α9α10-nAChR in the pituitary gland has been shown (Elgoyhen et al., 1994), though no functional studies have been performed. Due to the paucity of information on the role of the α9α10-nAChR in the stress response and affective disorders, the possible impact of α9α10-nAChR-inhibiting analgesics on affective state has never before been considered. The function of the α9α10-nAChR in nervous-endocrine-immune communication may account for an alternative mechanism of analgesia, or alternatively might be a source of unforeseen side effects of such analgesics.
1.8.1 The HPA-AXIS STRESS RESPONSE

The main components of the HPA-axis are the hypothalamus, primarily producing corticotropin releasing hormone (CRH) and vasopressin, the anterior pituitary gland, which responds to CRH stimulation by secreting adrenocorticotropic hormone (ACTH), and the adrenal gland, which responds to ACTH with the production of corticosteroids such as glucocorticoids and mineralocorticoids (Figure 1.9). Glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) (Figure 1.10) are widely distributed both peripherally and centrally, and are responsible for the regulation of gene expression in response to stressors, and are vital for the negative feedback control of the HPA-axis stress response.

The HPA-axis is vital for successful appraisal of a stressful situation, adapting behaviour to the present stress and preparing for subsequent stressors (Sapolsky et al., 2000). Both physical and emotional stressors initiate central and peripheral responses that are designed to preserve homeostasis. Centrally, neural pathways mediating arousal, alertness, vigilance and cognition are activated. Peripherally, changes occur that promote adaptive redirection of energy, for example the redirection of vital substrates (oxygen, nutrients) to the CNS and stressed body site(s), sympathetic NS changes such as elevation of blood pressure and HR, gluconeogenesis and lipolysis (Chrousos and Gold, 1992).

The key to a healthy response to stress is adaptation. HPA-axis responses must adapt to the variable durations and intensities of stressors, while functioning to promote physiological and psychological adaptation to those stressors. Pathologies arise when what is adaptive and temporally limited in the generalised stress response, becomes quantitatively and qualitatively altered to be prolonged and maladaptive. Thus, arousal becomes dysphoric hyper-arousal and anxiety; vigilance becomes hyper-vigilance and insomnia.
Figure 1.9. Major components of the HPA axis. A stressful trigger initiates corticotropin-releasing hormone (CRH) to be released from the hypothalamus. CRH acts on the anterior pituitary gland, which releases adrenocorticotropic hormone (ACTH). ACTH triggers corticosterone to be released from the adrenal gland, which acts as a negative feedback signal that dampens the CRH and ACTH production.
Lipophilic glucocorticoids readily cross into cytoplasm

Glucocorticoid binds to the GR. Heat shock protein (hsp) complex dissociates. GR conformation changes to activated state and receptor is translocated to nucleus.

Figure 1.10. Glucocorticoid receptor (GR)-regulated gene expression. Corticosteroids (e.g. glucocorticoids) enter the target cell by passive diffusion, and bind to GRs in the cytoplasm. GRs dissociate from heat shock protein (hsp) complex and undergo conformational change to the activated state. Activated GRs are translocated to the nucleus where they bind as dimers to glucocorticoid-responsive elements (GREs) in the promoter region of the target gene. Transcription of the target, hormone-regulated gene is induced.
1.8.2. The HPA axis in stress & affective disorders

The HPA axis is vital for mediating and regulating physiological and behavioural responses to stress. Stressors may be physical (hypoglycaemia, dehydration, temperature extremes, physical exertion, illness, infection and pain) or psychological (fear, social anxieties, bereavement).

Affective disorders, such as major depressive disorder, are marked by dysregulation of the HPA-axis. The peripheral hormones that define HPA-axis function are ACTH and glucocorticoid (corticosterone in rodents, cortisol in humans), both of which are dysregulated in clinically depressed patients (Holsboer et al., 1984). The majority of patients with diagnosed depression have abnormal levels of circulating cortisol; approximately 50% have chronically elevated circulating cortisol and the other 50% have either lowered baseline cortisol or are eucortisolaemic. This variation in the putative stress marker reflects the heterogeneity of the patient population, as well as the complexity of disorders that fit under the umbrella term of “depression” (Blackburn-Munro and Blackburn-Munro, 2001).

Dysregulation of glucocorticoid secretion reflects either faulty negative feedback circuitry or an altered central drive on the pituitary gland. Normalisation of HPA-axis activity precedes clinical remission from depression, and patients whose depressive symptoms have appeased but still have HPA-axis dysregulation are at high risk of relapse (Holsboer et al., 1982; Zobel et al., 2001). Normalisation is achieved by increasing GRs in the brain, which restores negative feedback and decreases central drive (Reul et al., 1993). Indeed clinical remission from depression resulting from antidepressant treatment is in part due to direct actions of antidepressants on HPA-feedback, through increasing glucocorticoid receptor (GR) expression in relevant brain regions such as the hypothalamus and amygdala (Pepin et al., 1989). New generation antidepressants that target the HPA-axis (e.g. GRs, CRH receptors), rather than the archetypal serotonin or noradrenergic enhancers, are showing promise (Murphy et al., 1998; Zobel et al., 2000).
Interestingly, such clinical outcomes in depressed patients are paralleled in experimental models of chronic stress-induced depression-like behaviour, the symptoms of which resolve with antidepressant treatment (Sapolsky et al., 1984b). This is of particular interest as depressive episodes are often triggered by stressful life events (Pepin et al., 1989).

1.8.3 The HPA-axis in chronic pain disorders

Similar cognitive and emotional processing is provoked by painful and stressful stimuli (Senba et al., 1993), thus emotional stress influences central pain mechanisms (Imbe et al., 2006). The HPA-axis has been shown to be involved in some of the most common chronic pain disorders including fibromyalgia, chronic lower back pain and rheumatoid arthritis. These patients show hypocortisolaemia, and abnormal glucocorticoid feedback (Eijsbouts et al., 2005; Lentjes et al., 1997).

Although mood disorders and chronic pain are treated as entirely separate illnesses in terms of psychiatric diagnostics, it is now well established that the two disorders not only co-occur, but are strong predictors of each other. The high rate of comorbidity of chronic pain and affective disorders has been mirrored in some, though not all (Urban et al., 2011), animal studies showing chronic stress-induced hyperalgesia (Imbe et al., 2006; Jorum, 1988; Quintero et al., 2003); (Vidal and Jacob, 1986) and chronic pain-induced depression- (Jesse et al., 2010) and anxiety-like behaviour (Benbouzid et al., 2008). The question of antecedence or consequence may never be answered due to the shared biological and environmental factors that contribute to the disorders (Fishbain et al., 1997; Maletic and Raison, 2009). Treatment strategies are more likely to succeed if the mechanistic overlap is considered. In the same way that normalisation of HPA-axis is predictive of lasting clinical outcomes of depression, so too is it necessary to treat chronic pain syndromes and depression concurrently for lasting success of both conditions (Dworkin and Gitlin, 1991; Wingenfeld et al., 2010).
Antidepressants give significant benefit to pain sufferers for a range of neuropathic pain conditions including diabetic neuropathy, postherpetic neuralgia, orofacial pain and central pain (McQuay et al., 1996) as well as other pain conditions such as fibromyalgia, arthritis, migraine and chronic headache (Smith, 1992). Estimates from meta-analyses of clinical studies predict that the average relief attained by antidepressants is 74% greater than placebo alone for chronic pain (Onghena and Vanhoudenhove, 1992).

Antidepressant analgesic relief is attained even in non-depressed patients and is believed to be through an analgesic effect rather than an unmasking of undiagnosed depression (McQuay et al., 1996; Onghena and Vanhoudenhove, 1992). Not all antidepressants have analgesic effects, with the less selective antidepressants (i.e. tri-cyclic antidepressants (TCAs), serotonin-noradrenaline reuptake inhibitors (SNRIs)) having superior efficacy in pain relief than selective inhibitors (i.e. selective serotonin reuptake inhibitors (SSRIs)) (Smith, 1998). Though the precise mechanisms of antidepressant analgesia have not yet been uncovered, significant progress has been made in this area (Sawynok, 2003; Sawynok et al., 2001). Both central and peripheral mechanisms are believed to take place and this may be the key to the superior efficacy of TCAs over more specific inhibitors, as they act on multiple nociceptive targets (Mico et al., 2006).

The antinociceptive effects of antidepressants require intact descending inhibitory bulbospinal pathways (Ardid et al., 1995) and act through alteration of spinal serotonin (Hwang and Wilcox, 1987; Millan, 2002; Mixcoatl-Zecuatl and Jolivalt, 2011) and norepinephrine (Proudfit and Hammond, 1981) tone, affecting the descending modulation of pain. Neuropathic injury causes elevation of spinal monoamine oxidase (MAO) enzymes that inactivate neurotransmitters such as 5-HT and noradrenaline (Villarinho et al., 2013), while spinal 5-HT is decreased (Vogel et al., 2003). Antidepressant analgesia may also act through normalisation of such changes.

In addition to serotonin- and noradrenaline-mechanisms (Iyengar et al., 2004), TCSs and SNRIs have been shown to produce analgesia via opioidergic (Botney
and Fields, 1983; Wattiez et al., 2011) and NMDA-mediated (Eisenach and Gebhart, 1995) mechanisms, and to act synergistically with opioid analgesics when both are administered together intrathecally (Pettersen et al., 2009).

Recently, clinically effective anticonvulsants have shown to have both analgesic and anxiolytic effects in animals that are believed to be through modulation of both the sensory and affective dimensions of pain (Munro et al., 2007; Siddall et al., 2006). Thus, delineation of sensory and affective components of pain and understanding the mechanism of adjuvant analgesics is an area of great interest.

### 1.8.4 OVERLAPPING TARGETS FOR PAIN, MOOD & INFLAMMATION

#### 1.8.4.1 CRH receptors

CRH is the major central secretagogue of the HPA axis. Activation of either of the two CRH receptor subtypes, CRH receptor type 1 (CRH-R1) and type 2 (CRH-R2), has been shown to alleviate pain in animal models of inflammatory (Hummel et al., 2010; Mousa et al., 2003), neuropathic (Hummel et al., 2010), somatic (Yarushkina et al., 2009) and visceral (Million et al., 2006) pain. However, distinct roles of the two-receptor subtypes are likely, with activation of CRH-R1 and CRH-R2 shown to respectively increase and decrease visceral pain (Million et al., 2006), while CRH-R1 inhibition or down-regulation reversed or prevented pain, respectively (Hummel et al., 2010). Peripheral CRH appears to act in concert with the endogenous opioid system and the immune system, to cause inhibition of opioid receptors, particularly via β-endorphin, on peripheral sensory afferents in inflamed tissue (Schafer et al., 1994; Schafer et al., 1997). Thus an intact immune system is vital for pain control (Schafer et al., 1996). Central CRH also leads to analgesia, however the mechanism of this is still unclear (Lariviere and Melzack, 2000). Central CRH likely explains the effects of stress on subjective pain, particularly accounting for the differential effects of acute and chronic stress. CRH reduction occurs in the hypothalamus after chronic stress, but rises after acute stress (Culman et al., 1991), and LC neurons become less sensitive to CRH after acute stress but sensitised to CRH after persistent stress (Curtis et al., 1995).
1.8.4.2 Substance P Neuropeptide Receptors

Neuropeptides, in particular substance P, that have key roles in pain transmission are being targeted for the treatment of depression- and anxiety-disorders. First identified in 1930’s (Gaddum and Schild, 1934; von Euler and Gaddum, 1931), substance P was shown to be expressed in pain-relevant central and peripheral nerves (Amin et al., 1954; Hokfelt et al., 1975a; Hokfelt et al., 1975b; Otsuka and Konishi, 1976), and to selectively excite nociceptors that terminate in the spinal dorsal horn (Randic and Miletic, 1977). The presence of substance P, and other peptides, are now used to biochemically define nerve fibre types in the periphery as peptidergic nociceptors that particularly contribute to neurogenic inflammation (Geppetti et al., 2008; Lembeck and Holzer, 1979). Interestingly, these substance P-expressing pain pathways signal information about the intensity of the painful stimulus, rather than site or type of stimulus, and terminate within the ventromedial hypothalamus and the central nucleus of the amygdala- two regions closely concerned with affective states (Hunt and Mantyh, 2001). The substance P-preferring neurokinin-1 (NK1) receptor is highly expressed in brain regions critical for affective behaviour and neurochemical responses to stress (Kramer et al., 1998). Pursuit of this link between substance P and affective manipulation has led to successful clinical trials of NK1 receptor antagonists for the treatment of anxiety and depression (Kramer et al., 1998; Kramer et al., 2004).

1.8.4.3 Cytokines

Inflammation is emerging as a common mechanism of numerous diseases, including chronic pain and neuropsychiatric diseases (Figure 1.11). Cytokines play a key role in the post-injury pain response as well as the “sickness behaviour” that affects physical (hypersomnia, weakness, depressed appetite and activity) and psychological (malaise, listlessness, loss of interest in social activities) responses to illness (Kelley et al., 1997). Following nerve injury, pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α are induced in the nerve and DRG. These cytokines are vital for the generation of the hyperexcitability in DRG neuronal cell bodies and axons that leads
to peripheral sensitization and are strongly implicated in neuropathic pain (Ji et al., 2009; Kawasaki et al., 2008; Schafers et al., 2003; Sommer and Kress, 2004). IL-1β, IL-6 and TNF-α can stimulate the HPA-axis both independently as well as in synergy with each other, while increased HPA-axis activity can suppress virtually all components of the immune response (Tsigos and Chrousos, 2002).

IL-1β is a pro-inflammatory cytokine with links to both chronic pain and the HPA-axis. In vivo, IL-1β administration or stimulation of endogenous IL-1β (Bluthe et al., 1992) can induce illness behaviour and symptoms of depressive illness, as well as reducing pain threshold (Wilhelm et al., 2012). IL-1β activates CRH-expressing regions in the brainstem that are activated by stress and that show alterations in clinical depression (Linthorst et al., 1995; Molina-Holgado et al., 1998). Additionally, IL-1β increases brain monoamines, such as serotonin, that are implicated in stress and depression (Linthorst et al., 1995).

Other cytokines that prove to be consistent biomarkers of depression are IL-6 and TNF-α, which are consistently elevated in patients (Dowlati et al., 2010) and which show abnormal circadian rhythmicity and physiological complexity (Alesci et al., 2005). IL-6 and TNF-α levels in depressed patients have been shown to correlate with treatment outcome, where patients with higher levels of cytokines prior to antidepressant-treatment have poorer outcomes (Lanquillon et al., 2000).

Animal models of psychological stress exposure show increases in pro-inflammatory cytokines (e.g. IL-1β, TNF-α) in brain regions involved in emotional regulation (Madrigal et al., 2002; O’Connor et al., 2003; Raison et al., 2006), and the behavioural manifestations of the stress response are inhibited by cytokine antagonists (Pugh et al., 1999).

The link between stress, affective disorders and chronic pain is very interesting and shows great potential for improved pharmacological treatments with greater clinical utility, but is yet to be fully understood.
Figure 1.11. Schematic representation of the interactions between the hypothalamic-pituitary-adrenal axis and the immune system. Image taken from Chrousos & Gold (1992).
1.9 Validity of Animal Models of Affective State

Studies of affective disorders in humans have practical and ethical limitations. They require large amounts of time, funding and manpower, and are burdened by high participant dropout rates (Shumake and Gonzalez-Lima, 2003). It is therefore imperative that sound and reliable animal models are available for screening potential pharmaceutical interventions and to elucidate predisposing factors for these disorders.

Perhaps the greatest limitation of animal models of psychiatric/psychological symptoms is that these symptoms often require verbal report, e.g. feelings of worthlessness, and suicidal thoughts in clinical depression. A method to circumvent this problem is to model core clinical symptoms that have observable, behavioural consequences. For example, the two core clinical symptoms of depression are anhedonia and depressed mood. While the mood of the animal cannot be measured, anhedonia is reliably simulated in animal models of depression.

Due to the complexity and heterogeneity of affective disorders, suggestions have been made to approach the diagnosis and treatment from discrete behavioural, physiological or neurochemical perspectives, as opposed to treating the entirety of the disorders (Cryan and Holmes, 2005). For example, anhedonia is a core symptom of depression which, as a measure of depression-like behaviour in rodents, has been established to have good construct validity in that sucrose intake correlates with sensitivity to reward (Papp et al., 1991; Sampson et al., 1992) and face validity, in its phenomenology and responsiveness to antidepressants (Muscat et al., 1992).

Concerns have arisen regarding the predictive validity of commonly used mouse models of depression and anxiety disorders. The most widely used animal tests of depression- (e.g. FST) and anxiety-like behaviour (EPM) grew in prominence based on their sensitivity to classic treatments (monoamine inhibitors and
benzodiazepines respectively), but may be less sensitive to newer generation treatments with novel mechanisms of action (Cryan and Holmes, 2005). In the same way that human affective disorders present as a spectrum of symptoms (e.g. depressed patients can show hypo- or hyperactivity according to DSM-IV), animal models of these disorders can show a range of behaviours that are strain, model, and test dependent. Thus it is advised to use more than one behavioural test before drawing definitive conclusions about treatment or genetic effects in any particular behavioural domain (Crawley, 2007; Mineur et al., 2006). Additionally, methods of obtaining valid internal controls are now known for depression-like behaviour in mice (Strekalova et al., 2011) that aptly reflect the pathogenesis of this disorder across the human population.
1.10 PROJECT GOALS & SIGNIFICANCE

The primary aims of this project were to determine the role of the α9α10-nAChR in pain, and to determine the value of the receptor as an analgesic target. A mouse strain with germline deletion of the α9-nAChR was utilised to this end, allowing characterisation of the functional role of this receptor at the organism level, both behaviourally and physiologically.

The specific goals of this project were to:

**Characterise the pain phenotype of α9-nAChR knockout mice compared with wildtype counterparts (Chapter 3).** A battery of behavioural pain testing was performed comparing WT and KO mice in both the naïve state and following the induction of inflammatory and neuropathic pain models. Numerous pain modalities were examined and the development and maintenance of chronic pain was compared between the two genotypes.

**Determine whether the α9α10-nAChR is involved in the immune and inflammatory responses to sciatic nerve injury (Chapter 4).** Histological examination of the pathology caused by a neuropathic pain model was compared in WT and KO mice. The reported role of the α9α10-nAChR in the development of the pathology was explored.

**Investigate the necessity of the α9α10-nAChR for the efficacy of specific analgesic α-conotoxins (Chapter 5).** The efficacy of analgesic α-conotoxins such as Vc1.1 and RgIA has controversially been attributed to α9α10-nAChR-inhibition. Whether these conotoxins function in α9-nAChR KO mice was investigated.

**Characterise behavioural and physiological effects of α9α10-nAChR deletion that may indicate potential side effect risks for α9α10-nAChR-inhibiting pharmacological agents (Chapters 6 and 7).** The functional
importance of the α9α10-nAChR in the HPA-axis was investigated behaviourally and physiologically using commonly assessed parameters such as anhedonia, arousal, anxiety-like behaviour, and the corticosterone stress-response.

The significance of this work is in the resolution of controversies regarding the validity of pursuing α9α10-nAChR-inhibitors as acute analgesics. The necessity for functional α9α10-nAChRs for the efficacy of analgesic α-conotoxins such as V1.1 and RglA is refuted. Furthermore, potential side effects of α9α10-nAChR-inhibition are identified for the first time.
CHAPTER 2

GENERAL

MATERIALS & METHODS
2.1 ANIMALS

All behavioural experiments were performed on mice, strictly adhering to the guidelines for the ethical use of animals for scientific purposes.

2.1.1 ETHICS APPROVAL

All animals used in these experiments were housed and maintained in accordance with the National Health and Medical Research Council (NHMRC) Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013) and all experiments were approved by the University of Sydney Animal Ethics Committee (AEC, ethics protocol number K00/12-11/3/5650).

2.1.2 MICE

Adult male C57Bl/6j or 129Sv/Ev mice aged 7-13 weeks were used. Animals were housed in groups of no more than 6 littermates, in Mouse Individually Ventilated Cages (IVC, Sealsafe Plus Green Line, Techniplast) with Smart Flow Air Handling Units (Techniplast), lined with corncob bedding. Mice were maintained on a 12:12 hour light:dark cycle (lights on at 6am), ambient temperature was maintained at 21 ± 2°C and relative humidity at 50 ± 5%. Standard rodent chow (Irradiated rat and mouse; Specialty Feeds, WA) and filtered water was available ad libitum in cages.

C57Bl/6j mice were ordered from Animal Resources Centre (ARC, WA, Australia) at 6 weeks of age on arrival. Out-bred animals were allowed 1 week to acclimatise prior to starting experiments.

129Sv/Ev mice were bred in-house. Litters were weaned and sexed at 4 weeks, and females sacrificed. Mating pairs were retired after 10 litters.

α9-nACHR KO (Chrna9<sup>-/-</sup>) mice (Vetter et al., 1999)) with a 129Sv/Ev background and their wild-type littermates were kindly provided by Douglas R. Vetter.
Heterozygous mice were used as breeding pairs, and genotypes were confirmed by PCR genotyping (see Section 2.1.3). KO mice had been maintained in the Vetter laboratory via backcrossing to F6 generation, and further backcrossed for two generations in our laboratory. Homozygous wildtype-controls were bred separately from homozygous knockout mice.

**2.1.3 GENOTYPING OF α9-\(\alpha\)ChR KO MICE**

DNA preparation:
Mouse tail clippings (1-2mm) were mixed with 300 μL of 10% Chelex and 2.2 μL/mL of Proteinase K and vortexed. Tissue was incubated for at least 3 hours at 55° C. After incubation, samples were vortexed for 10 sec, and then heated to 95-100° C for 8-10 mins. Samples were vortexed for 10 sec and then spun on a bench-top centrifuge at top speed for 5 mins. The supernatant was then used for PCR.

PCR reaction mix:
1 μL DNA extraction; 8.2 μL H₂O, 0.8 μL of 10 μM Primer mix, 2 x 10 μL Biomix (Taq polymerase).

PCR primers:

| KO: 151 bp | Noe F: AGAAAGTATCCATCATGGC |
| Noe R: TCTTCGTCCAGATCATCC |
| WT: 481 bp | Nach9 8F: GTACGATGGGCCTCATCACC |
| NaCh9 8R: CAGAGGGACGTTTTCTGAGG |

**2.1.4 EUTHANASIA**

At the completion of all experiments, or if any individual animal demonstrated signs of illness or complications from the experimental procedures, animals were humanely sacrificed.

Euthanasia was by gradual carbon dioxide (CO₂) overdose through inhalation of over 70% CO₂ in oxygen. This is a commonly used method of euthanasia, as it has rapid anaesthetic effects (45-60 sec), which proceeds to respiratory arrest and
death after prolonged exposure (5-6 mins). To ensure successful euthanasia, following CO₂ inhalation, cervical dislocation was performed on unconscious mice. The skull and brain were separated from the spinal cord by applying anteriorly directed pressure to the base of the skull, while pulling the tail caudally.

Euthanasia was confirmed by cessation of respiration and heartbeat, absence of palpebral and toe-pinch reflexes, and loss of colour in the eyes.
2.2 Anaesthesia and General Surgical Procedures

The induction of all pain models was performed using general anaesthesia, to minimise the suffering of the animals. All surgeries were performed under aseptic conditions to minimise the risk of post-surgical infections.

2.2.1 Anaesthetic

Anaesthesia was induced and maintained using isoflurane inhaled anaesthetic (Laser Animal Health, Pharmachem, QLD). Anaesthesia was maintained throughout all surgeries and minor procedures using an anaesthetic vaporiser breathing circuit (Tec 3, Key Fill, Advanced Anaesthesia Specialists, NSW), with the isoflurane adjusted to 2 - 2.5% in oxygen, and the flow rate at 0.2 L/min.

2.2.2 Induction and Recovery

The open-drop method of isoflurane delivery (Risling et al., 2012; Zardooz et al., 2010) was used to induce initial anaesthesia, as this allows rapid induction. Tissue soaked in 0.65 mL/L isoflurane was placed into the chamber and allowed to vaporise. Animals were removed one at a time from the home cage and placed in the anaesthetic induction chamber. Anaesthesia was considered induced when the animal lost its righting reflex. The anaesthetised animal was then rapidly removed from the induction chamber and placed prone, on the surgical bench and the snout placed into the nose-cone of the anaesthetic breathing circuit.

The animals' respiration rate and foot withdrawal reflexes were monitored until stable, deep anaesthesia was achieved. When foot withdrawal and corneal reflexes were lost, the animals were ready to commence surgical or minor procedures.

After the completion of the procedures, animals were transferred to a recovery chamber (a clean holding cage) until able to stand and ambulate, before being returned to their holding cages.
2.3 Pain models

It should be noted that the pain models used here produce sensory signs (allodynia and hyperalgesia) that are consistent with chronic pain states in humans and have strong therapeutic predictive validity but they do not produce ongoing distress, particularly in mice (Urban et al., 2011). Both neuropathic and inflammatory pain models were used (Figure 2.1).

2.3.1 Neuropathic pain

Each of the neuropathic pain surgeries used here are relatively rapid procedures, performed within 10-15 mins, which ensures minimal anaesthesia time. We have established that development of sensory signs of allodynia and hyperalgesia are maximal 7-14 days after injury.

2.3.1.1 General surgical procedures for sciatic nerve pain models

Once deep anaesthesia was confirmed, the hindquarter was shaved and the skin sterilised with a swab saturated with 70% ethanol and allowed to dry. A small incision was made into the skin with scissors at the approximate level of the sciatic nerve, and the incision was widened further by blunt dissection. The dermis was further separated from the underlying muscle by blunt dissection of the connective tissue layers. The natural cavity between the biceps femoris and the gluteus superficialis muscles was exposed by blunt dissection of the connective tissue between the two muscles, within which the sciatic nerve is visible. A drop of sterile saline (0.9%) was placed into the cavity to prevent the exposed tissue and nerve from drying out. The nerve was isolated from surrounding fascia using fine forceps.

For detailed procedures of the various nerve injury models, see Sections 2.3.1.2, 2.3.1.3, and 2.3.1.4 below. Sham operated animals had their sciatic nerves exposed, without further intervention.
Upon completion of the injury or sham procedure, the surgical wound was closed with Vetbond (3M, North Ryde, NSW, Australia) tissue adhesive. Mice were administered an antibiotic (Amoxicillin, 150 mg/kg i.m.) and allowed to recover, before being returned to their home cage.

2.3.1.2 Partial Nerve Ligation
The partial nerve ligation (PNL) model of neuropathic pain was first described in 1990 (Seltzer et al., 1990) and has been used as a standard procedure in pain research worldwide. The surgery was developed to model a partial nerve injury, which was described as the main cause of causalgiform pain disorders in humans (Seltzer et al., 1990).

In the PNL model, approximately 1/3 to 1/2 of the dorsal sciatic nerve was ligated with a size 6-0 silk suture (6-0 sterile Silk, Johnson & Johnson Medical Pty. Ltd., Australia), proximal to the sciatic nerve trifurcation. The suture was tied tightly, trapping that portion of the sciatic nerve, loose ends of the suture trimmed, and the wound was closed.

2.3.1.3 Chronic Constriction Injury
The chronic constriction injury (CCI) model of neuropathic pain was first developed by Bennett and Xie (1988), which has many features characteristic of human neuropathic pain and provides different behavioural outcomes to the PNL model (Basbaum et al., 1991).

In the CCI model, 3 loosely constrictive chromic catgut (6-0 sterile chromic gut suture, Johnson & Johnson Medical Pty. Ltd., Australia) ligatures were placed around the common sciatic nerve, causing a slight constriction to the entire nerve. The first ligature was placed above the first bifurcation in the nerve. The ligatures were tight enough to visually constrict the nerve, but without occluding the blood flow through the superficial epineurial vasculature. Two more ligatures were tied above the first, at 1 mm intervals, with a maximum of 5 mm
length of nerve covered by all 3 ligatures. Loose ends of the catgut were trimmed, leaving enough length to avoid unravelling of the knots.

2.3.1.4 Sciatic Nerve Cuff variant of the CCI model
A further modified injury model was implemented that was adapted from Mosconi & Kruger (1996) and Bendouzid et al (2008). This injury model produced a chronic constriction of the nerve that was uniform across the nerve and was standardised for each subject (see Discussion of models in Section 1.7.2).

Polyethylene cuffs (PE-20 polyethylene tubing, Clay Adams® BD & Co. Maryland; inner diameter of 0.38 mm and outer diameter of 1.09 mm) were precisely cut to 2mm in length and split longitudinally. Immediately prior to use, cuffs were sterilised in 70% ethanol, allowed to dry and rinsed in sterile saline (0.9%). Cuffs were placed around the same region of the common arm of the sciatic nerve as the PNL and CCI injuries. Cuffs were gently pressed with toothed forceps to ensure the split was closed.

The cuff neuropathic pain model does not produce a thermal hyperalgesia (see hotplate results; Section 3.3.2), and thus models a pure neuropathic pain phenotype, rather than a mixed neuropathic-inflammatory pain model, which is reflective of clinical populations with nerve injury (Jorum et al., 2003). Since the cuff model did not induce thermal pain, the CFA model was used to ensure that any involvement of the α9α10-nAChR in inflammatory pain was thoroughly investigated.

2.3.2 Inflammatory pain
The inflammatory pain model used here takes less than 5 mins after induction of anaesthesia with animals recovering within minutes. We have established that sensory allodynia and hyperalgesia are maximal 3 days after induction of inflammation, which wanes after 7 days. Experiments are therefore performed 3-4 days after pain induction.
2.3.2.1 Complete Freund’s adjuvant

Once deep anaesthesia was confirmed the plantar surface of the left hind paw was swabbed with ethanol (70%), and allowed to dry. A 29 G needle was inserted into the distal region of the plantar surface of the paw, directing it toward the heel. The translucent dermis allowed the needle to be visible through the skin. Taking care to keep the needle horizontal, and avoiding contact with underlying anatomy, the needle was pushed approximately 1 cm beyond the insertion point, approaching the vicinity of the heel. 50 μL of complete Freund’s adjuvant (CFA) was then injected sub-dermally. Time was given for the CFA to disperse, before slowly retracting the needle. The foot and bench were wiped clean with ethanol after each injection.

Sham animals underwent the same procedure, but were injected with 50 μL saline, instead of CFA.

Animals were allowed to recover from anaesthesia in isolation while being monitored, before being returned to their home cage.
Figure 2.1. Chronic neuropathic pain models of the sciatic nerve in the mouse. A| The sciatic nerve is accessed via the natural cavity between the biceps femoris and the gluteus superficialis muscles. B| Nerve injury is induced via silk sutures (PNL), chromic catgut (CCI) or polyethylene cuff (Cuff). Sham nerves do not undergo any injury.
2.4 **BEHAVIOURAL PAIN TESTING**

A battery of pain tests was used to determine the pain phenotype of the animals. Tests were chosen to specifically measure differences in pain thresholds, examining non-evoked responses, as well as evoked responses to non-noxious and noxious stimuli in mechanical and thermal pain modalities.

2.4.1 **HABITUATION AND HANDLING**

As described in Section 2.1.2, animals were housed in IVC cages, in which the circulating air differs significantly from the atmosphere in the testing environment, and which dampen light and sounds (Hawkins *et al.*, 2003). These cages are primarily designed for optimal health of animal colonies, by providing non-contaminated, consistent airflow to each cage (Kemppinen *et al.*, 2008). However the contrasting environments of internal home-cage and external test settings necessitates considerable habituation to testing conditions.

In order to avoid unfamiliar sensory stimuli such as odours, light and sounds that might be present in the testing environment from confounding experimental results, mice were habituated in the testing room prior to testing. Habituation was for 8 hours per day for 2 days prior to first handling, and for 3 subsequent days during which mice were handled. To acclimatise mice to the testing environment, the ventilation filters were removed from the animals' home cages while the cages remained closed, thus allowing ambient air, light and sounds to suffuse the internal cage, without removal of the animals from the familiar environment.

Mice were handled for at least 3 days prior to testing. Animals that had been habituated to the testing room were removed from the cage one at a time, and handled with gloved hands in approximately 10 minute bouts, until the mice appeared comfortable with the handler. Habituation to the experimenter and handling was judged by absence of escape behaviour, decreased exploratory behaviour and increased resting and grooming while on the experimenters'
gloved hands. For experiments requiring scruffing, the scruff of the neck was manipulated until the animal tolerated a gentle grip. This was gradually increased to a full scruff, which was repeated until the mouse was at relative ease with the procedure.

**2.4.2 MEASUREMENT OF HOT AND COLD NOCICEPTION**

Increased sensitivity to extreme temperatures is a hallmark of neuropathic and inflammatory pain. Both alldynia and hyperalgesia may be observed in response to changes in temperature.

**2.4.2.1 HOTPLATE TEST**

Thermal nociception and hyperalgesia were tested using the hotplate test. The hotplate (IITC Life Sciences) consists of a square plate (275 mm x 263 mm x 15 mm), upon which an open-topped clear Perspex chamber (diameter: 10 cm, height: 15 cm) is placed to limit ambulation. The hotplate was allowed to warm to the desired noxious temperature (range tested was 52-58 ± 0.2° C). With minimal animal-handler interaction, mice were taken from their home-cages and placed onto the hotplate within the Perspex chamber, and the timer was started via foot-pedal. The animals’ behaviour was closely observed through the sides of the Perspex chamber, and the foot-pedal timer stopped when the first nocifensive behavioural endpoint was exhibited. Endpoints that terminated the test were shaking or licking of the hind-paws, or attempted jumping from the chamber. The animal was removed from the hotplate immediately, upon displaying nocifensive behaviour.

**2.4.2.2 ACETONE TEST**

Cold alldynia was assessed by measuring the acute nocifensive responses to acetone-evoked evaporative cooling. For two days prior to testing, and on testing days, mice were habituated in testing chambers. On test day, mice were placed onto fine (1 mm) wire mesh flooring, raised 50 cm off the lab-bench, and confined to an opaque cylindrical chamber (8 x 15 cm). To test, 10 µL of acetone
(analytical grade, Bacto laboratories) was propelled onto the plantar surface of the left hind-paw. The air burst from a 100 μL pipette was used to project the acetone, thus avoiding mechanical stimulation of the paw with the pipette tip. The time spent lifting, licking or shaking the hind-paw over a 60 second time period was recorded using a hand-held stop-watch. Acetone was applied 3 times with at least 5 minute intervals and the average of the three scores was used.
Figure 2.2. Hot and cold pain threshold tests. A| Thermal hyperalgesia tested on the hotplate test. B| Cold alldynia tested with the acetone test.
2.4.3 Measurement of Mechanical Allodynia

Mechanical allodynia is a common complaint in chronic pain disorders of widely varying aetiologies. Allodynia is evoked by normally non-noxious stimuli, such as light touch or brushing of the skin, for example by clothing.

2.4.3.1 Mechanical Incapacitance Test

Differences in static weight distribution across hind paws was assessed using a dual channel weight average, the Linton Incapacitance Tester (MJS Technology Ltd., Herfordshire, UK), as described previously in rats (Clayton et al., 1997) and mice (Inglis et al., 2008). Mice were placed at the opening of a transparent acrylic chamber, which they freely enter. The chamber design encourages an upright posture, with the majority of weight placed on the hind-paws, and forepaws resting in an inclined plane, for balance and support. The floor of the chamber is split equally into two electronic weighing scales, and measurements are taken when each hind-paw rests symmetrically on each scale. The average of six measurements were taken and represented as a ratio of ipsilateral (left hind-paw) to contralateral (right hind-paw) weight distribution. Decreased ratio indicates less weight placed on the injured hind-paw. This decreased weight bearing is considered to indicate mechanical allodynia in the present experiments, although it should be noted that this behaviour may indicate more complex processes. The Incapacitance test has been validated as a test with less subjectivity and variability than other tests of clinically relevant pain (Clayton et al., 1997).

2.4.3.2 Von Frey Test

The von Frey test was used to assess the development of mechanical allodynia. For 2 days prior to testing and on testing days, mice were habituated in testing chambers for 1 hour. In order to determine mechanical alldynia, a calibrated von Frey filament (Stoelting Co. Chicago, IL) was pressed perpendicularly to the plantar surface of both ipsilateral and contralateral mouse hind-paws, until the filament just buckled. The stimulus was applied to the central part of the footpad, avoiding the protrusions. A single grade of filament of 3.61 g (0.4 mN) was
chosen, that elicited mean response frequencies of approximately 15% at baseline (Bortalanza et al., 2002). The filament was applied 10 times for a duration of 5 seconds, with an interval of at least 5 mins between each stimulation. Responses of brisk withdrawal (flinches, vigorous shaking/kicking) and licking were considered as positive nociceptive responses. Scores are represented as a percentage of positive responses.
Figure 2.3. **Non-noxious mechanical threshold tests.**

A | Altered weight bearing tested on the Incapacitance test.  

B | Tactile allodynia tested with the von Frey test.
2.4.4 Measurement of mechanical hyperalgesia

Hyperalgesia is mechanistically distinct from allodynia, and results in an exaggerated pain response to a normally noxious stimulus.

2.4.4.1 Paw pressure test

Mechanical nociceptive thresholds were measured using a Pressure Application Measurement (PAM) analgesymeter with Paw Pressure Transducer (Ugo Basile). Analogous to the Randall-Siletto test for rats (Randall and Selitto, 1957), mice were gently restrained and a mechanical pressure was applied to their left hind-paw. The plantar surface was supported against the flat base of the paw pressure transducer, while the blunt conical probe was pressed against the dorsal surface at a linearly increasing force (maximum 600 g). The PAM device automatically recorded the force at which paw withdrawal is elicited. The peak forces of 3 trials were averaged for each mouse, with at least 5 min inter-trial interval.
Figure 2.4. Noxious mechanical threshold test. Mechanical hyperalgesia tested on the PAM paw pressure test.
2.5 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Differences in injured sciatic nerve morphology and immune cell infiltration of α9-nAChR KO mice compared to WT mice was investigated using histology and immunohistochemistry.

2.5.1. ANTIBODIES & REAGENTS

Antibodies against T-cells (CD3ζ positive) and acetylcholine-producing (ChAT positive) cells were used.

Primary antibodies:
Alexa Fluor® 488 anti-mouse CD3ε (100321, BioLegend, San Diego, CA)
Anti-ChAT antibody (ab144P, Merck Millipore, Darmastast, Germany)

Secondary antibody:
Anti goat (rabbit) Alexa Fluor 650

For haematoxylin and eosin (H & E) staining, reagents used were Scott’s bluing reagent, acid alcohol (Kinetik Pty Ltd, Qld, Australia); Haematoxylin, ethanol, xylene and eosin (1% alcohol) (Point of Care Diagnostic Healthcare, NSW, Australia); and Pertex ® (Grale HDS, NSW, Australia).

For immunohistochemistry:

**PBS (1x)** (0.1M, pH 7.2-7.4): NaH₂PO₄.2H₂O 3.95 g, Na₂HPO₄ 15.36 g, NaCl 9 g in 1 L distilled H₂O.

**Fix:** 1 L 1x PBS + 40 g paraformaldehyde. NaOH was added to help dissolve the paraformaldehyde and the solution was heated to a maximum of 60° C, stirring. The fix was cooled to room temperature, filtered using a vacuum pump and pH adjusted to 7.4.

**Flush:** NaCl 9 g, NaNO₂ 5 g, heparin 3000 i.u. in 1 L distilled water.
2.5.2. TISSUE DISSECTION, FIXATION & SECTIONING

2.5.2.1 CARDIAC PERFUSION & TISSUE PREPARATION

Perfusions were performed in a lamina-flow fume hood. Tubing, comprising of three arms connected with a three-way tap (3-way Luer stopcock, Cole-Parmer, John Morris Scientific Pty Ltd., Sydney, Australia), was assembled. Two short arms were each placed into the fix and flush solutions, and a long arm was passed through a peristaltic pump (Gilson Miniplus 3, John Morris Scientific Pty Ltd., Sydney, Australia), and fitted at the end with a 1 mL syringe and 23 G x 1¼’’ needle. The tubes were prepared for perfusions by first passing fix through, until tubes were filled and free of air bubbles. With the pump continuing, the three-way tap was quickly turned so that flush passed through, replacing any fix in the tube distal to the tap. Air bubbles were again removed. Flow rate was adjusted to approximately 90 mL/hour.

Animals were anaesthetised using isoflurane (see open drop-method in section 2.2.2). Once the righting reflex was lost, animals were injected intraperitoneally with 5 mg/kg of Lethabar (pentobarbitone sodium, Virbac Animal Health; approximately 100 μL diluted 2:10 in saline). Animals were placed singly in a cage until toe-pinch and ocular reflexes were lost.

Anaesthetised animals were placed supine onto a Styrofoam block that was inclined to allow for drainage, to which the four limbs were secured using needles. The abdominal skin was cut and pulled back to reveal the thoracic and peritoneal membrane surfaces. Using forceps to hold the xiphoid process of the sternum, scissors were used to cut the peritoneal wall separating diaphragm from abdominal cavity and opening up the ribcage on lateral sides, revealing viscera and exposing the heart. The reflected ribcage was pinned to the Styrofoam so as to prevent the cardiac cavity from being obscured. In rapid succession, the right atrium was cut and, with the flush flowing, the perfusion needle was inserted into the bottom of the left ventricle. Flush was infused until the fluid exiting the right atrium ran clear (10 – 25 mL). The clarity of the liver gave a good indication of the quality of the perfusion, with the organ completely
blanching in a good perfusion. The three-way tap was then turned to run fix through, until the animal became stiff (50 - 100 mL). The liver again gave a good indication of fixation, with a fixed liver becoming firm to the touch. The pump flow-rate was decreased as the fixation progressed, which prevented the rise in pressure due to the stiffening vessels from rupturing capillaries and damaging tissue.

Upon fixation, the pump was stopped, needle removed from the heart, and the mice were removed from the Styrofoam mount. Mice were placed prone onto bench protector and the sciatic nerves were dissected out. The skin was cut to expose the leg musculature. The natural cavity between the biceps femoris and gluteus superficialis muscles was opened by blunt dissection of the connective tissue between the two muscles, to expose the sciatic nerve. A drop of fix was placed into the cavity to avoid drying out of the tissue and to assist fixation. Sciatic nerves were separated from the underlying tissue using fine forceps and dissected out, cutting as proximally as possible at the proximal end and distal to the post-femoral trifurcation at the distal end.

**2.5.2.2 Post-fix, cryoprotection & freezing**

The dissected nerves were placed into fix and post-fixed for 1 hour at room temperature, followed by a rinse in phosphate buffered saline (PBS). Under a dissection microscope, cuffs were removed from relevant nerves, and fat and connective tissue was conservatively removed, avoiding any distortion or damage to the nerves. Nerves were then cryoprotected by placing in 30% sucrose in PBS overnight at 4° C.

Following post-fixation and cryoprotection, sciatic nerves were frozen, ready for sectioning. In order to snap-freeze the nerves as straight as possible and in parallel orientation, foil cubic moulds (approximately 15 mm x 12 mm x 5 mm) were made. The foil moulds were labelled on the base with the specimen identification, and on one wall to denote the orientation. A small streak of super-glue was spread along one long edge of the base of the mould. Fixed sciatic
nerves were placed one-at-a-time in the mould, with the proximal tip of the nerve secured into place by the super-glue. Once all of the nerves were secured by their proximal ends, any residual liquid was mopped from the mould using the corner of a Kimwipe and super-glue was streaked on the opposite long-edge of the mould. The trifurcated distal ends of the nerves were glued to the mould ensuring the trunk of the nerve was as straight as possible. The mould was then filled with O.C.T. Compound embedding medium (Tissue-Tek®, ProSciTech, Thuringowa, QLD). The mould was placed into a receptacle that was lowered over liquid nitrogen, snap-freezing the tissue. Frozen blocks of tissue were stored at -80°C until ready to section.

2.5.2.3 Cryostat sectioning & mounting
Frozen tissue specimens were stored at -20°C for two days prior to sectioning. On the day of sectioning, nerves were placed into the cryostat (CM1520, Leica Biosystems, Sydney, Australia) for 30 mins prior to sectioning. The foil was peeled away from the O.C.T. block, and mounted to the cog using a small amount of O.C.T. medium. The block was sectioned longitudinally at a thickness of 15 μm. Sections were aligned on the cryostat stage and directly mounted onto Superfrost Plus slides (Menzel-Gläzer, Grale HDS, Ringwood, VIC) that had a permanent positive charged surface. Slides were stored at -20°C until staining.

2.5.3 Staining
2.5.3.1 Haematoxylin & eosin staining
Mounted sections were washed in PBS 3 times for 10 mins each, and dried in an oven for 5 mins at 60°C. Dry slides were placed in a staining rack and stained in haematoxylin (POCD healthcare, NSW, Australia) for 2 mins. Slides were then washed in deionised water until excess dye was washed out, de-colourised in acid alcohol (Kinetik Pty Ltd, Qld, Australia) for approximately 5 sec, and again washed in deionised water. Slides were dipped into blueing solution (Scott’s bluing reagent, Kinetik Pty Ltd, Qld, Australia) for 30 sec, briefly washed in deionised water, briefly washed in 95% alcohol, and then stained in 1% eosin.
(1% alcoholic eosin, POCD healthcare, NSW, Australia) for 2 mins. Sections were dehydrated in 100% ethanol three times for 1 min each, and then cleared in xylene (POCD healthcare, NSW, Australia) two times for 2 mins each. Slides were coverslipped with Pertex® (Histolab, Grale HDS, NSW, Australia) in a fume hood.

2.5.3.2 IMMUNOHISTOCHEMICAL STAINING

Mounted sections were washed in PBS 3 times for 10 mins each. A barrier was then drawn around the perimeter of the slide with a PAP pen (ADI-950-233-0001, Enzo Life Sciences, NY), encircling the sections. Slides were laid flat and blocking medium (10% normal horse serum and 0.3% triton-X 100 (Sigma-Aldrich Co. LLC) in PBS) was gently pipetted into the enclosed section. Sections were blocked for 30 - 60 mins. The blocking medium was discarded, and replaced with (labelled) primary antibody, diluted in PBS with 0.4% triton-X 100. To ensure the antibody did not evaporate over the duration of incubation, the slides were placed on an elevated platform inside a sealed box, with damp paper towels lining the bottom. Slides were concealed from light and remained on the lab bench overnight, at room temperature. After primary antibody staining, slides were wash 3 times for 10 mins each. Where un-labelled primary antibody was used, secondary antibody was applied for 90 mins, protected from light, at room temperature. Slides were washed 3 times for 10 mins each. Finally, slides were dipped into 50:50 PBS:dH2O, and allowed to dry, flat at room temperature, protected from light.

To coverslip the slides, 100 µL VECTASHIELD® Mounting Medium with DAPI (H-1200, Vector Laboratories, CA) was placed across the slide, in approximately 4 drops of 25 µL each, and the coverslip was gently placed over the mounting medium. Coverslips were permanently sealed around the perimeter with nail polish. Slides were stored at 4°C until imaging.
2.5.4 Imaging

H&E staining was imaged on an upright light microscope (Olympus BX50) in brightfield. Sections were imaged at 4x, 10x and 20x objective magnifications using Qcapture Imaging Software.

Fluorescence images were scanned on a confocal microscope (Olympus FV1000) and imaged using Fluoview Viewer software (FV10-ASW 3.1).

For T-cell imaging: 2 channels were used: Channel 1 = 405 laser for DAPI at 10% with HV = 450 v, Gain at 1x, offset at 6%. Channel 2 = 473 laser for A488 at 10% with HV = 580 v, Gain at 1x, and offset at 18%.

Stacks of 29 images, with a step size of 0.5 μm was used.

For ChAT-immunoreactivity, 2 channels were used: Channel 1 = 405 laser for DAPI at 10% with HV = 495 v, Gain at 1x, offset at 10%. Channel 3 = 650 laser for A647 at 20% with HV = 700 v, Gain at 1x, and offset at 10%.

Stacks of 15 images with a step size of 1 μm was used.

2.5.5 Quantification

Quantification was performed using ImageJ software (ImageJ 1.43u, National Institutes of Health, USA). The Polygon Selection tool was used to create a fixed-area region of interest (ROI) on stacked images of each nerve. The ROI was drawn around the portion of intact nerve just proximal to the cuff, beginning at the proximal side of the depression made by the cuff. Depressed areas were not included. The ROI continued along the perimeter of the nerve, forming a polygon that extended along the length of the nerve as far as a fixed area of 300 000 units² allowed. Quantification was performed by the experimenter who was not blinded.

2.5.5.1 Cell density

The number of CD3ξ- and ChAT-immunopositive cells within the sciatic nerve was counted using the Multi-Point Selection tool. The number of cells in the fixed-area ROI was averaged across groups to give mean cell densities.
2.5.5.2 Oedema

The extent of oedema was calculated according to the length of the ROIs created (see Section 2.5.5 above). The Straight Line Selection tool was used to measure the length of nerve contained in the ROI. The line was created along the midline of the nerve, meeting the ROI perimeter at either end. Since the ROIs were of a fixed area, larger diameter nerves had shorter ROIs, while ROIs of narrower nerves were able to span longer lengths. Thus, the lengths of the ROI were inversely proportional to the diameter of the nerves. To avoid potential confusion of decreasing lengths denoting increased oedema and vice versa, the inverse length of ROIs was reported, as this was proportional to the extent of oedema.
2.6 Drug Administration

Due to the potential instability, susceptibility to enzymatic degradation and unknown intestinal absorption of the peptide compounds of interest here, enteral administration was not possible. Mice were therefore administered either drug or vehicle via injection using hypodermic needle (Figure 2.5). Prior to injections, animals were handled and scuffed multiple times to minimise any anxiety or distress caused by the procedure.

Insulin syringes were used to eliminate wastage of drugs due to dead space in needle tips. 0.5 mL syringes with 29 G needles were used, with injection volume determined by injection site. A new syringe and needle was used for each animal, which reduces discomfort to the animal due to blunting of needles, and reduces the risk of infections at the injection site. Isotonic saline was used as vehicle to avoid irritation to the injection site.

Sterilisation of the injection site is largely ineffective and unnecessarily prolongs the duration of restraint, so was not used.

All drugs were prepared as close to the time of testing as possible.

2.6.1 Scruffing

Animals that had been acclimatised to the testing room and handler were gradually introduced to scruffing. To scruff, mice were placed onto a clean lab gown, providing a soft surface to grip, as well as darkened folds to hide in. With one hand, mice were held securely by the tail, which was gently pulled back to encourage forepaw toe-grip of the gown and elongation of the animals’ torso. Gentle pressure was applied to the dorsal side of the animal and the skin of the nape of the neck was gathered with the free hand, using thumb and the outside edge of the forefinger. Once a grip was attained, fingers were repositioned to a grip using the finger pads of the thumb and forefinger. The animal was raised off the gown, using one hand to lift the scruff and the other to lift the tail, and the tail was tucked under the 5th finger of the hand gripping the scruff. This grip allowed
full restraint of the animal, and complete access to the scruff and ventral side of the animal.

2.6.2 Sub-cutaneous injections

Sub-cutaneous (s.c.) administration of solutions causes minimal pain and discomfort and is easy to perform.

The animal was restrained securely by the scruff-method, and the needle was inserted into the loose skin over the interscapular area, directing the needle to the base of the tented skin to avoid inadvertent injury to fingers with the needle. The needle was advanced several millimetres through the subcutaneous tissue to minimise leakage, before depressing the plunger. The needle was slowly removed and animal returned to the holding cage.

A volume of 200 µL was injected.

2.6.3 Intra-peritoneal injections

Intra-peritoneal (i.p.) injections are easy to employ and allows rapid absorption, due to the large surface area and abundant blood supply.

The animal was restrained securely by the scruff-method, and the animal held in supine position. The needle was inserted into the lower quadrant of the abdomen at an angle of approximately 10° to the abdominal surface (Shimizu, 2004). The needle was advanced several millimetres through the subcutaneous tissue before being inserted into the abdominal wall, in order to avoid puncturing viscera. The syringe was briefly aspirated to ensure correct placement of the needle, before depressing the plunger. The needle was slowly removed and animal returned to the holding cage.

A volume of 200 µL was injected.
2.6.4 Intra-muscular injections

Intra-muscular (i.m.) injections are not recommended in mice, due to the small capacity of the musculature. If necessary, volumes of < 0.05 mL may be used (Shimizu, 2004).

Mice were restrained by the scruff method, however, instead of securing the tail with the 5th finger, the foot of the leg to be injected was secured between the 5th finger and lower thumb. It is imperative that the animal is securely restrained and unable to move or kick, as this risks injury to the animal or experimenter.

The needle was inserted, bevel up, into the vastus lateralis muscle, pointing the needle caudally rather than cranially, to avoid puncturing the sciatic nerve. The needle was inserted at an angle 45° to the thigh, aspirated to ensure a blood vessel was not punctured, and the plunger slowly depressed at approximately 10 µL/sec. The needle was slowly removed and animal returned to the holding cage. A volume of 50 µL was injected, which is the maximum recommended volume for IM injections in mice (Shimizu, 2004).
Figure 2.5. Routes of drug administration in the mouse. The mouse is restrained with one hand (A) and drugs administered via hypodermic needle with the other hand via sub-cutaneous (B), intra-peritoneal (C) or intra-muscular (D) routes.
2.7 INTELLICAGE

The IntelliCage (NewBehaviour, Switzerland) is an automated monitoring system that can be used to study cognitive and learning behaviours in laboratory mice over an extended period of time. Animals are group-housed in the IntelliCage and electronic recording units in each of the cage corners monitor natural and learned behaviours of individual mice. Animals access drinking bottles in each corner, so that the need for water is the principle motivator for the observed behaviours (Figure 2.6).

2.7.1 THE INTELLICAGE SYSTEM

The IntelliCage was used to determine any phenotypic differences between α9-nAChR KO mice and WT mice. Both natural behaviour (circadian rhythms, general activity) and cognitive behaviour (learning, aversion, hedonic preference) were investigated.

2.7.1.1 CAGE COMPOSITION AND SOFTWARE

Each IntelliCage comprised of a large home-cage (207 (h) x 579 x 417 mm), containing corncob bedding, one large food hopper and a tetragonal shelter. Standard rodent chow (Specialty Feeds, WA) was available ad libitum. The cage had 4 “intelligent corners” which were identical operant conditioning corners that held the drinking bottles, and accommodated only one mouse at a time (Figure 2.7). The cage had sensors to monitor temperature and illumination over the course of the experiment, ensuring consistency in the testing environment within and across experiments. A circuitous network of tubes connected air-puff valves in the corners with a pressurised air supply.

A controlling computer automatically monitored, recorded and stored all behavioural data. Cages were connected in series to the controller, fitted with IntelliCage Plus Software. The software consisted of three parts: Designer, Controller, and Analyzer.
The Designer software was used to define protocols used in the experiments. Initially, hardware settings were specified (see Appendix A). Animal lists were created, matching the transponder numbers with the genotypes and groups. Finally, the experimental design was created and specific modules were assigned to the animals.

The Controller software was used for general monitoring of the progress of the experiments, checking that adequate drinking and locomotor behaviour was present and sustained for all animals, and to assign the correct modules when appropriate.

The Analyzer software was used to filter the data output, bin the output by specified time duration, and export desired data into Excel for more thorough analysis.
Figure 2.6. The IntelliCage system. A large home-cage monitoring system, equipped with four “intelligent” corners that monitor the activity and drinking behaviour of each individual mouse. A central housing dome is placed centrally. Up to 16 animals may be group-housed, for observation of naturalistic home-cage behaviour.
2.7.1.2 Intelligent corner composition

Each “intelligent corner” allowed for operant conditioning, with water as the reward. The behaviour of the mice could be shaped according to individual reinforcement and conditioning protocols (Figure 2.7).

Each corner was equipped with detection devices that recorded information about Visits. An antenna recognised the individual mouse entering the corner and a temperature sensor confirmed the animals’ presence in the corner. Once in the corner, the animal was faced with two motorised doors (left and right) that controlled access to the two drinking bottles, and were triggered by Nosepokes on the respective side. The Nosepoke was a learned behaviour, defined as the insertion of the mouses’ snout into the aperture, across which a light beam passed. The Nosepoke broke the light beam and was recorded by the IntelliCage system. Multi-coloured LED lights above each door were used as conditional stimuli in choice discrimination tasks, and air-puff valves were used as negative reinforcement.

The IntelliCage was fitted with lickometer technology, and was able to detect with precision, the Licks, an index of drinking behaviour of each mouse. The lickometer accurately detected number and duration of tongue contacts with the tip of the water bottle. This tongue contact changed the capacitance between the nozzle and the surrounding metal support, which was detected by the system.
Figure 2.7. IntelliCage “intelligent” corners. A| Each intelligent corner is equipped with two drinking bottles that are accessed through doors that can be triggered by nosepoking behaviour. B| Each corner has the capacity to hold one mouse at a time.
2.7.1.3 RFID transponder implantation

The radio-frequency identification (RFID) transponders employing electronic tagging technology were used. This system facilitated data transfer between transponder and a reader (antenna) built into the intelligent corner. The RFID transponder was implanted subcutaneously, and allowed each individual animal's behaviour to be profiled, while in the group-housed environment.

To implant the RFID transponder, mice were lightly anaesthetised using inhaled isoflurane anaesthetic in an induction chamber set at 2% in oxygen. When the righting-reflex was lost, animals were quickly removed from the chamber and laid prone on a sterile workbench. The scruff of the neck was gathered in one hand, causing a natural rise in the skin at 90° to the spine. The transponder, loaded in a sterile syringe and injection gun, was then implanted immediately below the operator's grip. The transponder must be injected towards the head of the animal and expelled between the scapulae. To ensure that the transponder retains its subcutaneous position, the skin was pinched while extracting the syringe. The transponder implantation procedure was performed in less than 5 mins.

Animals were kept under observation for at least 48 hours after transponder implantation. A hand held scanner was used to confirm that the transponder was held in its position prior to introduction of animals into the IntelliCage.

2.7.2 Experimental design

The IntelliCage is capable of housing up to 16 animals per cage. A number of regular holding cages were therefore combined per IntelliCage for the experiments. To allow the animals to adjust to the change in social environment and establish dominance hierarchies, mice were rehoused in rat-sized holding cages for 1 week prior to commencement of IntelliCage experiments. Mice were then introduced to the IntelliCage and the appropriate module started on the Controller software.
Cages were cleaned every 2 weeks. Drinking bottles were changed every few days. Great care was taken not to touch the sippers, so that any odour cues that may affect drinking behaviour were undisturbed, and the same sipper was always used in the same corner for the duration of the experiment.

The modules were progressively changed as follows:

Mice began on a Free Adaptation (FA) protocol, where the doors that permit access to the drinking bottles were open, and mice were free to explore the cage.

In the Nosepoke Adaptation (NPA) protocol the doors were closed, which forced mice to learn to Nosepoke to access the drinking bottles.

Drinking Session Adaptation (DSA) was introduced, where access to water was only permitted during selected hours of the day. This was to ensure that all relevant behaviours were being performed in a discrete timeframe.

The Sucrose Adaptation (SA) module introduced a palatable sucrose (10%) solution in the right hand side (RHS) bottle in each corner, while water remained on the left hand side (LHS). Sucrose preference was inferred by calculating the percent fluid intake that was of sucrose compared to the water bottles.

An Impulsivity (Imp) task was introduced that required sophisticated cognitive reasoning. Mice were required to Nosepoke twice on the RHS with incrementally increasing delays between first and second Nosepoke, in order to access sucrose. Water was available through usual Nosepoke on the LHS.

Sucrose Adaptation was repeated (SAII) to re-establish sucrose preference.

An Aversion task was also implemented that introduced an aversive air-puff upon drinking from the sucrose bottle. The extent to which the mice sought the preferable sucrose despite the aversive air-puff was recorded.
See Appendix B for experiment notes and detailed descriptions.

2.7.2.1 General activity & circadian rhythms
General home cage activity was observed in 129Sv/Ev mice of WT and α9-nAChR KO genotypes. The primary parameters recorded being Visits, Nosepokes and Licks. With the IntelliCage controller software set to FA, circadian rhythms were inferred from the number of Visits made per hour, indicating the average level of activity of each animal across time.

2.7.2.2 Socio-environmental effects of group housing
Due to the group-housing of the IntelliCage system, any phenotypic differences observed between WT and α9-nAChR KO mice are amenable to socio-environmental influences of cage-mates and intra-cage conditions. With this in mind the IntelliCage experiment was performed twice, with genotypes separate-housed (WTs in one cage, KOs in another) in Run1 and with genotypes mixed-housed (both WT and KOs housed in the same cage) in Run2. The sequence of modules for Run1 was FA, NPA, DSA, sucrose adaptation I (SAI), impulsivity I (ImpI), sucrose adaptation II (SAII), impulsivity II (ImpII), sucrose adaptation III (SAIII), Aversion. The sequence of modules for Run2 was FA, NPA, DSA, SAI, Imp, SAII, Aversion.

2.7.2.3 Sucrose preference
Hedonic discrimination was determined using the sucrose preference test. The preference for 10% sucrose over water was determined by calculating the percentage of Licks each animal made from sucrose bottles as a percentage of total Licks. Sucrose preference was tested after stressful changes in environmental predictability to identify any stress-induced anhedonia. Stress-induced anhedonia is known to develop in animals following chronic stress (Papp et al., 1991; Sampson et al., 1992; Willner et al., 1992).
2.7.2.4 Learning tasks: Impulsivity & Aversion

Potential phenotypic differences between WT and α9-nAChR KOs in their responses to cognitive and emotional challenges were assessed. For both tasks, a drastic change in the environmental reinforcement schedule was introduced. In the Impulsivity task, access to the rewarding, palatable sucrose was changed from simple, relatively low-cost access via a single Nosepoke, to a task requiring high cognitive cost for the same outcome. In the Aversion module, environmental predictability was again drastically changed, with the rewarding sucrose paired with an aversive air-puff.

2.7.3 IntelliCage for Greater Bench-to-Bedside Translation

Analysis of animal behaviour can be extremely difficult to standardise (Wahlsten et al., 2006). However, the IntelliCage has been shown to generate highly reproducible results between laboratories (Krackow et al., 2010). With more reproducible and reliable data, the number of animals required for each experiment may be reduced.

There are two key features of the IntelliCage that proffer advantage over other monitoring systems, or experimental designs. The first feature is the ability to collect data on group-housed animals, rather than individually housed, through the use of individual radio-frequency tags. Mice are social animals and are negatively impacted by isolation (Bartolomucci et al., 2003), which some monitoring systems employ, usually relying on detecting breaks in photobeams e.g. AccuScan, Digiscan (Tang and Sanford, 2005); PhenoTyper system, Noldus Information Tech. (Kas et al., 2008); DiLog Instruments (Goulding et al., 2008). It is therefore optimal for observations to be made during communal living. The second advantageous feature of the IntelliCage is the absence of experimenter handling, or disturbance to the animals (apart from cage cleaning) for the duration of the experiment. Handling is a stressor (Balcombe et al., 2004) that is difficult to avoid in traditional behavioural experiments, but that introduces artefacts and reduces reliability (Wolfer et al., 2012). The undisturbed group housing in the IntelliCage allows the collection of data that reflects natural,
undisturbed behaviours, the benefits of which are reflected in high reproducibility and inter-laboratory consistency (Lipp et al., 2005; Würbel et al.).

Automated home cage monitoring systems that allow group housing, such as the IntelliCage, offer more ethologically sound testing conditions than conventional behavioural tests. Rather than contrived, stressful testing conditions, where animals are separated to avoid inter-subject communication, behaviour in the IntelliCage better reflects real-world behaviours. Such systems can be used for observing spontaneous behaviour, spatial conditioning, operant conditioning, and taste preference/aversion tasks. Any impairment or enhancement of these behaviours arising from pharmacological agents, disease models or genetic mutations can be studied with greater translatability to humans that are rarely removed from social environments. IntelliCage data may, therefore, more accurately reflect the effects that experimental manipulations in mouse models will have on humans, and allow better experimental design and screening.

Hence, the continued use of this system may assist in development of more humane endpoints for scientific studies of mice, with greater bench-to-bedside translation.
2.8 STRESS & AFFECTIVE DISORDERS

The phenotypic effect of α9-nAChR subunit deletion on the stress response was investigated using established models of stress, and tests of depression- and anxiety-like behaviour. Corticosterone is the primary stress hormone in rodents and was used as a physiological marker for stress.

2.8.1 ELEVATED PLUS MAZE

The premise behind the elevated plus maze (EPM) is that environmental novelty evokes both fear and curiosity in rodents (Rodgers and Dalvi, 1997). The mice are thus in conflict between an innate curiosity and instinctual exploratory behaviour, and their aversion to exposed and brightly lit areas. The EPM is described as a psychogenic stressor (Anisman et al., 2001), due to exposure to the novel apparatus, and the conflict of interest.

The plus maze apparatus comprised of two open and two closed arms, which intersected in a central area. The arms being identical in all aspects but for the presence or absence of walls ensured that the same exploratory drive applied in each arm. The differentiating factor of the open arms engendering higher levels of fear compared to the closed arms allowed discrimination of different degrees of anxiety-like behaviour between test groups.

The maze was elevated to 42 cm high. Each of the arms were 30 x 5 cm, with a 5 x 5 cm central area, and the walls of the closed arms were 15 cm high. A camera was placed above the apparatus and recorded each run. The testing room was illuminated with overhead lighting that provided 100 lux for the closed arms and 400-550 lux for the open arms. The movements of the mice were automatically tracked using TopScan software (CleverSys, VA, USA). Animals started in the central area, facing an open arm and remained in the maze for 5 mins. The experimenter was not present in the room for the duration of testing.
Figure 2.8. The elevated plus maze (EPM). The EPM consists of two open arms and two closed arms. Exploratory behaviour of the animal is recorded by a camera positioned above the maze.
2.8.2 Forced swim test

The forced swim test (FST) is one of the most commonly used stressors, having a physical, psychological and metabolic component (Bowers et al., 2008; Cryan et al., 2002). The test, also known as the Porsolt swim test, was originally designed as a screen for antidepressant drugs (Porsolt et al., 1977), but is now commonly used as a measure of depression-like behaviour in animals and is also considered to examine arousal and agitation (Butler et al., 1990). During testing, animals display increasing immobility over time, described as “behavioural despair”, which is used to indicate depression-like behaviour, and which can be ameliorated by pharmacological antidepressants (Porsolt et al., 1977).

Animals were placed in glass cylinders (15 x 25 cm) containing 14 cm deep tap water, adjusted to approximately 20° C. Fresh water was used for each mouse to eliminate odours from previously tested animals (Carr et al., 1970; Zalaquett and Thiessen, 1991). The animals remained in the water for a total of six minutes and their activity was recorded for later analysis. Mice were then removed from the water and dried with paper towels before being placed in a recovery cage which was warmed by a heat pad set to 37° C. Animals remained in the recovery cage until blood collection. The last 4 mins of the video recordings were scored for immobility time, with mobility defined as any movement beyond that which is necessary to stay afloat and balanced.
Figure 2.9. The forced swim test (FST). Mice swim in fresh water for 6 minutes while recorded for later analysis. The last 4 minutes of swim behaviour is manually analysed.
2.8.3 Restraint stress

Restraint stress was chosen to induce chronic stress, as this is a well-established model, with robust and repeatable physiological effects. Restraint stress is a psychological stressor that is painless and causes no physical harm, thus allowing any observed effects to be attributed to the stress model, rather than any stress resulting from long-term physical injury (Buynitsky and Mostofsky, 2009; Voorhees et al., 2013).

Mice were placed in a restraint device (Harvard Apparatus, Holliston, MA, USA), which consisted of a clear acrylic plastic cylinder (9.5 cm x 2.5 cm) with a plug that adjusts for the length of each animal. Restraint was applied for 2 hours per day for 4 days. The time of day that restraint was applied was always changed to maintain an element of unpredictability, and minimise habituation (Mineur et al., 2006).
Figure 2.10. **Mouse restraint.** Restraint stress was applied using a restraint apparatus for 2 hours per day, for 4 days, at variable times of day.
2.8.4 Physiological stress response

Corticosterone is a primary stress hormone in rodents, generally peaking 15 minutes after a stressor. It is routinely used as a marker for stress.

2.8.4.1 Blood collection

Isoflurane anaesthesia was used to minimise the distress caused to the animals by decapitation. Although isoflurane anaesthesia causes distress in itself (Valentine et al., 2012), the rapid completion of anaesthesia and decapitation compared to the time-course of corticosterone release makes this isoflurane effect negligible. Additionally, isoflurane has been shown to have the least impact on stress and metabolic parameters compared to other commonly used anaesthetics (Zardooz et al., 2010).

Blood was collected 15 mins after the start of the FST for stressed animals, while control animals remained in the holding room until blood collection, and collection was within 1 minute of cage disturbance. Anaesthesia was induced via isoflurane inhalation (see open drop-method in Section 2.2.2), and animals were removed when the righting reflex was lost. Large, sharp scissors were used to decapitate the mouse, and trunk blood was collected in 1.5 mL Eppendorf tubes (Eppendorf AG, Hamburg, Germany). This method of blood collection yields arterial blood (Fukuta, 2004).

2.8.4.2 Corticosterone measurement

Blood samples were allowed to clot at room temperature, centrifuged at 6000 rpm for 20 min at 16°C, and serum was stored at -30°C until analysed. Serum corticosterone levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Enzo Life Sciences, NY), according to the manufacturers instructions. Samples were tested in triplicate and quantified using a multiplate reader (FLUOstar Omega, BMG Labtech).
CHAPTER 3

PAIN PHENOTYPE OF

α9-nAChR KO MICE
3.1 INTRODUCTION

Just over a decade ago, the involvement of neuronal nAChRs in the efficacy of analgesic α-conotoxin, Vc1.1, was first suggested (Sandall et al., 2003). Initially, α3 subunit-containing receptors were thought to be involved, but the α9α10-nAChR was subsequently implicated a few years later (Vincler et al., 2006). Following the isolation of α9α10-nAChR-inhibiting analgesic conotoxins, much focus has been placed on the synthesis of novel α9α10-nAChR-inhibiting compounds to treat pain (McIntosh et al., 2009; Vincler and McIntosh, 2007). However, no direct evidence for the involvement of this receptor subunit in pain has been documented. As discussed in greater detail in Section 1.6, inhibition of α9α10-nAChRs is not necessary or sufficient for analgesia, as several analogues of the analgesic conotoxins that are equipotent at the α9α10-nAChR produce no analgesia in a rat model of neuropathic pain (Nevin et al., 2007). Furthermore, no pain-relevant anatomical site of α9α10-nAChR expression has been confirmed.

Some non-peptide, small molecule α9α10-nAChR antagonists have been reported that reduce mechanical hypersensitivity in chronic pain models (CCI or vincristine), as well as attenuate the development of neuropathies (vincristine, phase II of formalin test) (Holtman et al., 2011; Wala et al., 2012; Zheng et al., 2011). Thus, the involvement of the α9α10-nAChR in pain continues to be suggested. However, as with the α-conotoxin studies, these assertions are only as reliable as the selectivity, pharmacokinetics and pharmacodynamics of the compounds used. Therefore, there is a significant risk of unknown functions of such compounds being misattributed to the known targets is great.

In order to directly investigate the involvement of the α9α10-nAChR in pain, I have investigated the pain phenotype of mice with a germline deletion of the α9-nAChR subunit. As the α10 subunit requires the presence of the α9 subunit to form functional receptors, deletion of the α9 subunit effectively deletes the functional α9α10-nAChR. A battery of pain tests was used to investigate several pain modalities in acute, inflammatory and neuropathic chronic pain states. The
results presented in this chapter have been published (Mohammadi and Christie, 2014) (see Appendix D).
3.2 METHODS

For General Materials and Methods used in this chapter, refer to Chapter 2 under the following section headings:

2.1 Animals
   2.1.1 Ethics approval
   2.1.2 Mice
   2.1.3 Genotyping of α9-nAChR KO mice
   2.1.4 Euthanasia

2.2 Anaesthesia and general surgical procedures
   2.2.1 Anaesthetic
   2.2.2 Induction and recovery

2.3 Pain models
   2.3.1 Neuropathic pain
       2.3.1.1 General surgical procedures for sciatic nerve pain models
       2.3.1.4 Sciatic nerve cuff variant of the CCI model
   2.3.2 Inflammatory pain
       2.3.2.1 Complete Freund's Adjuvant

2.4 Behavioural pain testing
   2.4.1 Habituation and handling
   2.4.2 Measurement of hot and cold nociception
       2.4.2.1 Hotplate test
       2.4.2.2 Acetone test
   2.4.3 Measurement of mechanical allodynia
       2.4.3.1 Mechanical Incapacitance test
       2.4.3.2 Von Frey hairs
   2.4.4 Measurement of mechanical hyperalgesia
       2.4.4.1 Paw pressure test
Specific Methods relating to this chapter:

3.2.1 PAIN TESTING PARADIGMS

For hot and cold nociception, and for mechanical hyperalgesia, animals were tested for one modality only. This minimised the time each individual animal spent in the injured state, as well as avoided potential confounds of combining multiple test manipulations. For mechanical allodynia, mice were tested on both the von Frey test and the Incapacitance test, as these are innocuous stimuli and are not prone to habituation effects at the frequency of testing performed here.

3.2.2 EXPRESSION OF DATA & STATISTICAL ANALYSES

Data were analysed using Prism (GraphPad Software Inc. version 6.0b for Mac OS X, San Diego, CA, USA) and SPSS (IBM® SPSS® Statistics, Version 21, Armonk, NY) software. All data are presented as mean ± SEM.

Student’s T-tests and one-, two- and three-way ANOVAs were performed using Prism software. The between-subjects main effects of genotype (WT vs. KO) and treatment (temperatures or injury) were evaluated with two-way ANOVAs. Within-subjects effects (days post-injury) were analysed with three-way ANOVAs comparing genotype and treatment groups with a fixed measure (normalised baseline score). When significant effects were observed, Bonferroni’s post-hoc tests were used. Time-course data were analyzed using SPSS general linear model with repeated measures, with time as a within subjects factor.
3.3 RESULTS

3.3.1 ACUTE NOCICEPTION IS UNAFFECTED BY DELETION OF THE α9-nACHr SUBUNIT

Acute thermal and mechanical nociceptive thresholds in α9-nACHr KO mice did not differ significantly from WT counterparts. In naïve animals mechanical nociception was normal when tested using the von Frey (Fig 3.1 A, $t(20) = 0.52, P = 0.61$) and paw pressure tests (Fig 3.1 B, $t(26) = 0.22, P = 0.82$). Thermal nociception was also unaffected in the KO mice when tested at three noxious temperatures on the hotplate (Fig 3.1 C, no significant genotype effect $F(1,73) = 0.76, P = 0.39$).

3.3.2 CHRONIC HOT AND COLD HYPERSENSITIVITY IS NORMAL IN α9-nACHr KO MICE

Changes to both hot and cold sensory modalities post-injury were tested in models of inflammatory (Freund’s complete adjuvant; CFA) and neuropathic (chronic constriction injury; CCI model) pain. Thermal hyperalgesia was tested on the hotplate. The inflammatory pain model produced a decreased response latency at 4 days post injury (Fig 3.2 A, significant treatment effect, $F(1,39) = 52.16, P < 0.0001$) that did not differ between WT and KO animals (no significant genotype effect, $F(1,39) = 0.008, P = 0.93$). The neuropathic pain model did not produce thermal hyperalgesia in either WT or KO animals (Fig 3.2 B, no significant treatment effect, $F(1,29) = 0.489, P = 0.49$ or genotype effect $F(1, 29) = 0.13, P = 0.72$). Cold allodynia evoked by the acetone test was observed after CCI (Fig 2C, significant treatment effect, $F(1,21) = 83.82, P < 0.0001$) and did not differ between WT and KO animals (Fig 3.2 C, no significant genotype effect, $F(1, 21) = 4.1, P = 0.06$).
Figure 3.1. Baseline pain thresholds for mechanical alldynia, mechanical hyperalgesia and thermal hyperalgesia. A| von Frey thresholds were comparable in naïve WT and KO mice. B| Mechanical pain thresholds were normal in naïve KO mice. C| Thermal pain thresholds were normal in naïve KO mice, at a range of noxious temperatures.

Figure 3.2. Hot and cold thermal hypersensitivity in chronic neuropathic and inflammatory pain models. A| The CFA chronic inflammatory pain model induced thermal hyperalgesia in WT and KO mice. B| The cuff variant of the CCI neuropathic pain model did not induce thermal hyperalgesia in either WT or KO mice. C| Cold alldynia developed in both WT and KO mice that had undergone the cuff CCI neuropathic pain model.
3.3.3 CHRONIC MECHANICAL ALLODYNIA DEVELOPS NORMALLY IN α9-nAChR KO ANIMALS, BUT MECHANICAL HYPERALGESIA SHOWS A UNIQUE PHENOTYPE

Chronic mechanical allodynia developed normally in α9-nAChR KO mice. Inflammatory (Fig. 3.3 A and C) and neuropathic (Fig. 3.3 B and D) pain models both induced mechanical allodynia as expected. Maximal von Frey responses were reached 4 days after CFA injection ($F(1,20) = 875.5, P < 0.0001$) and 7 days after CCI ($F(1,38) = 75.85, P < 0.0001$) compared with control groups. Continuation of testing over a 21-day period after CCI showed sustained allodynia that did not differ between WT and KO (no significant genotype effect, $F(1, 38) = 0.13, P = 0.72$), with sustained increased responsiveness to the von Frey test on the injured hind-paw, but not on the contralateral hind-paw over 21 days (Fig. 3.3 B). For the Incapacitance test, L:R ratio of weight bearing on the hind paws remained lowered after CCI but not sham surgery (Fig. 3.3 D) and did not differ between WT and KO over 21 days (no significant genotype effect, $F(1,24) = 2.79, P = 0.10$).

Mechanical hyperalgesia developed in both genotypes, however the magnitude of hypersensitivity was less in α9-nAChR KO animals compared to WTs in both inflammatory (significant genotype effect, $F(1,42) = 10.35, P = 0.003$ using two-way ANOVA; multiple comparisons between WT and KO CFA, $P < 0.001$) (Fig 3.4 A) and neuropathic (significant genotype effect $F(1,39) = 18.14, P = 0.0001$ using two-way ANOVA by treatment; multiple comparisons between WT and KO CCI, $P = 0.05$) (Fig 3.4 B) pain models at 4 and 7 days post injury respectively. Continuation of testing weekly after cuff surgery showed that mechanical hyperalgesia persisted in WT mice for at least 21 days ($P < 0.0001$ for each test day compared to normalised baseline, three-way ANOVA with multiple comparisons and Bonferroni post-hoc test), whereas the KO strain exhibited significant recovery by 14 days post injury ($P < 0.0001$ on D7, $P < 0.05$ at D14 and D21 compared to baseline) (Figure 3.4 B i).
**Figure 3.3. Mechanical hyperalgesia in chronic neuropathic and inflammatory pain models.** The CFA inflammatory pain model (A and C) and the cuff variant of CCI neuropathic pain model (B and D) induced mechanical allodynia, as tested using the von Frey (A and B) and Incapacitance (C and D) tests.

**Figure 3.4. Mechanical hyperalgesia in chronic neuropathic and inflammatory pain.** Inflammatory (A) and neuropathic (B) pain models produced mechanical hyperalgesia in both WT and KO mice, however, the magnitude of hyperalgesia was less in α9-nAChR KO mice. Injured KO mice showed recovery from mechanical hyperalgesia by 14 days post surgery (B i), while sham KO mice showed resistance to the repeated testing-induced mechanical hyperalgesia that developed in WT mice (B ii). ## P < 0.01, ### P < 0.001, #### P < 0.0001 compared to baseline. * P < 0.05, ** P < 0.01, *** P < 0.001 compared to WT mice.
3.3.4 Repeated testing-induced hypersensitivity does not develop normally in α9-nAChR KO animals

The noxious nature of the mechanical stimulus used repeatedly in the paw pressure test induced a mild mechanical hyperalgesia in the sham-operated WT mice, which was significant at day 14 (P < 0.01) and day 21 (P < 0.001, three-way ANOVA with multiple comparisons and Bonferroni post-hoc test) compared to baseline scores (Fig. 3.4 B ii). The α9-nAChR KO mouse strain was resistant to this repeated-testing effect (no significant change from baseline for KO animals on all post-injury test days, P > 0.05, three-way ANOVA with multiple comparisons). The decrease in threshold of WT sham mice was not due to a delayed effect of the sham surgery, as separate groups of sham-operated WT and KO mice tested only on post-injury day 21 did not differ from one another and had thresholds comparable to raw baseline scores of sham animals (F(3,42) = 0.98, P = 0.41, one-way ANOVA).

3.3.5 Further evidence for abnormal responses of α9-nAChR KO animals to repeated nociceptive testing

Further evidence for abnormal pain responses in α9-nAChR KO mice was observed in our lab by another researcher, during preliminary testing that used this strain as well as C57Bl/6j mice. The α9-nAChR KO mice exhibited a repeated-measures-induced analgesia in response to a thermal stimulus (Figure 3.5), an effect that was absent in WT C57Bl/6j mice. At test frequencies that do not cause sensitisation in WT C57Bl/6j mice, α9-nAChR KO mice appeared to become desensitised to acutely (i.e. within a single testing session) repeated hotplate testing. These observations were made on a far narrower time-scale (hours) than the repeated-measures testing of the pain phenotyping experiments (weeks). Although strain differences causing these divergent behaviours cannot be ruled out, the unexpected responses of the KO mice were reminiscent of the repeated-testing induced mechanical hyperalgesia described in Section 3.3.4, above. Since naïve and injured KO mice were shown to display normal pain responses to thermal stimuli, the effects appear more likely to be a stress-induced analgesia. This suggests that higher order pain processing is
altered in KO mice. Whether this involves the HPA-axis or other as yet unidentified mechanisms is yet to be determined but this observation prompted further experiments, described in Chapter 7, to examine stress responses in these animals.
Figure 3.5. α9-nAChR KO mice exhibit repeated-measures-induced desensitisation to the hotplate test compared with WT C57Bl/6j (C57) mice. After two tests (at 40 minutes), saline-injected α9-nAChR KO mice showed significantly prolonged response latency to the noxious 54° hotplate compared with saline-injected WT C57Bl/6j mice, which was sustained for the duration of testing. * P < 0.05, ** P < 0.01, *** P < 0.001; 2-way ANOVA with repeated measures and Bonferroni post hoc analysis. Morphine (30 mg/kg) produced sustained analgesia for the duration of the experiment. Data is from experiments performed by Anna Wang.
3.4 DISCUSSION

The discovery of analgesic α-conotoxins, such as Vc1.1 and RgIA, initiated interest in the α9α10-nAChR as a potential target for pain relief. Here, I have investigated the behavioural consequences of α9-nAChR subunit deletion in mouse models of chronic pain. I found that α9-nAChR KO mice display a largely normal pain phenotype, with acute nociception and chronic allodynia indistinguishable from WT mice. Differences between the two genotypes were detected only in the domain of chronic mechanical hyperalgesia, wherein α9-nAChR KO mice were found to be more resistant to the development of mechanical hyperalgesia, and this lessened hyperalgesia resolved more rapidly than in the WT counterparts.

3.4.1 DISADVANTAGES OF PHARMACOLOGICAL PROBES

The present results clearly demonstrate that the absence of functional α9-nAChR subunits does not abolish nociception, nor prevent the development of chronic pain. Thus, the clinical relevance of α9α10-nAChR inhibition is limited to a specific modality of chronic pain, wherein mechanical hyperalgesia may be prevented and alleviated.

The notion of inhibiting α9α10-nAChRs for analgesia initially garnered enthusiasm following the success of α-conotoxin Vc1.1 in acutely alleviating mechanical hyperalgesia in the rat CCI model of chronic neuropathic pain (Satkunanathan et al., 2005), along with the selectivity of Vc1.1 for α9α10-nAChRs (Vincler and McIntosh, 2007). α9α10-nAChR inhibition may have contributed to such effects, as supported by the present findings. Conversely, much of the literature that puts into doubt the involvement of α9α10-nAChRs in conotoxin analgesia has investigated mechanical allodynia as the primary measure of analgesic effect (Clark et al., 2010; Klimis et al., 2011; Napier et al., 2012; Nevin et al., 2007), which, as the present results show, does not require functional α9α10-nAChRs. Thus, my findings offer a possible explanation to the
early confusion surrounding the involvement of the α9α10-nAChR in pain, wherein the receptor contributes to mechanical hyperalgesia but not allodynia.

Studies on Vc1.1 and RgIA continue to demonstrate beneficial effects in animal models of nerve injury and attribute their analgesic mechanisms of action (both anti-hyperalgesic and anti-allodynic) to α9α10-nAChR inhibition (Mannelli et al., 2014). However, my results show a very limited analgesic phenotype in the α9-nAChR KO, which is likely due to the presence of additional mechanism(s) of analgesia beyond α9α10-nAChR inhibition, such as the inhibition of N-type Ca\(^{2+}\) channels via GABA\(_B\)Rs in peripheral sensory afferents (Cuny et al., 2012; Grishin et al., 2013; Klimis et al., 2011; Nevin et al., 2007). Vc1.1-analogues that retain the α9α10-nAChR inhibitory properties but not the GABA\(_B\)-receptor-dependent mechanism (Callaghan and Adams, 2010) do not have acute analgesic effects in animal models (Nevin et al., 2007). This dual mechanism of action of Vc1.1 and RgIA likely masked the dissociation between α9α10-nAChR-specific effects and other mechanism(s). Inhibition of the α9α10-nAChR may be conferring an additional attenuating and restorative capacity to the conotoxins, alongside the acute analgesic effects via GABA\(_B\)-dependent N-type Ca\(^{2+}\) channel inhibition (Callaghan and Adams, 2010; Klimis et al., 2011).

Some studies have reported specific pharmacological block of the α9α10-nAChR with quaternary ammonium analogues of nicotine, finding those compounds to be effective at attenuating the development of vincristine-induced pain (von Frey and paw pressure vocalisation threshold) and phase II formalin-pain, as well as acutely relieving CCI- and vincristine-pain (paw pressure vocalisation threshold) at high doses (Holtman et al., 2011; Wala et al., 2012). It remains possible that off-target non-CNS effects of these small molecule antagonists explain the discrepancy between the broad analgesic effects reported and the very selective effect on mechanical hyperalgesia observed in the present study. These studies have also noted motor incoordination as a side effect of their compounds, which has never been seen in Vc1.1 or RgIA studies, further suggesting that these small molecule agents have off-target mechanisms.
3.4.2 Disadvantages of gene knockout models

The use of gene knockout models in basic research comes with undeniable drawbacks. The knockout mouse presents an extreme phenotype that results from the life-long absence of functional protein production. This is in contrast with pharmacological studies that examine the effect of compounds in animals that have undergone normal embryogenesis, and wherein the compound in question may be withdrawn to restore normal function (Xiao et al., 2003). The results presented here examine the necessity of the α9α10-nAChR in nociception and in the development and maintenance of chronic pain. The functional development of the α9-nAChR KO mice may have been subject to compensation by other nAChR subtypes, and the role of such compensation in the pain phenotype observed here cannot be ruled out.

3.4.3 α9-nAChR KO mice show dissociation between mechanical allodynia & hyperalgesia

The results presented here suggest a sensory neuron type specific effect of α9α10-nAChR analgesia, where high threshold mechanoresponsive C fibres (Schmidt et al., 1995) are likely to be involved only under conditions of chronic inflammatory or neuropathic pain.

The molecular mechanisms of mechanotransduction are poorly understood. Primary sensory neurons are highly heterogenous, both functionally and molecularly, and distinct subsets of neurons selectively mediate different pain modalities (Cavanaugh et al., 2009). Thus it is likely that the key transduction molecules that initiate noxious mechanical sensation are equally numerous and varied, and await identification, as discussed below.

The mechanotransduction molecules that have been so far identified require more detailed understanding. The Mqprd family are GPCRs expressed in non-peptidergic, nociceptive sensory afferents that exclusively innervate the epidermis (Zylka et al., 2005), but their precise role in painful touch is not well understood. Progress is being made for K2p family ion channels, with the
mechanism of TRAAK channel gating by membrane-tension now well understood (Brohawn et al., 2014). Similarly, the role of Piezo2 in light-touch sensation is becoming increasingly well characterised, and its role in touch but not pain indicates yet unknown transducers are responsible for noxious mechanical sensation (Ranade et al., 2014). Advancements in techniques to classify the vast range of heterogenous primary sensory nerves, such as transcriptome analysis, suggest that there are at least 11 types of primary afferents (Usoskin et al., 2015). It is possible that the α9α10-nAChR may be expressed on one of these types, presumably a subset of high-threshold mechanosensitive nociceptors that are yet to be classified, and are thus selectively affected in the pain phenotype of α9-nAChR KO mice. Alternatively, α9α10-nAChR expressed on another cell type may play a specific role in induction of hyperalgesia in high threshold mechanosensitive nerves (see Chapter 4).

The attenuation of the development of mechanical hyperalgesia in KO mice may indicate a beneficial effect of prophylactic treatments with α9α10-nAChR antagonists. However, the benefits of such treatments would have to be explored in vivo further.

### 3.4.4 ANATOMICAL SITE OF ACTION OF α9α10-NACHR-INHIBITING ANALGESICS

The mechanism responsible for the selective disruption of mechanical hyperalgesia seen here by deletion of the α9-nAChR subunit is unknown. The anatomical site of the α9-nAChR's involvement in pain remains uncertain. The majority of studies investigating this receptor subtype have been concerned with audition and the cochlea. In pain-related cell types, either an absence of expression is reported or findings are conflicting. The α9α10-nAChR is not expressed in the CNS (Elgoyhen et al., 1994). In the periphery, expression in sensory afferent axons appears to be absent (Elgoyhen et al., 1994; Lips et al., 2002), while expression in DRGs has been shown at various levels of the spinal cord. However, studies of expression in DRGs are limited to mRNA detection
(Haberberger et al., 2004; Lips et al., 2002), and α9-nAChR protein has not been identified via immunochemical methods (Callaghan and Adams, 2010). Inhibition of immune cell activity, presumably inhibiting the downstream release of inflammatory and algogenic molecules, has been suggested as a potential target of α9α10-nAChR antagonists (Mannelli et al., 2014; Vincler et al., 2006). A reduction in immune cell infiltration of injured sciatic nerves was shown in Vc1.1-treated animals compared to controls, which was attributed by the authors to α9α10-nAChR inhibition. Whether the same result is seen when comparing WT and α9-nAChR KO animals is addressed in Chapter 4. In the present study, the CFA model of inflammatory pain did show significantly less hyperalgesia in KOs than WT, though Vc1.1 has shown only weak activity in this model (McCracken, 2005). Studies have shown expression of α9-nAChR RNA in human blood lymphocytes (Peng et al., 2004) and protein in mouse splenic B-lymphocytes (Koval et al., 2011), however, no demonstrable functional α9α10-nAChR response to ACh has been achieved in lymphocytes (Peng et al., 2004). Indeed, many attempts to induce α9α10-nAChR-mediated ACh responses were made by adjusting recording conditions (with or without magnesium, altered Ca\textsuperscript{2+} concentrations, varying holding potentials, changing intracellular media, and altering ACh application time) and using the Jurkat immortalised T lymphocyte cell line, all to no avail (Peng et al., 2004). Thus, the support for immune-cell α9α10-nAChRs as the target of α-conotoxin analgesia is scant and must be considered controversial.

### 3.4.5 Potential Neuroendocrine Involvement of α9α10-nAChR in Pain

An alternative possibility for the mechanism of α9α10-nAChR involvement in mechanical hyperalgesia is via the neuroendocrine system. The widely varying aetiologies of neuropathy (neuropathic (Clark et al., 2010; Klimis et al., 2011; Livett et al., and issued 10 October 2002; Nevin et al., 2007; Satkunananthan et al., 2005; Vincler et al., 2006), inflammatory (McCracken, 2005), chemogenic (Wala et al., 2012), metabolic (McCracken, 2005)) that benefit from α9α10-nAChR inhibition might suggest a higher-order system effect. The resistance of the KO
mice to repeated testing-induced hypersensitivity also suggests that higher order pain processing, rather than nociception, is altered in this genotype. It would be of interest in future studies to test whether the repeated testing anti-nociception observed in the α9-nAChR KO mice in our laboratory was an opioid or endocannabinoid-mediated effect by examining the effects of antagonists of these systems. The α9α10-nAChR is expressed in the anterior pituitary and adrenal glands (Elgoyhen et al., 1994), and is upregulated in the adrenal gland in cold-stressed animals (Colomer et al., 2010). Hypothalamic-pituitary-adrenal (HPA) axis dysregulation is associated with chronic pain (Blackburn-Munro and Blackburn-Munro, 2001; Imbe et al., 2006), and α9α10-nAChR activity in the HPA-axis might contribute to the enhanced and sustained pain by affecting changes in circulating stress hormones such as corticotrophin and catecholamines. Whether or not HPA-axis regulation is affected in the α9-nACh KO mice is explored in Chapter 7.

3.4.6 Potential side effects of α9α10-nAChR-inhibiting analgesics

No deleterious side effects of α9α10-nAChR-inhibiting conotoxins have so far been reported. However, α9α10-nAChRs are expressed in a range of tissues, which may make α9α10-nAChR-inhibiting therapeutics vulnerable to side effects. The α9α10-nAChR is best understood in the auditory system, where it is the primary receptor in efferent auditory feedback. Inhibition of auditory α9α10-nAChRs may cause predisposition to noise-induced hearing loss (Elgoyhen et al., 2009), however this will require drugs to cross the blood-cochlear barrier (Swan et al., 2008), which is a challenge in itself. α9α10-nAChRs play a role in epithelial cell proliferation and are upregulated in cancers (breast (Lee et al., 2011) and lung (Chikova and Grando, 2011)), though this upregulation occurs only after nicotine exposure and has more relevant implications for tobacco smoking. Expression in the HPA axis (Colomer et al., 2010; Elgoyhen et al., 1994) may impart α9α10-inhibitors with susceptibility to mood-altering side effects. This is an aspect that requires greater attention if α9α10-nAChR antagonists are to be pursued.
3.4.7 Unusual properties of α9α10-nAChRs

The results presented here suggest that the inhibition of the α9α10-nAChR may have been erroneously attributed to be the mechanism of acute α-conotoxin analgesia. Such misattribution may be due to the unusual properties of the α9α10-nAChR, leading to misinterpretation of data. Nicotine, an α9α10-nAChR antagonist, has been routinely used as an agonist in α-conotoxin specificity and efficacy studies (Clark et al., 2006; Clark et al., 2010; Lang et al., 2005; Sandall et al., 2003; Townsend et al., 2009). Such assays continue to be used in the context of Vc1.1 analgesia (Clark et al., 2010), however the nAChR subunit target in these assays is unknown (for a list of known nAChR combinations, see Millar and Gotti (2009)). In many of these assays (Clark et al., 2006; Clark et al., 2010; Sandall et al., 2003; Townsend et al., 2009), the α9α10-nAChR may have been rendered inactive in the tissue preparation process as collagenase, the primary digestive enzyme used, uncouples the α9α10-nAChR from small conductance Ca\(^{2+}\)-dependent K\(^+\) channel (SK2) which is a complex that is necessary for receptor function (Kong et al., 2008; Zhou et al., 2013). However, it is possible that the requirement of α9α10-nAChR/SK2 coupling is specific to the cochlear and vestibular hair cell types, since functional α9α10-nAChRs have been recombinantly expressed in X. laevis oocytes (Clark et al., 2006; Clark et al., 2010; Halai et al., 2009; Napier et al., 2012; Nevin et al., 2007; Vincler et al., 2006).

3.4.8 Conclusions

Germline deletion of the α9 subunit of nACh receptors produces an unusual pain phenotype in mice. Thermal hyperalgesia is unaffected and both thermal and mechanical allodynia develop normally in both inflammatory and neuropathic pain models. By contrast, mechanical hyperalgesia is attenuated and recovers more rapidly in KO mice. Although the α9α10-nAChR was first implicated in pain as the mechanism of action of some analgesic α-conotoxins, the present study shows that inhibition of this receptor alone cannot account for the analgesic effects of Vc1.1 and RgIA. α9α10-nAChRs may be a valid target for pharmacological compounds that prevent or alleviate long-term mechanical
hyperalgesia, perhaps via promoting recovery. The precise mechanism and anatomical location of the α9α10-nAChRs involved remains to be determined.
CHAPTER 4

IMMUNOHISTOCHEMICAL COMPARISON OF WT AND KO INJURED SCIATIC NERVES


4.1 INTRODUCTION

Analgesic α-conotoxins such as Vc1.1 and RgIA have numerous reported benefits in nerve-injured animals. Their analgesic properties are well documented, but their disease-modifying effects have been less widely investigated. Early publications demonstrated that these conotoxins have actions above and beyond attenuation of nociceptive signals, and the mechanisms of these actions warrant further investigation.

Since α-conotoxins such as Vc1.1 and RgIA were first tested in vivo, there have been indications that they attenuate the pathology underlying neuropathic pain. Satkunanathan et al. (2005) demonstrated that in addition to the acute (1, 3 hours), short term (24hrs) and long term (1 week post-treatment) analgesic effects, Vc1.1 also accelerated functional recovery. 8 weeks after the induction of the CCI neuropathic pain model (approximately 5 weeks post-cessation of Vc1.1 treatment), rats were tested for the functional recovery of the injured sciatic nerve using the blister model of neurogenic inflammation. Blisters induced on the glabrous skin of the rat hindlimb were monitored by laser Doppler flowmetry while superfused with Ringer’s solution. The vascular response of the blistered skin to substance P, a potent vasodilator released from C-fibres (White and Helme, 1985), was measured. CCI rats treated with Vc1.1 had substance P-induced vascular responses significantly closer to uninjured rats than saline treated animals.

A mechanism of action for this α-conotoxin-mediated protective/restorative capacity was soon proposed whereby α9α10-nAChRs on immune cells are targeted, inhibiting the functioning of these cells (Vincler et al., 2006). It was suggested that the resultant reduction in inflammatory response leads to attenuation of injury and allows for more rapid recovery from pathology. This mechanism has been proposed based solely on two studies that show Vc1.1- and RgIA-mediated analgesia in a rat model of neuropathic pain (CCI), with
additional conotoxin-mediated changes in histological parameters of the injured nerve, DRGs and spinal cord (Mannelli et al., 2014; Vincler et al., 2006).

Initial suggestions of an immune cell-mediated mechanism came from Vincler et al. (2006) findings that the peripheral immune response in RgIA-treated rats was altered. The authors found that RgIA (and presumably Vc1.1) decreased CCI injury-induced infiltration of immune cells (CD2+ T lymphocytes, CD68+ macrophages) and ChAT+ cells, which potentially drive the cholinergic immune response. This conotoxin-mediated inhibition of immune cell build-up at the site of nerve injury was interpreted as a mechanism for both antinociception, and accelerated functional recovery, and was presumed to be mediated by α9α10-nAChRs.

More detailed histological investigation of the disease modifying effects of α-conotoxins has revealed additional benefits. Mannelli et al. (2014) observed that daily administration of RgIA over 14 days had progressive disease modifying effects. Behaviourally, rat sensitivity to both sub- (von Frey, Incapacitance test) and supra-threshold (paw pressure test) mechanical stimuli was decreased. Histologically, this chronic RgIA treatment altered CCI-induced pathology of the sciatic nerve, preserving axon fibre number, myelin thickness and axon diameter. The extent of oedema in the injured nerve was reduced by 60%. In the spinal cord, RgIA prevented injury-induced increases in microglia and astrocyte numbers. Once again, α9α10-nAChR-inhibition was attributed to be the mechanism of action.

It is apparent that α-conotoxins Vc1.1 and RgIA impede the maladaptive changes that progress into the pathology of chronic neuropathic pain and are neurorestorative, making them very promising and attractive compounds that could revolutionise the way pain is treated. If these disease-modifying properties are indeed mediated by the α9α10-nAChR, the same histological changes seen in conotoxin-treated animals should be observed after CCI in KO mice.
To investigate this, CCI or sham surgery was performed on WT and α9-nAChR KO animals and any alteration in immune cell infiltration into the sciatic nerve was compared. Nerve injury also results in oedema, containing inflammatory infiltrate, which is also reduced by α-conotoxin treatment (Mannelli et al., 2014). Whether this reduction might be α9α10-nAChR-mediated was also investigated by comparing WT and α9-nAChR KO mice.
4.2 Methods

For General Materials and Methods used in this chapter, refer to Chapter 2 under the following section headings:

2.1 Animals
  2.1.1 Ethics approval
  2.1.2 Mice
  2.1.3 Genotyping of α9-nAChR KO mice
  2.1.4 Euthanasia

2.2 Anaesthesia and general surgical procedures
  2.2.1 Anaesthetic
  2.2.2 Induction and recovery

2.3 Pain models
  2.3.1 Neuropathic pain
    2.3.1.1 General surgical procedures for sciatic nerve pain models
    2.3.1.4 Sciatic nerve cuff variant of the CCI model

2.5 Histology & immunohistochemistry
  2.5.1 Antibodies & reagents
  2.5.2 Tissue dissection, fixation & sectioning
    2.5.2.1 Cardiac perfusion & tissue preparation
    2.5.2.2 Post-fix, cryoprotection & freezing
    2.5.2.3 Cryostat sectioning & mounting
  2.5.3 Staining
    2.5.3.1 Haematoxylin & eosin staining
    2.5.3.2 Immunohistochemical staining
  2.5.4 Imaging
  2.5.5 Quantification
    2.5.5.1 Cell density
    2.5.5.2 Oedema
Specific Methods relating to this chapter:

4.2.1 Timing of Nerve Collection
Sciatic nerves were dissected from animals at 7, 14 and 21 days after the placement of the CCI cuff or sham surgery. For H & E histology, naïve nerves were used instead of sham as the nerve morphology was unchanged between the two groups. Four nerves per group were collected and analysed.

4.2.2 Regions of Interest (ROIs) for Quantification.
The intact portion of the sciatic nerve just proximal to the CCI cuff was chosen for analysis, as described in Section 2.5.5, as a visual scan of the cell distribution indicated that greater cell infiltration occurred here (see Figures 4.1, 4.3 and 4.4). Additionally, this is likely to be the anatomical region most involved in the initiation and propagation of abnormal pain signals. Spontaneous pain, a hallmark of chronic neuropathic pain conditions, is due to the spontaneous activity of intact nerve fibres that remain after injury (Djouhri et al., 2006) and this spontaneous activity originates from regions proximal to the injury, particularly the cell soma (Kajander et al., 1992).
4.3 Results

4.3.1 Nerve morphology

Haematoxylin and eosin staining of dissected sciatic nerves revealed distinct changes in morphology resulting from the placement of the CCI cuff. A distinct depression is apparent along the length of nerve that the cuff enclosed, in nerves from both WT and α9-nAChR KO mice, whereas sham or naïve nerves are comparatively straight and uniform along the entire length. Figure 4.1 shows representative images of naïve and cuffed nerves at several magnifications.

Increased numbers of nuclei are apparent in the injured nerves from mice of both genotypes. The highest density of nuclei is at the region of nerve just proximal to the cuff, gradually decreasing in density in the proximal direction.
Figure 4.1. H & E staining of naïve and cuffed sciatic nerves. Sagittal sections of a naive nerve (A) and a cuffed nerve 14 days after injury (B) are shown at magnifications of 4x (Ai, Bi), 10x (Aii, Bii) and 20x (Aiii, Biii).
4.4.2 Oedema

Significant oedema, as measured by the inverse of the ROI length (see Section 2.5.5.2), resulted from the constriction injury induced by the cuff in the sciatic nerves of both WT and KO mice.

There was no significant difference in the extent of oedema between WT and KO genotypes at any time-point either before or after surgery (P > 0.05, two-way ANOVA with Bonferroni’s post-hoc pairwise comparison) (Figure 4.2).

The extent of oedema increased at 7 (P < 0.05, t = 3.44 for WT; P < 0.0001, t = 5.229 for KO), 14 (P < 0.0001, t = 6.514 for WT; P < 0.01, t = 4.339 for KO) and 21 (P < 0.0001, t = 11.04 for WT; P < 0.0001, t = 8.06 for KO) days after CCI surgery for both genotypes.

Two-way ANOVA with Bonferroni’s post-hoc multiple comparisons test revealed a significant main effect of time post-injury (P < 0.0001, F(3, 72) = 59.62), accounting for 65.7% of variation, and a small interaction effect (P < 0.05, F(3, 72) = 3.014) between genotype and time post-injury, accounting for 3.3% of variation. This indicates a potential misalignment in the rate of increase of oedema between the WT and KO animals.
Figure 4.2. Oedema in sciatic nerves of WT and α9-nAChR KO mice. The extent of oedema was taken as the inverse of the length of the ROI analysed. ROIs were of a fixed area, therefore the lengths of the ROI were inversely proportional to the diameter of the nerves.
4.3.3 IMMUNE CELL INFLTRATION OF SCIATIC NERVES

Immune cell infiltration of sciatic nerves increased after injury. This immune cell infiltration has been reported to be attenuated in animals treated with α-conotoxins Vc1.1 (Vincler et al., 2006) and RgIA (Mannelli et al., 2014; Vincler et al., 2006), potentially via inhibiting α9α10-nAChR on immune cells.

4.3.3.1 LYMPHOCYTE INFILTRATION

Lymphocyte infiltration of the sciatic nerve was quantified by Immunohistochemical staining with an anti-CD3ε antibody (Figure 4.3). The number of CD3ε-immunoreactive T-cells in the sciatic nerve increased considerably, after the application of a constricting cuff.

In sham-operated nerves, T-cell density was very low in the ROI (6 ± 2.7 for WTs, 2 ± 0.5 for KOs). After CCI surgery, T-cell density increased significantly above sham values at 7 (P < 0.001, t = 4.91 for WT; P < 0.0001, t = 6.20 for KO), 14 (P < 0.0001, t = 6.021 for WT; P < 0.0001, t = 6.262 for KO) and 21 (P < 0.0001, t = 8.751 for WT; P < 0.0001, t = 7.925 for KO) days after CCI surgery (Figure 4.5 A).

A significant main effect of time post injury (P < 0.0001) was observed for lymphocytes (F(3, 58) = 47.8), which accounted for 65.7% of variation. A small genotype effect (P = 0.05, F(1, 58) = 4.8) was observed accounting for 2.2% of variation indicating a slightly increased amount of lymphocyte infiltration in the KO nerves compared to WT nerves.
Figure 4.3. Representative lymphocyte (CD3ε) staining in injured and naïve WT sciatic nerves. 

A | Sciatic nerves were constricted by cuffs (cuff) or remained intact (sham). ROIs (inset) were analysed in each nerve. 

B | The ROI of injured nerves was just proximal to the cuff (yellow). 

C | The ROI of sham nerves was a representative section of the nerve.
Figure 4.4. Representative staining of ChAT-immunoreactive cells in injured and sham sciatic nerves. Images are of WT mouse nerves 14 days after CCI surgery. A| Region of injured sciatic nerve just proximal to the cuffed region. B| Segment of sham nerve. C and D show the ROI boundaries used for analysis.
4.3.3.2 ChAT immunoreactive cell infiltration

ChAT-immunoreactive (IR) cell density (Figure 4.4) was significantly increased above sham values at 7 (P < 0.0001, t = 6.873 for WT; P < 0.0001, t = 8.703 for KO), 14 (P < 0.0001, t = 15.68 for WT; P < 0.0001, t = 18.17 for KO) and 21 (P < 0.0001, t = 15.64 for WT; P < 0.0001, t = 18.13 for KO) days after CCI surgery (Figure 4.5 B).

A significant main effect of time post injury (P < 0.0001) was observed for ChAT-IR cells (F(3, 121) = 265.0), which accounted for 85.4% of variation. A small genotype effect (* P < 0.05, F = (1, 121) = 4.7) was observed accounting for 0.5% of variation indicating a small increase in ChAT positive cell infiltration in the KO mouse nerves when compared to WT nerves.
Figure 4.5. Immune cell infiltration of sciatic nerves. A| Density of lymphocyte infiltration into sham sciatic nerves and injured nerves at weekly intervals after surgery. B| ChAT-immunoreactive (IR) cell density in sham and injured sciatic nerves.
4.4 DISCUSSION

Neuropathic pain management is currently directed at reducing symptoms, generally by suppressing neurotransmission. It would be far more beneficial if treatments modified the pathology itself, preventing and/or reversing the damage that develops into persistent pain. Modulating the immune response to nerve injury and painful inflammatory conditions may provide opportunities for such disease modification (Ji et al., 2009; Scholz and Woolf, 2007), and indeed analgesic α-conotoxins such as Vc1.1 and RgIA have been proposed to do so.

4.4.1 THE α9α10-nAChR IS NOT NECESSARY FOR INFLAMMATORY RESPONSE IN INJURED SCIATIC NERVES

The large amount of oedema that occurs after nerve injury, as well as the marked immune cell infiltration of the injury site, have been proposed to be α9α10-nAChR-mediated (Mannelli et al., 2014; Vincler et al., 2006). The present results demonstrate that the α9α10-nAChR is not necessary for either of these phenomena. While α9α10-nAChR-inhibiting α-conotoxins have been shown to suppress oedema and immune-cell infiltration of CCI-injured sciatic nerves, I found no such suppressive effects of α9α10-nAChR deletion in mice.

The absence of α9α10-nAChRs in KO mice did not prevent either the magnitude or progression of oedema, nor the infiltration of lymphocyte or ChAT-IR cells in the weeks following surgery. Significant effects of genotype on immune-cell infiltration indicated that KO mice, in fact, have slightly increased immune cell infiltration into the sciatic nerve after injury. However, this increased immune cell infiltration observed in KO injured nerves did not coincide with increased oedema or result in any increased susceptibility to pain (see Chapter 3).

Although the α9α10-nAChR is expressed on B- and T-lymphocytes (Koval et al., 2011; Peng et al., 2004), the role of the α9α10-nAChR on immune cells is not fully understood. As described in Section 1.5.4.5, α9α10-mediated ACh responses
have not been evoked in either human B or T-lymphocytes or Jurkat immortalised T cells (Peng et al., 2004). While protein and gene expression is highly dynamic and responsive to cell activation in immune cells (Scholz and Woolf, 2007), α9α10-nAChRs are not upregulated in activated B-lymphocytes (Koval et al., 2011).

4.4.2 α9α10-nAChR DELETION IN MICE MAY BE SUBJECT TO FUNCTIONAL COMPENSATION

A commonly acknowledged flaw of transgenic studies is the potential for compensatory mechanisms to confound interpretation of the observed phenotype. Consequently, the notion that gene targeting will reveal the in vivo function of the gene of interest has been questioned (Routtenberg, 1995). Genetic redundancy can cause up/down regulation of genes other than the one(s) targeted. This could either mask the phenotypic effect of the mutation, or cause secondary phenotypical changes to be misattributed to the absence of the targeted protein itself (Gerlai, 2001).

Nevertheless, there is great value in gene targeting studies. In particular, combining gene targeting with other methods such as behavioural pharmacology can enable the unmasking of novel biochemical pathways and the functional interactions between the targeted gene and other genes (e.g. Chen et al. (1995)). Attempts to determine the efficacy of analgesic α-conotoxins in α9-nAChR KO mice were inconclusive (see Chapter 5) and must be repeated. However, the possibility must be considered that there may be some compensatory mechanisms during development in the α9-nAChR KO mice that affect the pain phenotype and account for the relatively normal histopathology observed here.

4.4.3 ODEMA & IMMUNE REACTIONS ARE NOT PAIN MODEL-DEPENDENT

A possible reason for why the current results demonstrated differing histopathology patterns to those of Vincler et al. (2006) and Mannelli et al. (2014) is the slight methodological difference in the neuropathic pain model
used. Both of the mentioned papers use a CCI model that implements chromic catgut to constrict the nerve. Here, an adapted version of CCI was used, where polyethylene cuffs formed the constriction. This tubing, having a fixed inner diameter, ensures that a more consistent constriction is applied across all animals, and therefore produces a standardised model (Benbouzid et al., 2008). Additionally, the notorious setbacks associated with chromic catgut are avoided, such as poor knotting and knot insecurity, unreliable tensile strength and difficulty in handling (Akinrinmade and Lawal, 2010), which all lead to variable amounts of constriction applied across animals.

While it is true that the two suture materials elicit differential changes in the microenvironment of the injured nerve (Maves et al., 1994), the cuff model does still have a neurogenic inflammatory component (Mosconi and Kruger, 1996). This is quite evident by the oedema and the enormous increase in immune cell infiltration in the injured nerves seen here in both genotypes. Also, a recent comparison of catgut with polypropylene (a polyolefin similar to the polyethylene used) sutures failed to show any increase in overall inflammatory response caused by the catgut over polypropylene (Akinrinmade and Lawal, 2010). In fact, the polypropylene caused slightly more inflammation than catgut in the first week after surgery, before levelling off in the subsequent weeks (ibid.) Furthermore, α-conotoxins have been shown to be highly effective in a range of painful pathologies of varying aetiologies, whether inflammation is a primary component or not (neuropathic (Clark et al., 2010; Klimis et al., 2011; Livett et al., and issued 10 October 2002; Nevin et al., 2007; Satkunanathan et al., 2005; Vincler et al., 2006), inflammatory (McCracken, 2005), chemogenic (Wala et al., 2012), metabolic (McCracken, 2005)). Thus, it is unlikely that this slight difference in methodology could account for the complete lack of difference seen between genotypes in our study if the immune cell inhibitory mechanism were α9α10-nAChR-mediated.

Further variation between the present results and those in the literature may have arisen due to differences in the regions of the sciatic nerve analysed. Here, only the proximal regions were examined, whereas Mannelli et al. (2014)
analysed cells from regions of the nerve that were proximal and distal to the constricted area (the regions examined by Vincler et al. (2006) were not specified). Changes at central terminals may also be present and this is an area that could be explored in the future. However, the α9α10-nAChRs on immune cells at the injury site are proposed to be the site of action of α9α10-nAChR-inhibiting analgesics, and changes at central sites would likely be indirect or downstream from α9α10-nAChR-inhibition.

4.4.4 CONCLUSIONS

There is no doubt that conotoxins Vc1.1 and RgIA (and presumably many more of the family yet to be discovered) have unique analgesic and restorative properties that could revolutionise pain management when properly harnessed. However, the assertion that the primary mechanism of conotoxin analgesia is via α9α10-nAChR inhibition has not been substantiated by a number of studies (see Sections 1.6, 3.4, 5.4.2). Moreover, the assertion that the mechanism of neuroprotection by these conotoxins is via inhibition of α9α10-nAChRs on immune cells is without direct evidence and the present results do not support the interpretation that that is the mechanisms of action. My results demonstrate that α9α10-nAChR expression is not necessary for these actions, suggesting that another mechanism is likely to be responsible for the potentially curative properties of these conotoxins and their effects on immune cell infiltration into injured nerves.
CHAPTER 5

α-CONOTOXINS IN

α9-nAChR KO MICE
5.1 INTRODUCTION

Conotoxins Vc1.1 and RgIA are two highly promising peptides for the treatment of pain disorders. Vc1.1 in particular has been extensively tested in rat models of chronic pain and has proven to be highly effective in a range of pain models, however, efficacy in mice has yet to be reported.

The highly promising Vc1.1 advanced to phase Ila of human clinical trials in 2006 (Metabolic, 2006). However, trials were discontinued when it emerged that the conotoxins’ affinity for the putative target, the $\alpha 9\alpha 10$-nAChR, was 100-fold lower at the human receptor than that of the rat (Azam and McIntosh, 2012) making pursuit of Vc1.1 cost-prohibitive (Metabolic, 2007). Since that time, increasing evidence has emerged that inhibition of the $\alpha 9\alpha 10$-nAChR is unlikely to be the primary mechanism of $\alpha$-conotoxin analgesia (see Sections 1.6 and 3.4.1). If indeed affinity for the $\alpha 9\alpha 10$-nAChR is not a determining factor for $\alpha$-conotoxin efficacy, Vc1.1, RgIA and other similar conotoxins warrant further investigation and testing at the clinical level, as they appear to act via a novel mechanism and may surpass existing analgesic drugs in their efficacy and side-effect profiles.

An alternative mechanism of $\alpha$-conotoxin analgesia is inhibition of N-type VGCCs through a GABA$_B$-dependent mechanism (Callaghan and Adams, 2010; Callaghan et al., 2008). Indeed it has been shown in rats that co-administration of a GABA$_B$-antagonist with Vc1.1 completely abolishes the analgesic effects of the conotoxin (Klimis et al., 2011).

I hypothesised that if $\alpha$-conotoxins such as Vc1.1 and RgIA achieve their analgesic effects through a mechanism independent of the $\alpha 9\alpha 10$-nAChR, then the peptides should proffer the same degree of analgesia in WT animals as in transgenic animals with the $\alpha 9$-nAChR subunit deleted. Additionally, if the proposed GABA$_B$R-dependent N-type VGCC inhibitory mechanism is indeed the primary mechanism of acute analgesia, the co-administration of a GABA$_B$-
antagonist should abolish the effects of the conotoxin in both WT and α9-nAChR-KO animals.

Since the *in vivo* efficacy of α-conotoxins in mice has yet to be reported, I first tested Vc1.1 in WT C57Bl/6j mice. I determined the optimal dose of GABA\(_B\)-antagonist that inhibited baclofen analgesia. My final aim was to test Vc1.1 in α9-nAChR-KO mice, and determine the antagonistic effects of the GABA\(_B\)-antagonist on the conotoxin.
5.2 Methods

For general materials and methods used in this chapter, refer to Chapter 2 under the following section headings:

2.1 Animals
   2.1.1 Ethics approval
   2.1.2 Mice
   2.1.3 Genotyping of α9-nAChR KO mice
   2.1.4 Euthanasia

2.2 Anaesthesia and general surgical procedures
   2.2.1 Anaesthetic
   2.2.2 Induction and recovery

2.3 Pain models
   2.3.1 Neuropathic pain
      2.3.1.1 General surgical procedures for sciatic nerve pain models
      2.3.1.2 Partial Nerve Ligation
      2.3.1.3 Chronic Constriction Injury
      2.3.1.4 Sciatic nerve cuff variant of the CCI model
   2.3.2 Inflammatory pain
      2.3.2.1 Complete Freund’s Adjuvant

2.4 Behavioural pain testing
   2.4.1 Habituation and handling
   2.4.2 Measurement of hot and cold nociception
      2.4.2.1 Hotplate test
      2.4.2.2 Acetone test
   2.4.3 Measurement of mechanical alldynia
      2.4.3.1 Mechanical Incapacitance test
      2.4.3.2 Von Frey test
   2.4.4 Measurement of mechanical hyperalgesia
2.4.4.1 Paw pressure test

1.1 Drug administration
   1.1.1 Scruffing
   1.1.2 Sub-cutaneous injections
   1.1.3 Intra-peritoneal injections
   1.1.4 Intra-muscular injections

Specific methods relating to this chapter:

5.2.1 GABA<sub>B</sub> receptor-ligands
A GABA<sub>B</sub>R agonist, (R)-baclofen (Sigma), was used to determine a submaximal dose and timecourse of a GABA<sub>B</sub>R antagonist, SCH50911 (Sigma). Determination of these parameters was needed for subsequent testing of the role of GABA<sub>B</sub>Rs in Vc1.1-mediated analgesia in WT and α9-nAChR-KO mice.

5.2.2 Toxin preparation
Conotoxins Vc1.1 (batch QC0610; 444 μM and batch QC25102011; 782 μM), RgIA (batch PUB-0582) and cyclic Vc1.1 (cVc1.1; batch PUB-2612a) were provided by Dr Richard Clark from the University of Queensland. Aliquots of conotoxins were stored as received at 4° C until ready to use. If dilution was necessary, it was done as close to the time of injection as possible.

5.2.2.1 Saline solvent
Conotoxins were dissolved in sterile 0.9% sodium chloride injection BP (Pfizer).

5.2.2.2 Conotoxin doses
For Vc1.1 testing, an initial dose of 0.5 mg/kg was used. A dose of \( \frac{1}{2} \log_{10} \) unit lower, of 0.17 mg/kg was then tested. The maximum dose of Vc1.1 tested was 1.5 mg/kg. Intramuscular injections used undiluted stock solution, as received from the synthetic chemists and were 0.2 mg/kg.
For RgIA testing, a single 0.8 μg/kg dose was used. The peptide was subcutaneously administered, and changes in weight bearing on the Incapacitance test were measured at hourly intervals for 5 hours.

For cVc1.1 testing, 0.3 mg/kg was administered subcutaneously. The cyclic peptide was administered once daily for 3 or 5 days, and anti-allodynic and anti-hyperalgesic effects were tested at 1 hour post injection using the von Frey and PAM tests respectively.

5.2.2.3 BSA
0.1% bovine serum albumin (BSA; Sigma) was added to a 0.5 mg/kg dose of Vc1.1 in some experiments, in an attempt to act as a carrier to prevent the peptide from potentially sticking to the plastic tubing and syringe.

5.2.3 PAIN TESTING PARADIGMS
Baseline thresholds were measured prior to PNL, CCI or cuff surgery. Animals were allowed to recover from the surgery for 7 days before being tested. All pain testing was conducted between 10:00 and 17:00.

5.2.3.1 CONOTOXIN ACUTE ADMINISTRATION
Vc1.1 and RgIA have been shown to produce acute analgesia in rat models of chronic neuropathic pain. The peak effect occurs about 1 hour after administration, but a prolonged effect also occurs (Nevin et al., 2007; Satkunanathan et al., 2005). I therefore initially tested mice at 30, 60 and 90 and 240 minutes after a single acute dose of conotoxin. Testing at 24 hours after injection was also performed. I was blinded to drug treatments for all experiments.

5.2.3.2 CONOTOXIN REPEATED ADMINISTRATION
Vc1.1 has been shown to have cumulative effects after repeated daily dosing in rat models of chronic neuropathic pain (Satkunanathan et al., 2005). Vc1.1 or
cVc1.1 was therefore administered for 2, 3 or 5 days and the effects tested daily, 1 hour after injection. The in vivo analgesic effects of Vc1.1 have been shown to remain well after the peptide has cleared (Satkunanathan et al., 2005). Thus, animals were re-tested at 7 days after the final drug administration. For PAM testing of cVc1.1, animals were also retested at 11 days after final drug administration.

5.2.4 Expression of data & statistical analyses

Data were analysed using Prism (GraphPad Software Inc. version 6.0b for Mac OS X, San Diego, CA, USA). Data are presented as mean ± SEM where appropriate.

Area under the curve (AUC) calculations, Student’s T-tests, and one- and two-way ANOVAs were performed using Prism software. P < 0.05 was considered significant. Significant effects are shown throughout as * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
Figure 5.1. Timelines of conotoxin testing.
5.3 **RESULTS**

5.3.1 **BACLOFEN ANALGESIA IS ATTENUATED BY SCH50911**

The dose-response relationship of baclofen was determined using the PNL pain model and Incapacitance test in C57Bl/6j mice in order to establish efficacious dose-response and time-response of the GABA$_B$R-antagonist SCH50911. A range of doses of baclofen (1, 2, 3, 4, and 5 mg/kg) was administered and tested over 180 mins. The AUC of the analgesic effects were calculated (Figure 5.2 A). Baclofen had significant analgesic effects compared to saline (F(5,24) = 20.7, P < 0.0001, ordinary one-way ANOVA with Fisher’s LSD test, comparing mean values for each dose to the saline-control group) at doses of 2 mg/kg (P < 0.05) and above (P < 0.001 for 3 mg/kg; P < 0.0001 for 4 and 5 mg/kg). However severe motor deficits (ataxia) occurred above 3 mg/kg. 2 mg/kg produced analgesia without motor side effects or sedation, and was therefore selected as the optimal dose. The peak analgesic effect of 2 mg/kg of baclofen occurred at 60 mins post-injection (Figure 5.2 B) and this time-point was used to test the effectiveness of GABA$_B$R-antagonist pre-treatment.

Pre-treatment with the long-acting GABA$_B$R-antagonist SCH50911 30 min prior to agonist treatment prevented baclofen (2 mg/kg)-induced analgesia in a dose-dependent manner. The AUC of the SCH50911-mediated inhibition over 180 min was calculated (Figure 5.2 C). SCH50911 inhibited baclofen-induced analgesia compared to saline (F(3,21) = 6.5, P < 0.01, ordinary one-way ANOVA with Fisher’s LSD test, comparing mean values for each dose to the saline-control group). Significant, near maximal, inhibition produced by 10 mg/kg (P < 0.01), and the time-course of this dose was assessed. 10 mg/kg SCH50911 significantly antagonised baclofen-induced analgesia compared to saline (F(3,31) = 9.5, P < 0.0001, ordinary one-way ANOVA with Fisher’s LSD test, comparing mean values for each dose to the control saline group), maintaining peak antagonism for at least 2 hours. By 4 hours post-treatment, inhibition of baclofen-analgesia was no longer significant (Figure 5.2 D).
Figure 5.2. Dose response relationships of GABAB receptor ligands. The PNL neuropathic pain model was applied to C57Bl/6j mice, and the degree of mechanical allodynia was measured on the Incapacitance test. 

A] Baclofen dose-dependently reversed mechanical allodynia. B] The time-course of 2 mg/kg baclofen was measured over 180 minutes. C] The dose-response relationship of SCH50911 inhibition of baclofen (2 mg/kg)-induced analgesia. D] Optimal SCH50911 pre-treatment time prior to baclofen (2 mg/kg) was determined.
5.3.2 α-Conotoxins in vivo in C57Bl/6j Mice

Initial tests of Vc1.1 in mice gave promising results, with 0.5 mg/kg and 0.17 mg/kg Vc1.1 reversing mechanical allodynia induced by PNL in C57Bl/6j mice (Figure 5.3 A). A dose of 0.5 mg/kg significantly reversed mechanical hyperalgesia compared to the saline-injected animals at each time-point, with maximal effects lasting for 4 hours (P < 0.001) and still significantly effective at 6 hours post-injection (P < 0.05, ordinary two-way ANOVA with Fisher’s LSD test). A dose of 0.17 mg/kg also produced significant analgesia, with peak effect at 4 hours post-injection (P < 0.001).

However, attempts to replicate the above findings were unsuccessful (Figure 5.3 B). The same batch, as well as another batch (QC25102011; data not shown) of Vc1.1 failed to produce any significant analgesia in the same pain model and test. Another analgesic conotoxin, RglA, was also unsuccessful at alleviating neuropathic pain either acutely (not shown), or after two consecutive days of administration (Figure 5.3 C). RglA was tested in the CCI model of neuropathic pain that used chromic catgut ligatures. CCI was comparable to PNL in the magnitude of pain induced, and was equally responsive to baclofen (Table 5.1).

Several potential causes for this loss of effect were investigated. In separate experiments, the route of injection was altered from s.c. to i.p. (Figure 5.3 D); BSA 0.1% was added to the injection to prevent any sticking of the peptide to the plastic during preparations (Figure 5.3 E); and the dose was increased 3-fold to 1.5 mg/kg (Figure 5.3 F). After numerous unsuccessful experiments, the peptide was sent to be re-tested by the synthetic chemists who provided us the drug. Integrity of the peptide was confirmed using HPLC.

Parameters of variability that were outside of the experimenters’ control were not tested, but are likely to have affected, if not accounted for, the inability to replicate testing. Mice are highly sensitive to environmental variability such as temperature, humidity, idiosyncrasies of seasonality etc. (Chesler et al., 2002; Ferguson and Maier, 2013; Wahlsten et al., 2006). The experimental environment at the time of testing was sub-optimal (air conditioning in the BMRI
mouse behaviour facility was inadequate), and future conotoxin testing will be performed in more adequate testing conditions.

**Table 5.1 Comparison of CCI and PNL pain models on the Incapacitance test**

<table>
<thead>
<tr>
<th>Time/treatment</th>
<th>Weight distribution ratio (R:L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CCI pain model</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.00 ± 0.01 (10)</td>
</tr>
<tr>
<td>Post-surgery</td>
<td>0.50 ± 0.02 (10)</td>
</tr>
<tr>
<td>Saline</td>
<td>0.55 ± 0.01 (4)</td>
</tr>
<tr>
<td>Baclofen</td>
<td>0.89 ± 0.03 (6)</td>
</tr>
</tbody>
</table>
Figure 5.3. Conotoxin testing in C57Bl/6j mice; Vc1.1 and RgIA. Conotoxins were tested in C57Bl/6j mice following induction of the PNL or CCI neuropathic pain models, and tested on the Incapacitance test. A| Vc1.1 dose-dependently reversed PNL-induced allodynia compared to pre-injection scores. B| Vc1.1-analgesia was unable to be reproduced. C| RgIA did not produce analgesia in the CCI neuropathic pain model. D-F| The PNL pain model was used for trouble-shooting experiments with n = 2 for all experiments. Vc1.1 did not produce analgesia after altering route of administration (D), adding 0.1% BSA (E), or after tripling the dose (F). Arrows indicate injection time. Injections were given subcutaneously (s.c.) unless otherwise stated.
5.3.3 Vc1.1 in vivo in 129Sv/Ev Mice

Vc1.1 was tested in the 129Sv/Ev strain of mice, onto which the α9-nAChR-KO mice were back-crossed. No baseline strain-dependent differences were observed between C57Bl/6J mice and 129Sv/Ev mice. The analgesic effect of Vc1.1 in the PNL pain model was tested, using an intra-muscular route of injection. This was the method used in all published Vc1.1 testing, which has been performed in rats, however, due to the small volume capacity of the mouse muscle, the upper limit of dose administered was limited to the stock concentration. 50 μL of undiluted 444 μm Vc1.1 stock gave a final dose of 0.2 mg/kg. The Incapacitance test revealed no effect of Vc1.1, either acutely, or after a repeated dosing approximately 24 hours later in either WT (Figure 5.4 A i) or KO (Figure 5.4 A ii) mice. However, the number of subjects used, particularly in the KO group where n = 1, were low and it is therefore not possible to judge whether the single animal in each group was representative. WT animals were also tested on the von Frey test, which revealed a small but significant effect 1 hour after the second dose (Figure 5.4 B) (P < 0.05, ordinary two-way ANOVA with Fisher’s LSD test). The von Frey and Incapacitance tests are highly correlated in our hands (Pearson’s correlation coefficient R² = 0.80, Figure 5.4 C) and have been consistently comparable in our lab in the past (Sadeghi et al., 2013), however, the present result suggest that the von Frey test may be slightly more sensitive for pharmacological studies than the Incapacitance test.
Figure 5.4. Vc1.1 testing in 129Sv/Ev WT and α9-nAChR KO mice. Intramuscularly administered Vc1.1 (0.2mg/kg) was tested in 129Sv/Ev WT and α9-nAChR KO mice. A] The Incapacitance test did not detect Vc1.1-induced analgesia in WT (i) or KO (ii) mice. B] In the same experiment, the von Frey test revealed a significant effect of Vc1.1 in WT mice at 1 hour after the second of two consecutive days of administration. C] The von Frey and Incapacitance tests were found to have a high degree of correlation. Note that in panel B, the number of subjects is too low to correctly interpret the behaviour. This data should be treated with caution.
5.3.4 cVc1.1 in vivo in 129Sv/Ev mice

The cyclised version of Vc1.1 (cVc1.1) was tested in the hope that the more stable peptide would yield more consistent analgesic effects. The cuff model of CCI neuropathic pain was used. For mechanical allodynia testing, the von Frey test was used, rather than the Incapacitance test, in anticipation of increased sensitivity to the pharmacological actions of cVc1.1 (see Section 5.3.3, above). For mechanical hyperalgesia testing, the PAM test was used. Doses of 0.3 mg/kg were given sub-cutaneously in both WT and KO mice. α-conotoxins are known to have cumulative effects when administered for multiple consecutive days, so cVc1.1 was administered for 3 (Figure 5.5 A) or 5 (Figure 5.5 B and Figure 5.6) days. Pain thresholds were tested prior to, and 1 hour after, injection. Follow-up allodynia (Figure 5.5) and hyperalgesia (Figure 5.6) testing was also performed at 7 days after the final dose of 5-day treatments. A further test at 11 days after final dose was performed for hyperalgesia testing (Figure 5.6).

Allodynia developed in both genotypes after surgery. Three consecutive days of cVc1.1 administration alleviated alldonya in WT animals at all post-treatment time-points (significant treatment effect $F(1,56) = 46.76, P < 0.0001$, Ordinary two-way ANOVA) (Figure 5.5 A i). KO animals did not show significant alleviation from allodynia (Figure 5.5 A ii), however the experiment was underpowered, and replication of the experiment may reveal significant effects. An a priori power analysis for repeated measures within-between interaction design indicates that numbers of 9 animals per group would be required to determine the analgesic effect of cVc1.1 in this experiment. Five consecutive days of cVc1.1 administration showed alleviation from allodynia in both genotypes (Figure 5.5 B i and ii). Again, these experiments were underpowered and should be cautiously interpreted without further replication. A priori power analysis indicates that groups of 7 animals would be required for this experiment.
Figure 5.5. cVc1.1 testing in 129Sv/Ev WT and α9-nAChR KO mice; mechanical allodynia. The anti-allodynic effects of subcutaneously administered cVc1.1 (0.3 mg/kg) was measured using the von Frey test. cVc1.1 was administered (indicated by arrows) daily for either 3 (A) or 5 (B) days, and mice were tested before and after administration. WT (A(i) and B(i)) and KO (A(ii) and B(ii)) data are separated for clarity. Note that in panel B, the number of subjects is too low to correctly interpret the behaviour. This data should be treated with caution.

Figure 5.6. cVc1.1 testing in 129Sv/Ev WT and α9-nAChR KO mice; mechanical hyperalgesia. The anti-hyperalgesic effects of subcutaneously administered cVc1.1 (0.3 mg/kg) was measured using the PAM paw pressure test. Paw withdrawal thresholds (PWT) were measured 1 hour after cVc1.1 daily administration (arrows) for 5 days. Animals were re-tested 7 and 11 days after the cessation of cVc1.1 administration. Both WT and KO genotypes were tested shown together A and separated by genotype in B and C.
Hyperalgesia was also investigated in a separate group of WT and KO mice using the PAM test (Figure 5.6). Unfortunately, the high frequency of 5-day testing with the extremely noxious stimulus caused a severe repeated-testing effect. This repeated-testing effect was not countered by the conotoxin, with both cVc1.1- and saline-treated animals showing a steady decrease in pain threshold over the 5 days of testing. When animals were re-tested 7 and 11 days after the final test day, pain thresholds had risen back towards the post-surgical thresholds. WT and KO animal thresholds did not significantly differ. This comparable repeated-testing-induced hyperalgesia between genotypes is in contrast to that seen in Chapter 3 (Section 3.3.4). The difference is speculated to be due to presumed greater tissue damage caused by the high frequency PAM testing in the current experiment, compared to the lower frequency of PAM testing in Chapter 3, wherein recovery may have occurred between tests. Future experiments must adapt to this rapid sensitisation of mouse responses.
5.4 Discussion

The α-conotoxin Vc1.1 is a promising analgesic that likely alleviates pain through a novel, and still controversial, mechanism. There is a large unmet clinical need for increased and improved pain management alternatives, which Vc1.1, and other mechanistically similar α-conotoxins, have the potential to fill. There is debate over the mechanistic target of Vc1.1, and similar α-conotoxins. The molecular target that was first identified was the α9α10-nAChR. However, there is robust evidence that inhibition of this receptor does not account for Vc1.1 analgesia. Here, I intended to test whether Vc1.1 analgesia occurs in mouse models of chronic neuropathic pain, irrespective of the presence of the α9α10-nAChR. Difficulty was encountered in attaining reproducible analgesia with α-conotoxins Vc1.1, RglA and cVc1.1. A major setback for the completion of conotoxin testing in mice was the sub-standard environmental conditions in the testing facility including defective climate control (temperature (as low as 14°C) and humidity) and high levels of noise. Dosing of conotoxins was also severely restricted by the limited availability of the peptides. Further experimentation is required in order to determine the efficacy of these α-conotoxins in mice, as well as in α9-nAChR KO mice, and after pre-treatment with a GABA_B antagonist.

5.4.1 Discontinuation of ACV1 Clinical Trials

In 2007, Metabolic Pharmaceuticals Pty Ltd (now a subsidiary of PolyNovo Ltd, formerly Calzada Ltd) deemed the continuation of trials of ACV1 (Vc1.1) cost prohibitive. The decision was made based on the finding that ACV1 had a 100-fold lower potency at the human over rat α9α10-nAChR (Azam and McIntosh, 2012). It was assumed that much larger doses would be needed in humans than in rats and the programme was announced as no longer tenable and phase 2A trials were stopped (Metabolic, 2007).

Seven years on from the decision to discontinue clinical trials of Vc1.1, new light has been shed on the molecular targets of the peptide. Not only does Vc1.1 potently and selectively inhibit α9α10-nAChRs, it inhibits N-type VGCCs in an
atypical GABA<sub>B</sub>-R-dependent manner (Callaghan and Adams, 2010; Callaghan et al., 2008). This atypical GABA<sub>B</sub>-R-dependent mechanism of action is unlike that of baclofen, which causes ataxia, and no such side effects of Vc1.1 or RgIA were observed here at any dose, nor have they been reported in other in vivo studies. It is possible that the decision to discontinue clinical trials was appropriate, and that low-affinity α9α10-nAChR-inhibitors would be unsuccessful analgesics in humans. However, VGCC-inhibiting mechanism was not considered at the time. The excellent in vivo results obtained from rat experiments may still be translatable, and are worth pursuing.

5.4.2 GABA<sub>B</sub> RECEPTOR-DEPENDENT Vc1.1 ANALGESIA

I was unable to determine the efficacy of Vc1.1 in mice consistently enough to test the ability of SCH50911 to inhibit Vc1.1-mediated analgesia. In vivo rat studies have shown SCH50911 to almost completely inhibit baclofen- and Vc1.1-mediated analgesia (Klimis et al., 2011). If the mechanism of action for α-conotoxins is indeed a GABA<sub>B</sub>-R-dependent inhibition of N-type VGCCS on sensory neurons, I anticipate the same results for both WT and KO 129Sv/Ev mice. Although Vc1.1 and similarly acting conotoxin analgesics are believed to act via a unique mechanism of GABA<sub>B</sub>R-binding, it is of interest to determine whether GABA<sub>B</sub>R-mediated analgesia such as with baclofen is altered in α9-nAChR-KO mice compared to WT mice. It is possible that GABA<sub>B</sub>Rs and α9α10-nAChRs interact to achieve the conotoxin-mediated analgesia.

5.4.3 PEPTIDE DRUG STABILITY

I found Vc1.1 to be an effective analgesic upon initial testing, however attempts to replicate this effect were largely unsuccessful (except perhaps for allodynia in Figure 5.5). Although integrity of the peptide was unlikely to account for the difficulties I encountered, stability of peptides is a major challenge for biologics in the pharmaceutical industry.

The main generic limitations that are associated with most peptides are their structural instability in reducing conditions, where the disulphide bond
connectivity is scrambled, and susceptibility to degradation by proteases. This can be overcome by methods such as backbone cyclisation (Clark et al., 2012; Clark et al., 2010), replacing disulphide bonds with non-reducible bonds (Armishaw et al., 2006; Chhabra et al., 2014; van Lierop et al., 2013), and conjugating the peptide to additional peptides with more favourable bioavailability (Belyea, 2006). The cyclised version of Vc1.1 has already been shown as highly effective in the CCI rat model of neuropathic pain (Clark et al., 2010), while efficacy in mice needs to be determined.

The ultimate aim of engineering highly stable versions of naturally occurring peptides is to achieve oral availability. Oral administration of analgesics is associated with greatest patient compliance and flexibility in dosing schedule, as well as being safer and cheaper than parenteral routes (Belyea, 2006). My initial experiments in mice, and the previous academic and patent literature in rats, have all confirmed Vc1.1 (Livett et al., and issued 10 October 2002; Nevin et al., 2007; Satkunanathan et al., 2005; Vincler et al., 2006) and cVc1.1 (Klimis et al., 2011) to be systemically active, and that cVc1.1 is orally active, which is encouraging for future development. It would be worthwhile in future to test the more stable cVc1.1 in the α9-nAChR KO models.

5.4.4 Binding site(s) of a-conotoxins on α9α10-nAChRs & species differences in affinity

Although there is little evidence that inhibition of α9α10-nAChRs is the primary mechanism of Vc1.1 analgesia, I have shown (see Chapter 3) that this receptor is involved in the development and maintenance of mechanical hyperalgesia in inflammatory and neuropathic pain states. It is, therefore, important to continue characterising the functional role of the α9α10-nAChR in pain and to delineate the role of the two molecular targets.

nAChR subunits have a primary face and a complementary face for ligand binding (Gotti et al., 2006; Taly et al., 2009). The primary component consists of a sequence of cystines adjacent to each other (vicinal), while the beta subunits
lack this sequence. Thus alpha subunits have both primary and complementary components, while beta subunits have only the complementary interfaces. The fact that functional α9α10-nAChRs are comprised of two alpha subunits is a unique instance of a heteromeric nAChR potentially possessing five binding sites per receptor, although no heteromeric nAChR has yet been reported to have this number of binding sites.

There is, as of yet, no agreed binding pocket of α9α10-nAChR-inhibiting conotoxins. Both Vc1.1 and RglA, which are presumed to have similar mechanisms of analgesic action, have been studied. In the (α9)2(α10)3 stoichiometry, the main question is whether the α9 subunit (α9α10) or the α10 subunit (α10α9) provides the principal face of the binding pocket (Pérez et al., 2009; Yu et al., 2013).

Studies of RglA receptor-binding have produced conflicting results. The first study used molecular modelling techniques to find that RglA binds to the α9α10 binding site, with RglA interacting with the Pro197 and Asp198 in the α9 principal face (Pérez et al., 2009). However, all residues in the α9α10 binding site, including those residues highlighted in that study, are conserved between rats and humans. This conflicted with the species difference in affinity of both RglA and Vc1.1, for the rat versus human α9α10-nAChR reported by Azam and McIntosh (2008) in a meeting abstract the previous year. The second publication (Azam and McIntosh, 2012) found through functional studies that RglA interacted with the α10α9 binding pocket, and a single residue within the complementary α9 face, position 56, was found to account for the species difference in potency of the conotoxin. The Thr56 of the rat subunit conferred higher sensitivity than the Ile56 of the human subunit. Homologous (e.g. Ser59 in α7 subunit, Arg57 of Ac-AChBP) or nearby (Thr59 in β2 subunit, Lys59 in the β4 subunit) residues in the complementary binding face of other proteins have been shown to be important for binding of other conotoxins (ImI, PnIA, MII and BuIA respectively) which adds support to these findings. Thus, RglA may bind to more than one site.
Combined computational and electrophysiological methods have revealed that Vc1.1 binds to the α10α9 binding site (Yu et al., 2013). Position 59 of the α9 subunit was found to confer specificity to the rat over human α9α10-nAChR.

No reports have yet detailed the homology of mouse α9 or α10 nAChR subunits with those of rat or human. A difference in the crucial residues for Vc1.1 and RgIA binding may account for the difficulty I encountered with testing. However, this is purely speculative and the success of initial experiments with Vc1.1 makes interpretation difficult. The compounds were not able to be re-evaluated in rats for logistical reasons, as the compound was expensive and only a small amount was available at the time.

Perhaps engineering bias toward one binding site or other (α9α10 or α10α9) will ultimately enhance favourable clinical outcomes (analgesia) and diminish potential on-target side effects (affective alterations, see Chapters 6 and 7) of analgesic α-conotoxins. Understanding the binding sites will further accelerate rational drug design, better elucidate species differences and optimise the progression of these α-conotoxins to future clinical trials.

5.4.5 Conclusions
Analgesic α-conotoxins such as Vc1.1, cVc1.1 and RgIA have proven to be effective analgesics in rat models of chronic neuropathic pain. This analgesia has been shown, in vivo, to require functional GABA\(_B\) receptors. Although Vc1.1 initially appeared to alleviate mechanical allodynia in WT C57Bl/6j mice in the present experiments, this effect was not consistently achieved. The role of GABA\(_B\)Rs in conotoxin analgesia in mice was unable to be tested, and further testing is required. Further testing is also needed to determine whether the conotoxins are equally effective in α9-nAChR KO mice, and their WT counterparts.
CHAPTER 6

BEHAVIOURAL PHENOTYPING OF α9-nAChR KO MICE IN THE INTELLICAGE
6.1 INTRODUCTION

During pain phenotyping (Chapter 3) and testing of conotoxins (Chapter 5) in WT and α9-nAChR KO mice, behavioural differences were observed between the two genotypes. Of note was the differing demeanour of the two genotypes, wherein KO mice appeared to be more calm than WT mice, and unaffected by handling, which is normally a situation of stress. Additionally, the noxious PAM testing evoked anticipatory behaviour in WT animals upon subsequent testing, which was not exhibited by KO animals. Other members of our lab found similar resistance to repeated testing-induced hypersensitivity (Section 3.3.4). These observations led me to investigate general activity profiles of the two genotypes, using naïve animals in their homecage environment.

Almost all organisms on earth have evolved intrinsic circadian “clocks” to deal with the 24-hour cycles of light and dark, from single-celled algae and fungi, to higher order animals including humans (Kalsbeek et al., 2012). The role of the circadian clock is to entrain organisms to environmental cues, and ultimately synchronise and control the phasing of internal physiological processes that strengthen homeostatic control mechanisms. Such synchronisation with the external environment ensures optimal anticipation of food availability, predation risk, likelihood of reproductive success etc. (Kalsbeek et al., 2012).

Circadian patterns exist for sleep (timing and quality), food consumption, and physical activity/arousal. Such rhythms are highly affected by socio-environmental factors that act on neural and endocrine systems. For example, the sleep-wake cycle is highly coupled to light exposure, which signals the "central pacemaker" in the suprachiasmatic nucleus (SCN) of the hypothalamus (Kalsbeek et al., 2012). Circadian cycles of the hypothalamic-pituitary-adrenal (HPA) axis stress hormones, including CRH, ACTH and corticosteroids, are controlled by endogenous stimuli driven by the SCN circadian clock, and are highly sensitive to stress (Balbo et al., 2010b).
Expression of the $\alpha 9\alpha 10$-nAChR in the HPA axis (pituitary and adrenal glands, see Section 1.5.4.6), suggest that this receptor could play a role in regulating circadian rhythms of this neuroendocrine stress axis. Therefore, behavioural phenotyping of WT and $\alpha 9$-nAChR KO mice was considered in the context of circadian rhythms. Since very little phenotyping of $\alpha 9$-nAChR KO mice has been reported (only in the context of audition (Brown and Vetter, 2009; May et al., 2002)), a broad approach of behavioural phenotyping was undertaken using the IntelliCage.

The IntelliCage is a home-cage monitoring system that allows the observation of laboratory mice over an extended period of time. The system can be used to monitor basic behaviours, such as activity levels and circadian rhythms, when comparing different treatment conditions such as gene-manipulation. The IntelliCage can also be used to study more complex behaviours through operant conditioning, and can offer insight into cognitive aspects of treatment groups, such as learning, memory and aversion. The system provides social enrichment through group housing, and environmental enrichment through the cognitive tasks applied.

Disruptions of biological rhythms commonly occur during stress, and are frequent in patients suffering from affective disorders such as major depressive disorder. One of the most significant effects of stress is to reduce the sensitivity to both positive and negative reinforcement, so that the former is not sought, and the latter is not avoided (Shumake and Gonzalez-Lima, 2003). Thus, I investigated whether $\alpha 9$-nAChR subunit deletion caused any alterations in response to positive and negative stimuli.

Hedonic deficits commonly result from chronic stress (Strekalova et al., 2004; Willner et al., 1992). The Diagnostic and Statistical Manual of Mental Disorders-V (DSM-V) diagnostic criteria lists “loss of interest or pleasure” as one of two core prerequisite symptoms, beside depressed mood, for the diagnosis of a major depressive episode (American Psychiatric Association, 2013). In rodents, stress induced anhedonia can be inferred from the sucrose preference test, wherein
Anhedonic animals show a reduced preference for normally palatable sucrose compared to water (Muscat et al., 1992). The IntelliCage is an ideal set-up for the sucrose preference test, as the exact amount of sucrose versus water consumed by each animal may be measured in a naturalistic setting. I first determined whether the α9-nAChR KO mice exhibited any hedonic deficits, and then whether the stress of changes in environmental predictability could induce such deficits.

Psychomotor retardation is the slowing of mental, physical and emotional reactions that often accompany affective disorders that are associated with HPA axis dysregulation (e.g. depression) (Shumake and Gonzalez-Lima, 2003). Two tasks were designed using the IntelliCage software that challenged psychomotor control; the Impulsivity tasks required cognitive effort, and the Aversion task evoked an emotional component.
6.2 METHODS

For general materials and methods used in this chapter, refer to Chapter 2 under the following section headings:

2.1 Animals
   2.1.1 Ethics approval
   2.1.2 Mice
   2.1.3 Genotyping of α9-nAChR KO mice
   2.1.4 Euthanasia

2.7 IntelliCage
   2.7.1 The IntelliCage system
      2.7.1.1 Cage composition and software
      2.7.1.2 Intelligent corner composition
      2.7.1.3 RFID transponder implantation
   2.7.2 Experimental design
      2.7.2.1 General activity & circadian rhythms
      2.7.2.2 Socio-environmental effects of group housing
      2.7.2.3 Sucrose preference
      2.7.2.4 Learning tasks: Impulsivity & Aversion

See also Appendix B for experiment notes and detailed descriptions.
6.3 RESULTS

6.3.1 The IntelliCage monitoring system measures gross activity levels: Visits, Nosepokes & Licks

Gross activity (Visits, Nosepokes and Licks) of WT and α9-nAChR KO mice was monitored, with animals housed either as separate-genotype (Run1) or mixed-genotype (Run2).

Initial high levels of gross activity were observed for both genotypes on IntelliCage day 1, as the mice acclimatised to the new environment. Levels of activity stabilised throughout the IntelliCage testing (Figure 6.1).

Overall, some variations emerged between genotypes. For separate-genotype housed mice in Run1, two-way ANOVA revealed that while general exploratory activity was comparable between genotypes (no significant main effect of genotype on Visits, $P = 0.07$, $F(1, 15) = 3.9$) (Figure 6.1 A), and number of Nosepokes ($P = 0.11$, $F(1, 15) = 2.9$) (Figure 6.1 C), KO mice performed a greater number of Licks overall (significant genotype effect on Licks, $P < 0.0001$, $F(1, 135) = 72.9$, accounting for 14.6% of variation) (Figure 6.1 E). These differences may reflect idiosyncrasies of the sample, or cage-effects, as the same trends were not seen in Run2, when genotypes were mixed-housed. On the contrary, Run2 showed a small but significant main effect of genotype on exploratory activity ($P < 0.05$, $F(1, 10) = 5.2$, accounting for 11.3% of variation) (Figure 6.1 B) with KO mice performing overall more Visits than WTs, but Nosepokes ($P = 0.2$, $F(1, 10) = 2.1$) (Figure 6.1 D) and Licks ($P = 0.2$, $F(1, 10) = 1.9$) (Figure 6.1 F) were comparable between genotypes.
**Figure 6.1. General activity measures over the course of IntelliCage testing.** Behavioural indicators of Visits, Nosepokes, and Licks per day during IntelliCage testing, for Run 1 (A, C, E) and Run 2 (B, D, F). Vertical lines denote module switches. Grey areas denote Sucrose Adaptation module.
6.3.2 Module switches in the IntelliCage have minimal effect on behaviour

The effect of each module on behaviour was determined by analysing activity binned by module. Overall, there was minimal effect of switching modules on gross activity levels. KO mice show a trend for greater levels of gross activity compared to WTs (Figure 6.2 A and B). In Run1 this is particularly apparent in the earlier modules, with KO activity levels converge to match WT activity levels in later modules. In Run2, KO mice consistently show a trend for greater levels of activity, although the differences are not significant for any module (Figure 6.2 B).

In Run1, KOs showed significantly higher activity levels than WTs for Visits during the DSA module (t(135) = 3.8, P < 0.05, two-way ANOVA with Bonferroni’s post hoc test) (Figure 6.2 A); for Nosepokes during SAI (t(135) = 3.9, P < 0.05) (Figure 6.2 C); and for Licks during the FA (t(135) = 7.7, P < 0.0001) and NPA (t(135) = 4.0, P < 0.05) modules (Figure 6.2 E). Since KO mice exhibited greater overall levels of activity (see Section 6.3.1), it is likely that these significant genotype differences were dependent on the acclimatisation time, rather than the module switch itself. Additionally, since the experiment was not repeated, cage-effects could not be ruled out.

In Run2, fewer differences were seen between genotypes for each module, with the only significant difference between genotypes being the number of Licks during FA (t (70) = 3.7, P < 0.05) (Figure 6.2 F).
Figure 6.2. Average activity measures per module. Behavioural indicators of Visits, Nosepokes, and Licks per Module during IntelliCage testing, for Run1 (A, C, E) and Run2 (B, D, F). FA, Free Adaptation; NPA, Nosepoke Adaptation; DSA, Drinking Session Adaptation; SA, Sucrose Adaptation; Imp, Impulsivity.
The effect of module switches within each genotype was also investigated. For Run1, WT exploratory activity was unaffected by the changes in modules over time (P > 0.05 for all comparisons, two-way ANOVA with Bonferroni post hoc analysis), while KOs showed significantly greater numbers of Visits during the earlier modules than later ones (Table A1). Nosepokes (Table A2) and Licks (Table A3) were also significantly higher in both genotypes for earlier modules. Again it is difficult to distinguish whether these differences are due to increased acclimatisation over time, or the modules themselves, and are likely to be strongly influenced by cage effects.

For Run2, no significant differences in Visits occurred within either genotype, as the modules were switched (Table A4). Nosepokes (Table A5) and Licks (Table A6) were significantly higher for only the first module, FA, compared to subsequent modules.

Overall, the impact of switching modules appeared to have minimal effect on overall gross activity, in terms of Visits, Nosepokes and Licks. Greater activity was apparent towards the start of testing, which likely reflects the time needed for complete acclimatisation.

6.3.3 CIRCADIAN PATTERNS ARE ALTERED IN α9-nAChR KO MICE BUT ARE SENSITIVE TO SOCIO-ENVIRONMENTAL FACTORS

Circadian patterns were inferred from the number of Visits performed by mice in the IntelliCage over time. Circadian patterns of the first 5 days of IntelliCage housing are shown in figure (Figure 6.3 A & C), with average activity levels for separate- and mixed-genotype housed animals (Run1 and Run2 respectively) binned by the hour. Activity on day 1 was not representative of daily activity, due to the novelty of the IntelliCage environment, and was therefore excluded from further analysis (Figure 6.3 B & D).
Figure 6.3. Circadian patterns of exploratory activity for WT and KO mice during the Free Adaptation module of IntelliCage testing. The numbers of Visits were binned by hour for the first 5 IntelliCage days for Run1 (A) and Run2 (C). Day 1 activity was not representative of daily activity and was excluded from the pooled and averaged hourly Visits for the Free Adaptation module for Run1 (B) and Run2 (D). Grey areas represent the dark cycle. Asterisks indicate significant differences between WT and KO Visits per hour; * P < 0.05, ** P < 0.01, **** < 0.0001.
When genotypes were housed separately (Run1; Figure 6.3 A and B.), KO mice demonstrated altered circadian patterns compared to WT mice. Both genotypes were relatively inactive during the light period of the light/dark-cycle. WT animals displayed a bi-phasic activity pattern typical of crepuscular animals, with peaks in activity occurring around lights-off and lights-on, and a drop in activity prior to the second peak. In contrast, KO animals had a relatively monophasic nocturnal activity pattern with high activity occurring throughout the dark-cycle. Two-way ANOVA revealed no main effect of genotype (P = 0.15, F(1, 6) = 2.8) indicating comparable activity levels for the two genotypes. However, significant interaction effects between time and genotype (P < 0.0001, F(23, 138) = 5.8, accounting for 13.6% of variation) indicate that the pattern of activity is dissimilar between WT and KO genotypes. Post hoc Bonferroni’s multiple comparisons test revealed significant differences between WT and KO genotypes at several time-points, indicated by asterisks in Figure 6.3 B.

When WT and KO genotypes were housed together (Run2; Figure 6.3 C and D.), the great impact of socio-environmental factors exerted changes on activity patterns of both genotypes compared to when genotypes are separate-housed. Mixed-housed WT and KO mice showed largely synchronous activity patterns. However, neither a typical crepuscular pattern, nor the sustained nocturnal activity of separate-housed KO mice was observed. Rather, a compromise between the crepuscular and nocturnal activity patterns was apparent with peaks around lights-off and lights-on, as well as an additional peak during the lights-off period. Unlike separate-housed KO mice, mixed-housed KOs adopted the pre-dawn nadir of single-genotype housed WT animal (Figure 6.3 D.). A significant main effect of genotype was observed for Run2 (P < 0.001, F (1, 6) = 41.6, accounting for 4.4% of variation), as KO mice showed overall greater activity levels than WTs. This is in agreement with overall increased activity levels of the KO animals in Run2 (see Section 6.3.1, above). As for Run1, Run2 revealed significant main effects of interaction between time and genotype (P < 0.0001, F(23, 138) = 6.0, accounting for 13.6% of variation), indicating that the genotypes maintain some variance in their circadian activity patterns, when mixed-housed. Significant differences between WT and KO genotypes were again
observed at various time-points, using Bonferroni’s post hoc multiple comparisons test (indicated by asterisks in Figure 6.3 D). However, many of these differences reflect the differences in the magnitude of activity levels of the genotypes, rather than in circadian activity, which remain relatively synchronous. Notably, a pre-dawn nadir as seen in separate-housed WTs was seen in both genotypes when mixed-housed, with no significant difference in magnitude.

6.3.4 Sucrose preference develops in WT & KO mice in the IntelliCage home-cage

A strong sucrose preference developed in both WT and KO genotypes within 3 days of sucrose availability, whether genotypes were housed separately (Run1, Figure 6.4 A.) or together (Run2; Figure 6.4 B.). When genotypes were housed separately, WT animals showed an 83 ± 7% preference for sucrose bottles over water bottles, while KO animals show a 81 ± 7% preference. There was no significant difference in preference between genotypes (P = 0.85, Unpaired Student’s T-test). When genotypes were housed together, WT animals show a 96 ± 2% preference for sucrose bottles over water bottles, while KO animals showed a 88 ± 4% preference. Again, there was no significant difference in preference between genotypes (P = 0.24, Unpaired Student’s T-test).
Figure 6.4. Sucrose preference developed in both WT and α9-nAChR KO mice. The preference of WT (green) and KO (purple) genotypes for sucrose over water was observed in Run1 (A) and Run2 (B). No significant difference in sucrose preference was observed between the two genotypes (P > 0.05).
6.3.5 Anhedonia develops in KO mice after cognitive challenge

After the establishment of sucrose preference in both genotypes, a cognitively challenging task was introduced. The Impulsivity test (day 28 for Run1, day 25 for Run2) required complex learning in order to attain the palatable, and preferable sucrose, compared to the freely available water. The difficulty of this task inhibited the ability of the animals to drink sucrose. This challenge did not significantly affect the overall drinking (total licks; see Section 6.3.2, Figure 6.2 E), but simply altered the side (left or right) of Nosepokes (Figure 6.5 C & D) and Licks (Figure 6.5 A & B).

After seven days of the Impulsivity test, freely available sucrose was reintroduced. In Run1, WT animals rapidly regained a strong and sustained sucrose preference for the duration of SAII. However, KO animals exhibited a sustained anhedonia, with a Lick preference for water over sucrose for the duration of SAII (significant main effect of genotype, P < 0.0001, F(1, 15) = 36.5, accounting for 57.9% of variation) (Figure 6.5 A). Multiple comparisons testing showed that the KO animals exhibited significantly (P < 0.0001, Bonferroni’s post hoc test) lower right-side preference for every day of SAII compared to the WT mice, except for the first (P > 0.05). The increase in Nosepokes and Licks on the RHS during SAII, compared to Impl, indicates that the availability of sucrose was perceived by the KOs, but the preference was not established. A second round of the Impulsivity test again reduced the Nosepoke and Lick preferences from the right side for both genotypes. The reintroduction of sucrose during SAIII revealed the same trend of strong sucrose preference in the WT mice but lack of such re-acquisition in the KO mice (significant main effect of genotype for RHS Lick preference, P < 0.0001, F(1, 15) = 42.3, accounting for 53.5% of variation). The significantly greater right-side Nosepoke preference in WT mice over KOs during both of the Impulsivity tasks (significant main effect of genotype for Impl (P < 0.01, F(1,15) = 10.4, 24.5% of variation) and ImpII (P < 0.001, F(1, 15) = 19.0, 39.6% of variation), two-way ANOVA for Impulsivity modules) suggests that the WT’s continued to seek the sucrose to a greater degree than the KOs.
Figure 6.5. Anhedonia developed in KO mice. The preference of animals for the RHS bottle, which contains sucrose during Sucrose Adaptation modules, and water during all other modules, was observed for Run1 (A, C) and Run2 (B, D). The Lick preference (A, B) and Nosepoke preference (C, D) per day was determined. Asterisks indicate significant differences between WT and KO preferences per IntelliCage day; * P < 0.05, ** P < 0.01, **** < 0.0001.
The anhedonia seen in KO mice was less pronounced in Run2, when the genotypes were housed together, wherein social and environmental factors affected the drinking behaviour of the two genotypes. However, the anhedonia was robust enough to overcome these strong socio-environmental cues, as the KOs displayed a slower re-acquisition of sucrose preference during SAll in Run2, indicated by the right-side preference for Licks (significant main effect of genotype, P < 0.001, F(1, 15) = 9.1, accounting for 18.5% of variation) and Nosepokes (significant main effect of genotype, P < 0.05, F(1, 15) = 6.1, accounting for 17.2% of variation)(Figure 6.5 B and D).

The air puff introduced in the Aversion module appeared to be exceedingly aversive and completely abolished RHS Nosepokes and Licks (data not shown). The responses of the two genotypes did not significantly differ in this module.
**6.4 DISCUSSION**

The α9 subunit of nAChRs is an evolutionarily old subunit, with highly unusual pharmacological and biophysical characteristics. It has a well characterised role in the cochlear and vestibular systems (Chen et al., 1996; Elgoyhen et al., 1994; Elgoyhen et al., 2001; Sgard et al., 2002), and has relatively recently been implicated in pain (Vincler et al., 2006) and stress (Colomer et al., 2010). A mouse strain with germ line deletion of the α9-nAChR has allowed more thorough characterisation of the functional role of this receptor. Phenotyping of α9-nAChR KO mice has previously been limited to traits related to auditory perception (May et al., 2002), and behavioural traits have not yet been considered. Here, I looked at how deletion of the α9-nAChR affected the general home-cage phenotype of mice, how robust those changes were to socio-environmental influences, as well as how the absence of the receptor affects coping behaviour upon environmental manipulation.

**6.4.1 KO mice showed variable increases in general activity compared to WTs**

Small but significant increases in certain measures of general activity were seen in KO mice compared to WTs. These differences were not consistent across the two runs, and might, therefore, not reflect true phenotypic differences. They may merely reflect idiosyncrasies of the two samples, and may have been influenced by extraneous environmental factors since the two runs began approximately 3 months apart. The fact that the differences were consistently increased in KO animal activity (Licks for Run1, Visits for Run2) may indicate a trend for greater metabolic activity in the α9-nAChR KO mice. Metabolic activity is comprised of several components such as resting energy expenditure, physical activity, thermoregulation and thermic effect of food (Tou and Wade, 2002). Basal metabolic rate, the minimal rate necessary for sustaining basic physiological processes in awake quiescent animals, is correlated with voluntary activity as well as other measures such as dietary consumption (Gebczynski and
Konarzewski, 2009) and may differ between WT and KO animals. However, this would require further investigation, such as through metabolic cages (Adamovich et al., 2014; Castelhano-Carlos et al., 2014) or telemetry (Clement et al., 1989; Williams et al., 2003).

6.4.2 KO MICE SHOWED AN ALTERED CIRCADIAN PATTERN OF ACTIVITY
Mice are predominantly crepuscular animals (Goulding et al., 2008), with their circadian activity patterns having two peaks, at dawn and at dusk (Aschoff, 1966). Crepuscular activity is believed to have given prey animals an evolutionary advantage through being less exposed to both diurnal and nocturnal predators (Daly et al., 1992). The most potent environmental cue for the regulation of circadian rhythms is light exposure, but non-photic influences also affect circadian clocks including physical activity, food intake and social interactions (Mrosovsky, 1996; Nelson et al., 1975).

Here, clear crepuscular activity patterns were observed in both WT and KO mice during the Free Adaptation module. However, The KO mice exhibit far more nocturnal behaviour than do WT animals. This alteration in IntelliCage activity patterns implicates altered sleep architecture in KO mice compared to WTs. Sleep disturbances are often linked with HPA-axis dysregulation (Balbo et al., 2010a). In particular HPA-axis hyperactivity can have negative effects on sleep physiology including fragmentation of sleep, decreased slow-wave (non-REM) sleep, and shortened overall sleep time. Furthermore, poor sleep can exacerbate HPA-axis dysfunction, worsening the cycle (Buckley and Schatzberg, 2005). These alterations in sleep architecture and HPA-axis are a characteristic of affective disorders, in particular major depression (Antonijevic, 2008; Spath-Schwalbe et al., 1991).

Circadian rhythm disturbances in depression are associated with disorganisation or blunting of daily rhythms, and desynchronisation of different biological rhythms (e.g. hormones, body temperature, sleep) from one another (Ehlers et al., 1988). However, the initiation of depression/affective disorders has long
been acknowledged to be influenced by social support systems, and social contacts exert a clear influence on the synchronisation of circadian rhythms (Brown et al., 1973). Furthermore, the underlying causes of sleep disorders are often socially determined and that sleep can therefore be thought of as a mechanism through which social factors affect health (Hale, 2010). The clear differences in nocturnal activity seen in Run1 were not apparent in Run2, where mixed genotype-housed WT and KO mice behaved in a more synchronised manner. It is likely that in Run2, the social factors that can synchronise circadian rhythms, particularly in cohabitating individuals (Ehlers et al., 1988) prevented the highly dysregulated circadian activity rhythms in the separate genotype-housed KO mice. The effect of environment on circadian patterns is well known. Indeed, environmental cues have been shown to influence the circadian activity of other rodents, which exhibit either nocturnal or crepuscular behaviour depending on whether they are captive or wild (Gattermann et al., 2008; Mrosovsky, 1999).

It is difficult to distinguish sleep habits from the effects of physiological circadian rhythms due to the tight concordance of habitual sleep-wake times with certain circadian phases (Balbo et al., 2010a). However, methods such as electroencephalographic (EEG) recordings through telemetry could be performed in addition to home-cage monitoring, for a more well rounded analysis (Tang and Sanford, 2002).

6.4.3 KO mice showed normal hedonic preferences, but were more susceptible to stress-induced anhedonia

When offered the choice between a palatable sucrose solution and water, both WT and α9-nAChR KO mice showed a strong and sustained sucrose preference. Thus the hedonic preferences of mice did not appear to be altered through α9-nAChR deletion, and the motivation to perform RHS Nosepokes was comparable between genotypes.
The cognitive challenge imposed by the highly difficult Impulsivity task appears to have caused a strong enough cognitive stress to induce an anhedonia-like response from the KO mice upon subsequent Sucrose Adaptation modules. The rewarding properties of normally pleasurable rewards are known to decrease after persistent stress, whether it be severe or mild (Papp et al., 1991). This decreased sensitivity to rewards is one of the most central features of depressive disorders (American Psychiatric Association, 2013; Cryan and Holmes, 2005).

Anhedonia has been explained through a learned helplessness theory of depression (Seligman et al., 1979) and lack of environmental predictability (Katz, 1981). Learned helplessness is the term given to the behavioural adaptation to stressful or aversive environments due to a perceived lack of internal control (helplessness) over that environment. This manifests as acquisition and performance deficits due to a history of ineffective coping (Katz, 1981). The depressive state is thought to result from a generalized belief in the uncontrollability of the environment, which is induced by a lack of contingency between the individual’s coping response and environmental outcome (Ehlers et al., 1988). Dysregulation of the HPA axis is involved in helpless behaviour (Edwards et al., 1990), in particular elevated corticosteroids are associated with depression (Zhao et al., 2008).

In the present experiments, environmental predictability changed from the initial positive pairing of NPs on RHS with palatable sucrose, to Impulsivity tasks that proved overly restrictive, and impeded the animals’ access to sucrose for 7 days. Such a persistent loss of reinforcement from the environment creates and then maintains depressive behaviour (Ehlers et al., 1988).

Once again, the distinct phenotypic difference seen between the WT and KO genotypes was less pronounced in Run2 compared to Run1. Unlike the baseline circadian rhythm differences, the development of anhedonia was a robust effect that, although diminished, was still present in the mixed genotype-housed experiment. As for circadian rhythms, social environment plays a crucial role in the aetiology of depression, both as provoking and predisposing factors (Brown
and Harris, 1986). Here, it seems that social factors were able to attenuate the development of depression-like anhedonic behaviour in the KO mice, but not to abolish it.

### 6.4.4 Future experiments: Improvements & new tasks

Impulsivity is known to be a symptom of depression, although not a major one, and likely reflects psychomotor agitation (Willner et al., 1992). Unfortunately, the impulsivity task designed here was too challenging for the animals to grasp, and no differences in the two genotypes could be discerned. Similarly, the test of aversion, wherein sucrose drinking was paired with an air-puff, was too aversive, and was unable to discern any differences between genotypes. Future improvement of paradigms is needed for impulsivity and aversion to be tested in the IntelliCage.

The experiments presented here evoke myriad further questions. Causal relationships are not known among sleep disturbances, HPA-axis dysregulation, and mood disorders. However, exploration of these features in α9-nAChR KO mice may further enlighten the field. It would be of great interest to pursue in more detail the circadian rhythm changes in α9-nAChR KO mice, as well as their predisposition, if any, for stress-induced depression-like behaviour.

In terms of circadian rhythmicity, it would be of interest to know whether other biological rhythms bedside activity patterns are altered. In particular, whether HPA-axis hormones are affected. Since a significant functional role of the α9α10-nAChR in the adrenal gland has been described (Colomer et al., 2010), a logical measure would be corticosterone periodicity. Corticosterone is the predominant stress hormone in mice, and release of this hormone from the adrenal gland is controlled by HPA-axis circuitry as well as by direct signalling from the SCN central pacemaker (Buijs et al., 1999; Moore and Eichler, 1972).

Corticosterone is only one component of a complex neuroendocrine system, comprising of many hormones, peptides, target receptors and target organs.
Although adrenal disruptions are the likely explanation for any HPA-axis disruptions in α9-nAChR KO mice, there is the potential that disruptions exist at any, and potentially many points in the HPA-axis. Other hormones, such as ACTH, have been implicated in sleep disturbances (Meerlo et al., 2002). Similarly, CRH release is directly affected by both SCN input and corticosteroid signalling to affect arousal (Buckley and Schatzberg, 2005). However, logistical difficulties of measuring CRH in mice or measuring the circadian pattern of release limit the exploration of these questions.

To determine whether the apparent anhedonia exhibited by the α9-nAChR KO mice is a true reflection of depression-like behaviour, it would be necessary to repeat the experiment and attempt reversal or prevention of the behaviour through antidepressant treatment (Willner et al., 1987). To continue to exploit the valuable group-housing paradigm of the IntelliCage, such pharmacological treatments would require passive administration such as through the drinking bottles, rather than through experimenter intervention (File and Hyde, 1978).

Further detailing of the α9-nAChR KO behavioural phenotype is valuable for determining not only the functional role of the receptor in as yet unexplored systems such as the HPA-axis, but also for maximising awareness of potential side effects that may arise if this receptor is to be targeted by any therapeutic interventions.

6.4.5 Conclusions

Behavioural phenotyping of α9-nAChR KO mice was conducted, investigating general home-cage activity, as well as responses to several complex tasks. The IntelliCage experiments revealed an alteration in circadian rhythms of activity. This alteration was highly sensitive to socio-environmental influences, and was normalised when KO mice were housed with WT animals. KO mice demonstrated a stress-induced anhedonia, whereby an initially normal sucrose preference was lost after a period of stressful cognitive challenge. This anhedonia was found to be robust in that the effect, although diminished, persisted in the KO mice when
housed in mixed-genotype environments with non-anhedonic WT mice. The phenotype uncovered here highlights the vulnerability of KO mice to circadian dysregulation and to stress-induced depression-like behaviour, which must be considered when targeting the α9α10-nAChR with pharmacological agents. This altered phenotype prompted further investigation into the vulnerability of α9-nAChR KO mice to stress-related phenotypic differences from WT mice, which is explored in Chapter 7.
CHAPTER 7

STRESS, ANXIETY &

THE α9α10-nAChR
7.1 Introduction

Investigation into the behavioural phenotype of WT and α9-nAChR KO mice in the IntelliCage (see Chapter 6) revealed that KO mice display altered stress reactivity. These results, in addition to my observations of the differing demeanour of KO mice in stressful testing environments (described in Section 6.1), and the differing pain responses to both noxious PAM testing and repeated hotplate testing (see Chapter 3) led me to consider the possibility that inhibition or absence of α9α10-nAChRs results in modulation of the HPA-axis stress response. Such involvement of the α9α10-nAChR in HPA-axis responses could contribute to the regulation of affective state, and to a system-wide modulation of the subjective pain experience.

α9α10-nAChR-inhibiting pharmacological agents have demonstrated analgesic activity in numerous animal pain models of widely varying aetiologies. These include pain of neuropathic- (Clark et al., 2010; Klimis et al., 2011; Livett et al., and issued 10 October 2002; Nevin et al., 2007; Satkunanathan et al., 2005; Vincler et al., 2006), inflammatory- (McCracken, 2005), chemogenic- (Wala et al., 2012), and metabolic- (McCracken, 2005) origin. Analgesic effects are particularly evident after prolonged administration of these α9α10-nAChR-inhibiting agents (Livett et al., and issued 10 October 2002). Although non-nAChR targets of those compounds may account for some or all of the analgesic effects, it cannot be ruled out that inhibition of α9α10-nAChRs may be contributing to this analgesia. If inhibition of α9α10-nAChRs does account for the diminished pain perception, the question remains as to how such a broad range of pain conditions are able to be relieved through a single mechanism. The lack of CNS expression (Elgoyhen et al., 1994) implies that the analgesic effect of inhibiting α9α10-nAChRs is not mediated by altering central processing of the pain signal. Similarly, no definitive support for any peripheral mechanism (inhibition of peripheral nerve or immune cell α9α10-nAChRs) has yet emerged as an established mechanism (see Section 1.6.2).
Pain is a stressor, which activates the HPA-axis (see Section 1.8.3). The α9α10-nAChR is expressed in both the pituitary (Elgoyhen et al., 1994) and adrenal glands of the HPA-axis, and has been shown to be upregulated in rat adrenal medulla after persistent stress (Colomer et al., 2010). Thus, there is already anatomical and behavioural evidence that this receptor is key to mounting a normal HPA-axis response to stressful insults such as pain. It is possible that pharmacological suppression of α9α10-nAChRs may dampen the acuity of the stress caused by chronic pain, and therefore decrease the subjective pain experience. The cumulative and prolonged analgesic effects of repeated administration of α9α10-nAChR-inhibiting compounds (e.g. Satkunanathan et al. (2005)), even after peptide clearance, lends support to such a hypothesis, in that modulation of HPA-axis feedback circuitry occurs both acutely and in the order of days (Kudielka et al., 2004; Tsigos and Chrousos, 2002).

I hypothesised that the α9-nAChR KO mice have disrupted HPA-axis feedback circuitry, resulting in decreased affective regulation. This could manifest as reduced emotional memories, and therefore reduced anticipation of pain during testing, and potentially, less depression and/or anxiety-like behaviour. In order to better understand the role of the α9α10-nAChR in the HPA-axis response to stress, behavioural and physiological responses were compared between WT and α9-nAChR-KO mice. Behavioural tests were used to assess the phenotype of baseline and stress-induced affective responses of the mice. Corticosterone, the primary stress hormone in mice, was measured as the physiological marker of the HPA-axis response to both acute and chronic stress.
7.2 METHODS

For General Materials and Methods used in this chapter, refer to Chapter 2 under the following section headings:

2.1 Animals
   2.1.1 Ethics approval
   2.1.2 Mice
   2.1.3 Genotyping of α9-nAChR KO mice
   2.1.4 Euthanasia

2.2 Anaesthesia and general surgical procedures
   2.2.1 Anaesthetic
   2.2.2 Induction and recovery

2.8 Stress & affective disorders
   2.8.1 Elevated plus maze
   2.8.2 Forced swim test
   2.8.3 Restraint stress
   2.8.4 Physiological stress response
      2.8.44 Blood collection
      2.8.44 Corticosterone measurement

Specific Methods relating to this chapter:

7.2.1 HOUSING CONSIDERATIONS
Animals were housed no more than 6 per cage, with stressed animals housed separately from control animals so as to avoid chemosignalling of emotional information from stressed conspecifics (Knapska et al., 2010; Zalaquett and Thiessen, 1991). Non-stressed animals were housed 2 per cage, which allowed for serum collection from each animal within minutes of cage-disturbance, while avoiding the social isolation of single housing.
7.2.2 Temporal Considerations of Stressors and Behavioural Tests

All testing was conducted during the light portion of the dark/light cycle. To limit the influence of circadian factors, application of the final stressor and all blood collection was undertaken between 0900 and 1100. Control animals remained in the holding room until blood collection, which was completed within 1-2 minutes of cage disturbance.

Habituation to stressors is common in rodents, wherein repeated exposure to a stressor that initially elicits a spike in stress hormones, loses its potency and no hormone spike is elicited. Habituation to stressors is avoided by using “unpredictable” timing, so that the stressful insult is unable to be anticipated, as well as by alternating the type of stressor (Grissom and Bhatnagar, 2009).

Here, stressors with physical (restraint, FST), psychogenic (restraint, FST, EPM) and metabolic (FST) components were used. The restraint stress was the only stressor to be repeated on consecutive days, and therefore followed an unpredictable timing schedule (Table 7.1).

Table 7.1. Timing schedule of stress paradigm.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Persistent</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FST</td>
</tr>
<tr>
<td>Stress</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>09:00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-11:00</td>
</tr>
<tr>
<td><strong>Acute</strong></td>
<td>Restraint stress</td>
<td>EPM</td>
<td>FST</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stress</td>
<td>19:00</td>
<td>12:00</td>
<td>07:00</td>
<td>15:00</td>
<td>11:15</td>
<td>09:00</td>
</tr>
<tr>
<td></td>
<td>-21:00</td>
<td>-14:00</td>
<td>-09:00</td>
<td>-17:00</td>
<td>-14:00</td>
<td>-11:00</td>
</tr>
</tbody>
</table>
7.2.3 STRESS PARADIGM

Stress triggers long-lasting anatomical remodelling of intracellular communication, which has functional implications (Colomer et al., 2012). Chronic stress, over periods of months, is associated with affective disorders. However, stress-induced changes in the expression levels of α9α10-nAChRs have been shown to occur within 5 days (Colomer et al., 2010). In order to minimise the distress caused to the animals, persistent stress of 6 days was used, rather than a standard chronic stress model of 28+ days (Willner, 1997). This was predicted to evince any differences in stress response between the two genotypes that may result from α9α10-nAChR upregulation, while ensuring minimal distress would be caused to the animals.

In acute-stress groups, animals performed the FST, and blood was collected after 15 mins. In persistent stress groups, animals underwent daily restraint stress for 4 consecutive days, the EPM on day 5, and finally, the FST followed by blood collection on day 6 (Table 7.1).

Restraint stress was performed at differing times of the day to avoid habituation.

7.2.4 EXPRESSION OF DATA & STATISTICAL ANALYSES

Data were analysed using Prism (GraphPad Software Inc. version 6.0b for Mac OS X, San Diego, CA, USA) and SPSS (IBM® SPSS® Statistics, Version 21, Armonk, NY) software. All data are presented as mean ± SEM.

Student’s T-tests and one-, and two-way ANOVAs were performed using Prism software. The between-subjects main effects of genotype (WT or KO) and treatment (non-stressed or stressed) were evaluated with two-way ANOVAs. When significant effects were observed, Bonferroni’s or Tukey’s post-hoc tests were used. P < 0.05 was considered significant. Significant effects are shown throughout as * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
7.3 RESULTS

7.3.1 BEHAVIOURAL STRESS RESPONSES

7.3.1.1 STRESS-INDUCED AROUSAL BEHAVIOUR IS DECREASED IN α9-nAChR KO MICE

Differences in stress-induced arousal were observed between WT and α9-nAChR KO mice in the FST and EPM.

In the FST, the time spent immobile did not differ between non-stressed WT and KO mice. After persistent stress, both genotypes spent significantly less time immobile (P < 0.05, F (1, 28) = 6.3, main effect of treatment, 2-way AVOVA, not RM), accounting for 17.92% of total variation (Figure. 7.1). Such increased activity is indicative of a heightened state of arousal, brought about by persistent stress. A trend for the KO animals to spend more time immobile than WTs was observed, indicating the KOs were less prone to stress-induced arousal. This difference did not reach significance (P > 0.05), however this is likely to be due to the experiment being underpowered. A post hoc power analysis for ANOVA design indicates that for a large effect size (f = 0.4), the achieved power for this experiment was 1 – beta = 0.59.

In the elevated plus maze, the level of locomotor activity did not significantly differ between the two genotypes, as measure by the total distance travelled (Figure 7.2). Persistent stress caused a slight increase in locomotor activity in both WT and KO groups (P < 0.05, F (1, 50) = 4.1 main effect of treatment, 2-way ANOVA) suggesting that both genotypes were affected by the persistent stress in a manner indicative of stress-induced arousal.
Figure 7.1. Time spent immobile during the forced swim test was altered in persistently stressed mice. Naive WT and KO genotypes spent similar amounts of time immobile, and, after persistent stress, spent significantly less time immobile (P < 0.05, main effect of treatment). Stressed KO mice appeared to spend more time immobile than WT mice indicating less stress-induced arousal, but differences were not significant (P > 0.05). Numbers of animals per group are shown within bars.

Figure 7.2. Locomotor activity on the elevated plus maze did not differ between WT and α9-nAChR KO mice. Persistent stress caused a significant increase in the total distance travelled in both WT and KO mice (P < 0.05, main effect of treatment), but no significant difference was observed between WT and KO genotypes for either naïve or stressed mice. Numbers of animals per group are shown within bars.
Stress-induced arousal was greater in stressed WT mice than in KO mice when the number of arm crosses in the EPM was considered. For WT animals, the number of arm crosses was significantly higher than both non-stressed counterparts ($P < 0.01$, $t = 3.7$; two-way ANOVA with Bonferroni's post-hoc multiple comparisons test) and stressed KO ($P < 0.05$, $t = 3.3$; two-way ANOVA with Bonferroni's post-hoc multiple comparisons test) animals (Figure 7.3). Stress-induced arousal was not observed on this behavioural score in the KO mice. Two-way ANOVA revealed significant main effects of genotype ($P < 0.01$, $F (1, 50) = 9.3$), treatment ($P < 0.01$, $F (1, 50) = 7.3$), and an interaction between genotype and treatment ($P < 0.05$, $F (1, 50) = 6.6$), accounting for 13.3%, 10.4% and 9.5% of total variance respectively.
Figure 7.3. Bouts of arm crosses were elevated in persistently stressed WT mice, but not KO mice. After stress, WT mice performed significantly more bouts of arm crosses compared to naïve WT animals (P < 0.01), and stressed KOs (P < 0.05). # symbol indicates significant difference between treatment groups; * symbol indicates significant difference between genotypes.
7.3.1.2 Anxiety-like behaviour is increased in α9-nAChR KO mice

Naïve WT and α9-nAChR KO mice show comparable behaviour on the EPM. The α9-nAChR KO genotype demonstrated a greater susceptibility to increased anxiety-like behaviour after persistent stress, compared to WT mice.

For naïve animals, anxiety-like behaviour did not differ between WT and α9-nAChR KO genotypes on any parameters of the EPM. The total distance travelled (Figure 7.2), total arm crosses (Figure 7.3) and time spent in different parts of the maze (Figure 7.4) did not differ.

After persistent stress, anxiety-like behaviour was observed in KO animals in the EPM, but not in WT. The time spent in the closed arms of the maze, as a percentage of total time spent in the maze was used as a measure of anxiety-like behaviour. Persistently stressed WT animals did not show any behavioural changes from naïve animals, while KO animals spent significantly more time in the closed arms of the maze (P < 0.05, q = 3.8; Two-way ANOVA with Tukey's multiple comparisons test) compared to stressed WTs (Figure 7.4). This increased time spent in the closed arm indicates that persistent stress causes an increase in the anxiety-like behaviour of KO mice, which the WTs are resilient to. Two-way ANOVA revealed a significant main effect of genotype (P < 0.05, F (1, 50) = 5.1), accounting for 8.6% of total variance, and a significant interaction effect between genotype and treatment (P < 0.05, F (1, 50) = 5.1), accounting for 8.6% of total variance.
Figure 7.4. Anxiety-like behaviour was increased in persistently stressed α9-nAChR KO mice. The percentage of time spent in the closed arms of the EPM did not differ between naïve WT and KO mice. After persistent stress, KO animals spent more time in the open arm. Two-way ANOVA revealed significant main effects of genotype (P < 0.05) and an interaction effect (P < 0.05).
7.3.2 Physiological stress response

7.3.2.1 The corticosterone stress-response is dysregulated in α9-nAChR KO mice

At rest, WT and α9-nAChR KO mice show comparable circulating corticosterone levels. After a stressful insult, both genotypes respond with a spike in serum corticosterone. This corticosterone spike is of consistent magnitude in WT mice, whether the stressor is acute, or follows 6 days of persistent stress. As discussed below, the α9-nAChR KO mice demonstrated a failure to regulate this corticosterone spike, with the magnitude differing according to the duration of stress.

WT and α9-nAChR KO animals had comparable mean basal circulating corticosterone levels that were below 5 ng/mL (3.3 ± 0.7 and 4.3 ± 0.8 respectively). 15 mins after commencing the FST, WT mice had a consistent spike in corticosterone levels, whether stress was experienced acutely (182.6 ± 19.7 ng/mL) or persistently over the preceding successive days (174.7 ± 7.3 ng/mL) (Figure 7.5). α9-nAChR KO mice displayed dysregulated corticosterone responses to stress, with acutely stressed mice exhibiting a significantly muted response (P < 0.05, t = 2.6, unpaired Student’s T-test) compared to their WT counterparts. Conversely, persistently stressed KO mice exhibited an exaggerated corticosterone response compared to acutely stressed mice (P < 0.01, t = 3.7, two-way ANOVA of KO mice with Bonferroni’s post-hoc multiple comparisons test). For stressed animals of both genotypes two-way ANOVA revealed significant main effects of the duration of the stress (P < 0.05, F (1, 20) = 6.0), accounting for 17.5% of total variance, and an interaction between duration of stress and genotype (P < 0.01, F (1, 20) = 8.3), accounting for 24.1% of total variance.

A summary of the behavioural and physiological responses of WT and α9-nAChR KO mice to acute and persistent stressors (Chapter 6 and Chapter 7) is shown in Table 7.2.
Figure 7.5. The physiological stress response of corticosterone release was dysregulated in α9-nAChR KO mice. Animals that had undergone “no-stress” had low serum corticosterone levels. WT mice that experienced an acute stressful insult (“A”) or an insult following persistent stress (“P”) had a consistent corticosterone spike. Acutely stressed KO animals had a muted corticosterone response compared to WT counterparts (P < 0.05), while after persistent stress, the KO corticosterone response was significantly exaggerated (P < 0.01). # symbol indicates significant difference between treatment groups; * symbol indicates significant difference between genotypes. N=6 for all groups.

Table 7.2. Summary of behavioural and physiological phenotype of WT and KO mice after acute and persistent stress. Dashes indicate behaviours that have not been tested.

<table>
<thead>
<tr>
<th>Stressor type</th>
<th>Depression-like behaviour</th>
<th>Anxiety-like behaviour</th>
<th>Stress-induced arousal</th>
<th>Corticosterone response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>Acute</td>
<td>-</td>
<td>-</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Persistent</td>
<td>Normal</td>
<td>↑</td>
<td>Normal</td>
<td>↑</td>
</tr>
</tbody>
</table>
7.4 DISCUSSION

There is a strong but poorly characterised correlation between chronic pain and HPA axis dysregulation. It was hypothesised that the α9α10-nAChR may play a role in the HPA axis response to the stress caused by chronic pain, as this may account for the difference in pain phenotype between WT and α9-nAChR KO mice. After investigating the role of the α9α10-nAChR in the stress response, it was indeed found that a dysregulation, both physiological and behavioural, occurs in the absence of the α9α10-nAChR, in response to acute and persistent stress. However, instead of the predicted universally subdued stress-response in KO mice, dysregulation was complex and varied according to stressor. Behaviourally, KO mice exhibited decreased stress-induced arousal and increased anxiety-like behaviour. Physiologically, a muted acute-stress and exaggerated persistent-stress response was observed.

7.4.1 DECREASED STRESS-INDUCED AROUSAL

The phenomenon of stress-induced arousal is commonly accepted as a normal response to stress. Quite common are sleepless nights during stressful times, and restlessness in anticipation of stressful events. In rodent models of stress, this increased arousal manifests as behavioural changes such as hyperlocomotion (Strekalova et al., 2005), and as physiological changes including the requirement of increased doses of sedatives (pentobarbital) to induce sleep, and reduced sedation time compared to non-stressed animals (Shibasaki et al., 1994a; Shibasaki et al., 1994b; Shibasaki et al., 1991).

Stress-induced arousal occurs when CRH activates the locus coeruleus-noradrenaline (LC-NA) system (Balbo et al., 2010a; Valentino et al., 1991), increasing the amount of circulating wake-promoting NA. The LC-NA system is a potent modulator of forebrain (e.g. amygdala, posterior hypothalamus) and behavioural activity states, and its activation results in increased arousal (Berridge and Waterhouse, 2003; Kovacs, 2013) and anxiety-like behaviour (Butler et al., 1990; Van Gaalen et al., 2002). Additional mechanisms are likely
involved beyond CRH, such as opioid (Shibasaki et al., 1993) and beta-adrenergic receptors (Shibasaki et al., 1994b). This includes initiating and sustaining periods of alert waking, enhancing the salience of relevant stimuli and increasing vigilance (Berridge and Waterhouse, 2003).

Thus, both WT and KO animals experienced stress-induced arousal, manifesting as a decrease in immobility time in the FST and increased distance travelled in the EPM. The slightly lesser degree of stress-induced arousal in the KO mice compared to WTs in the post-stress FST, although not significant, suggests that the psychomotor response of the KO mice to the prolonged stress differs from WT animals. Since KO mice respond normally to acute FST, it appears that the prolonged stress-induced adaptations that constitute normal coping mechanisms are altered in the KOs, causing the altered post-stress FST responses.

7.4.2 INCREASED ANXIETY-LIKE BEHAVIOUR

The deletion of the α9-nAChR subunit caused stress-induced changes in anxiety-like behaviour compared to WT mice. No differences were seen in general locomotor activity, as indicated by the total distance travelled, between genotypes, which might otherwise obscure the interpretation of anxiety-related behaviour. This comparable exploratory activity level in the EPM is in agreement with the gross activity levels seen in Run1 of the IntelliCage as measured by corner Visits (Section 6.3.1), where animals were housed by genotype. Baseline levels of anxiety-like behaviour were normal in the KO mice, but the reduced number of open arm entries after 4 days of persistent restraint-stress indicated an anxiety-like phenotype (Dedic et al., 2012).

Contrasting effects of corticosteroids on anxiety have been described in the literature, and these contrasts are due to the presence of two distinct receptor-targets; mineralocorticoid (MR) and glucocorticoid (GR) receptors. MRs and GRs have overlapping expression in the CNS, particularly those regions involved in anxiety such as hippocampus and amygdala. Corticosterone has a 6 to 10-fold
higher affinity for MRs than GRs, and has differing actions on anxiety depending on the balance of MR and GR occupation (Korte, 2001; Reul and Dekloet, 1985).

Corticosterone binding to hippocampal MRs is implicated in acute unconditioned fear, manifesting as increased anxiety-like behaviour on the EPM in animal studies (Bitran et al., 1998; Weiss et al., 1970). Due to the very high affinity of corticosterone for MRs, low levels of circulating adrenal steroids cause greater occupancy of MRs over GRs, resulting in permissive actions of hippocampal MRs on acute fear-related EPM behaviour (Korte, 2001). If α9-nAChR KO mice had consistently muted corticosterone reactions in response to the daily restraint stress applied here, they conceivably experienced high MR to GR occupancy ratios compared to WT mice, leading to the generalised anxiety-like behaviour seen after persistent stress. Conversely, high levels of glucocorticoids, and therefore increased GR occupancy, also lead to increased anxiety-like behaviour in rodents. This is believed to be the mechanism of fear-potentiated behaviour that follows stressful insults (Korte et al., 1995; Roozendaal et al., 1996). Thus, different corticosteroid mechanisms are involved in different aspects of fear and anxiety.

Further investigation into the stress-phenotype of α9-nAChR KO mice is required to determine the mechanisms of the HPA-axis dysregulation observed here. Outstanding questions include whether the anxiety-like behaviour on the EPM seen in the KO mice results from hypo-secretion of corticosterone, and therefore occurs through passive MR-mediated mechanisms, or from corticosterone hyper-secretion and thus mediated by GR-mechanisms that potentiate fear and anxiety. If the latter is the case, it is an intriguing question as to what point the persistent restraint stress caused the switch from hypocortisolaemic responses to hypercortisolaemic.

7.4.3 Dysregulation of physiological stress response
Corticosterone is the primary stress hormone in rodents and is routinely measured as a marker of stress. The corticosterone response to acute stress
(FST) was measured here, either in naïve animals or in animals that had undergone five preceding consecutive days of persistent stress. When naïve animals were tested, the corticosterone response of KO mice was significantly muted. Although this did not correlate to any noticeable differences in baseline behavioural indices (EPM or FST), this subdued physiological stress response may account for the perceived “placid” nature of the KO mice during handling. Since only a limited number of behavioural tests were performed, any baseline differences in anxiety- or depression-like behaviour may have been undetected. Indeed, different aspects of stress-related pathological behaviour are known to be targeted by different behavioural tests (Van Gaalen et al., 2002).

After undergoing 5 days of persistent stress, KO mice exhibited an exaggerated corticosterone response to the FST, while the corticosterone response of WT animals remained consistent, whether the stressor was acute, or followed a week of persistent stress. It is apparent that the α9α10-nAChR is necessary for normal coping mechanisms that adapt during prolonged stressful periods, ensuring that future stressful encounters are met with an appropriate magnitude of response.

This potential α9α10-nAChR-mediated coping mechanism may rely on adequate corticosterone release. If corticosteroid levels are too low (e.g. after adrenalectomy), hypothalamic parvocellular cells become sensitised to stress. Thus, appropriate glucocorticoid levels act as a protective mechanism to prevent inappropriate activation of the HPA-axis in response to environmental stressors (Ma and Aguilera, 1999). Alternatively, the lack of stress adaptation in KO mice may be due to a defective hippocampal "off switch" in the corticosterone feedback loop, due to the absence of α9α10-nAChRs in the pituitary and adrenal glands. Under normal circumstances, the hippocampus is one of the main nuclei responsible for the termination of the stress response. Prolonged elevation of corticosterone (either exogenously administered or through prolonged stress) causes downregulation of GRs in the hippocampus. This downregulation is a coping mechanism associated with consolidating salient memories, which allows for dynamic coping skills upon future stressors (Sapolsky et al., 1984a; Sapolsky et al., 1984b), and may be absent in the KO mice.
7.4.4 HPA-axis dysregulation vs. pain phenotype in α9-nAChR KO mice

Investigation into the stress responses of α9-nAChR KO mice was initiated to explore the correlations between HPA-axis dysregulation and chronic pain. Have these experiments shed any light on the altered responses of KO mice to pain? The points of difference observed between WT and KO mouse pain phenotypes were restricted to the noxious mechanical testing using the PAM test. One aspect of KO pain phenotype in particular appears to be consistent with HPA-axis dysregulation. KO mice were resistant to the repeated testing-induced mechanical hyperalgesia seen in the WTs. Normal responses to salient stimuli are modulated by the HPA-axis, leading to avoidance and escape tendencies, increased motor output of subjective arousal (escape behaviour) and facilitation of acquisition of new information for optimal performance (anticipation of pain on future tests) (Heinrichs and Koob, 2004). The sham-operated WT animals plainly show such behaviour across the duration of PAM testing, with experimenter observations of evident fearful anticipatory behaviour. Similar mechanisms may be responsible for the increased stress-induced arousal seen in the WT animals in the FST. α9-nAChR KO mice show deficits in their avoidance learning (no repeated-measures hyperalgesia), and slightly reduced post-stress motor output, shown by the lesser stress-induced arousal during the FST. This may reflect abnormal hippocampal corticosterone levels attenuating memory formation (Sapolsky et al., 1984a).

The results presented in this chapter are unlikely to account for the large differences in the magnitude or duration of mechanical hyperalgesia in the neuropathic or inflammatory pain models (Chapter 3) between the two genotypes. It is likely that those differences are due to mechanisms of nociception such as alterations in specific nerve fibre subtypes. An obvious question remains as to whether the chronic pain models used here constitute severe enough stressors to reveal phenotypic differences in affective states between WT and α9-nAChR KO mice.
There is no consensus as to whether mice are able to adequately model the full spectrum of symptoms that result from chronic pain in humans. Changes to quality-of-life, including affective and motivational states, sleep and mobility are major concerns for patients. Whether the same may be said for mice is contested. Urban et al. (2011) have shown that after the induction of common mouse pain models (CCI, CFA and spinal nerve ligation (SNL)), quality-of-life measures such as food and water intake, circadian patterns, time-budgets (eating vs. moving vs. inactive vs. other (awake but not moving)), and locomotor activity are all unchanged compared to sham animals. Anxiety and depression-like behaviour has been shown to be unchanged (Hasnie et al., 2007). In rat pain models, CCI produced no HPA-axis alterations typical of chronic stress (Ulrich-Lai et al., 2006). Conversely, evident anxiety- and depression-like behaviour in injured mice has been shown in numerous studies (Benbouzid et al., 2008; Jesse et al., 2010; Narita et al., 2006; Suzuki et al., 2007), in addition to suppressed appetite (Stevenson et al., 2006), and reduced social interactions (Benbouzid et al., 2008). Exploration of the effect of the cuff model on stress is needed in future studies.

7.4.5 CONCLUSIONS
The effect of α9-nAChR subunit deletion on stress-responses was investigated both behaviourally and physiologically. The behavioural responses to stress were comparable between naïve KO and WT mice, however the physiological response of the KO mice to acute stress was muted. Following prolonged physical stress, KO mice demonstrated multiple changes in behavioural stress responses, as well as physiological. Persistently stressed KO mice exhibited decreased stress-induced arousal, increased anxiety-like behaviour, and an exaggerated corticosterone response to acute stress. These changes in KO mice suggest that pharmacological agents that inhibit the α9α10-nAChR may cause or exacerbate physiological stress responses and affective disorders in patients.
CHAPTER 8

GENERAL DISCUSSION
8.1 Advantages of α-Conotixin Analgesics

As discussed in Chapters 1 and 5, α-conotoxins such as Vc1.1 and RgIA have emerged as promising, novel analgesics that appear to act via a unique mechanism(s) of action. Although the mechanism(s) of action is yet to be fully characterised, the fact that such a novel class of analgesics may become clinically available shows promise for helping to resolve the growing chronic pain burden that is challenging clinical care. In particular, the efficacy of analgesic α-conotoxins in alleviating neuropathic pain (Satkunanathan et al., 2005), which is notoriously intractable to conventional treatments (Arner and Meyerson, 1988), suggests that these peptides will be invaluable for future pharmacological development of analgesics.

In addition to the acute analgesic effects, α-conotoxins proffer restorative/neuroprotective properties. For example, RgIA has been said to exert its effects on the origins of neuropathic pain and, thus, offers a means to directly modulate nervous signaling and to prevent maladaptive alterations that may lead to chronic pain conditions (Mannelli et al., 2014) (See Section 1.4.3.1, Section 1.5, Section 1.6, Chapter 4).

Furthermore, no addictive liability of analgesic α-conotoxins has been detected. Repeated administration of conotoxins such as Vc1.1 and RgIA does not decrease the acute actions of subsequent doses (Mannelli et al., 2014; Satkunanathan et al., 2005), indicating that no tolerance develops. To the contrary, the analgesic effects are potentiated with repeated dosing.

It is likely that Vc1.1 and RgIA are just the first of many in their class of analgesic α-conotoxins that act via similar mechanisms. Further exploration of the many hundreds of conotoxin species is likely to identify similarly promising and clinically valuable peptides. However, these peptides potentially have multiple mechanisms of action, including the GABA<sub>B</sub>R-dependent inhibition of N-type Ca<sup>2+</sup> channels (see Chapter 5).
Pain physiology is complex. Pain mechanisms encompass all levels of the nervous system, from peripheral sensory nerves to the spinal cord, the subcortical regions and cortex (Backonja, 2012). This complexity suggests that single targets are unlikely to resolve chronic clinical conditions. The experiments presented here have highlighted the difficulty in targeting a single receptor for the treatment of pain, in that the α9α10-nAChR was found to be important for only one pain modality: mechanical hyperalgesia. No essential contribution of the α9α10-nAChR in acute pain sensation, mechanical allodynia, or hot or cold hypersensitivity was observed (see Chapter 3). Thus, pure α9α10-nAChR-inhibiting compounds may only be partially effective in clinical pain conditions.

The analgesic α-conotoxins, Vc1.1 and RgIa that initially implicated the α9α10-nAChR in pain and analgesia are now known to possess other mechanisms of action that likely mediate their actions. The inhibition of N-type VGCCs via GABA_B Rs likely accounts for the acute analgesic effects that are seen in animal models of neuropathic pain (Callaghan et al., 2008) (see Sections 1.6 and 5.1). However, other properties of these conotoxins, such as their disease modifying and restorative actions, as well as the sustained and cumulative effects of these conotoxins may act through α9α10-nAChRs or via as yet unidentified actions.

The ideal experiment to resolve the question of α9α10-nAChRs in the analgesic actions of Vc1.1 and RgIa is to test them in α9-nAChR KO mice. As described in Chapter 5, initial testing of Vc1.1 in mice produced clear, dose-dependent analgesia that was comparable with rat studies (e.g. Satkunanathan et al. (2005)). The lack of success in reproducing those effects was disappointing and may be due to a number of reasons, such as poor environmental consistency in the animal house and thus increased stress in the local cage environment, or the instability of the peptide. The limited availability of the peptides restricted the progress and completion of testing, but further testing is planned upon the availability of the peptides in more recently developed testing facilities.
Based on the results of Chapter 3, wherein the pain phenotype of α9α10-nAChR KO mice was largely normal, I predict that the conotoxins will be equally effective in the KO as the WT mouse genotype. A difference may be seen in the mechanical hyperalgesia pain modality, however this remains to be tested.

Based on the present findings, it appears that for optimal and comprehensive pain management, α9α10-nAChR inhibitors alone are unlikely to alleviate clinical pain conditions and additional targets must be considered.
8.3 The anatomical sites of action of α9α10-nAChRs in analgesia

The potential anatomical sites of action of α9α10-nAChR-inhibiting analgesic compounds, whether conotoxin or otherwise, is unknown. While predictions have been made, the main candidate sites share the problem of not expressing functional α9α10-nAChRs.

The two primary candidate sites reported for Vc1.1 and RgIA-mediated analgesia are immune cells at the site of injury (e.g. Vincler et al. (2006)), and sensory afferent nerves that transmit the pain signal centrally (e.g. (Livett et al., 2002)). Whether the α-conotoxin analgesia relies on α9α10-nAChR expression at either of these sites is still unknown. Unfortunately, no functional α9α10-nAChR expression has been identified at either site. α9α10-nAChR-mediated responses to ACh are absent in immune cells (Peng et al., 2004), and while mRNA for the subunit has been detected in DRG neurons, sensory afferent nerves do not express translated, cell-surface protein (Callaghan and Adams, 2010).

In the developing inner ear, vestibular and cochlear hair cells express α9α-nAChR mRNA long before cell surface expression. The protein is present during embryological development, but is only inserted into the membrane following the arrival of cholinergic terminals during the early postnatal period (Simmons and Morley, 2011). In this system, functional cell surface expression of α9α10-nAChRs requires the presence of cholinergic inputs. The same may be so for functional α9α10-nAChR expression on immune and peripheral nerve cells, which have been reported to express α9-nAChR mRNA (Callaghan and Adams, 2010; Peng et al., 2004), but not functional receptors. Painful injury and inflammation cause enormous changes to the local environment, with the infiltration of pro-nociceptive cytokines, peptides and signalling molecules, and changes in protein expression in neuronal, immune and auxiliary cells (Sommer and Kress, 2004; White et al., 2005; Woolf and Mannion, 1999). Whether α9α10-nAChR-expressing immune and neuronal cells exhibit alterations in α9α10-
nAChR expression and function during pain states still needs to be resolved. If any of the actions of the analgesic α-conotoxins are found in future to be mediated by α9α10-nAChRs it will be important to address these anatomical questions in more detail.

8.3.1 α9α10-nAChRs ON IMMUNE CELLS

The initial response of immune cells after injury is the release of chemoattractants and cytokines that promote further infiltration of immune cells to the injury site. Macrophages release matrix metalloproteases (MMP) that degrade the integrity of the endoneurial blood vessels, causing an interruption of the blood-nerve barrier (Ji et al., 2009) and further immune cell infiltration.

Activation of immune cells, for example by exposure to pro-inflammatory cytokines (Schafers et al., 2003), could possibly induce a response from latent α9-nAChRs. Although the α9-nAChR is not upregulated upon activation of B lymphocytes (Koval et al., 2011), other α9α10-nAChR-expressing cell types such as T lymphocytes and macrophages might show different phenotypes upon activation.

The precise nAChR subtypes involved in pain are still unknown. α4β2- and α7-nAChRs are the predominant subtypes of the CNS and are also the predominant subunits expressed in resting and activated B-cells. The α7-nAChR is the major nAChR subtype involved in regulating the B lymphocyte mitogenic CD40-stimulated response, but the α9 nAChR has been suggested to compensate for some α7 function in α7−/− mice (Koval et al., 2011).

α-Conotoxin (Vc1.1 and/or RgIA) treatment has been reported to reduce CD86+ antigen presenting cells (CD86+ cells interact with T cells, including macrophages) (Mannelli et al., 2014; Vincler et al., 2006), CD2+ T cells (Vincler et al., 2006), as well as ChAT immunoreactive cells. Since no reductions in immune or ChAT-immunoreactive cells were found here (Chapter 4), the findings of Vincler et al. (2006) and Mannelli et al. (2014) of reduced immune cell
infiltration into the injured sciatic nerve may be a consequence of another mechanism of α-conotoxin-induced healing, rather than direct inhibition of α9α10-nAChRs on these immune cells. The lack of involvement of the α9α10-nAChR shown in Chapter 4 implies that the healing properties of α-conotoxins are likely due to the other mechanisms of actions they possess. Infiltration of immune cells is the body’s response to injury and in itself facilitates healing. Immune cells release anti-inflammatory modulators, in addition to pro-inflammatory ones, and their presence does not necessarily imply greater nociception. In fact, it has been shown that inflammation, and the coinciding increase in immune cells (e.g. macrophages) and inflammatory mediators (e.g. IL-1), promote regeneration of injured nerves (Dahlin, 1992; Lu and Richardson, 1991).

8.3.2 α9α10-nAChRs ON NEURONAL CELLS

The involvement of the α9α10-nAChR in mechanical hyperalgesia, but not other pain modalities (Chapter 3), points to the possibility that these receptors are expressed only on a subset of nerve fibres or that indirect mechanisms (e.g. immune interactions) interact only with a subset of fibres. Perhaps they are selectively expressed on high threshold mechanical nociceptive fibres. Evidence is lacking for the functional expression of α9α10-nAChRs on peripheral nerve cell bodies and/or their axons and terminals, and only mRNA has been detected (Callaghan and Adams, 2010; Haberberger et al., 2004; Lips et al., 2002). It remains possible that expression emerges in tissue that has undergone an inflammatory or neuropathic pain state. This is yet to be investigated and should be an important focus of future studies.

An alternative site of action that warrants investigation is the sympathetic nervous system. Sympathetic efferent nerves play a role in the modulation of C-fibre sensitivity in inflammatory and neuropathic pain (Ren et al., 2005). It has been proposed that α-conotoxins such as Vc1.1 may attenuate painful transmission through inhibiting the sympathetic post-ganglionic neurons that supply C-fibres (Satkunanathan et al., 2005). This proposition is yet to be
explored in the context of pain, however α9α10-nAChR expression has been confirmed at the synapse between sympathetic splanchnic nerves and chromaffin cells of the adrenal medulla (Colomer et al., 2010). Thus, α9α10-nAChRs may play a role in neuroendocrine homeostasis through sympathetic efferent neurons and may be the site of action of α9α10-nAChR-inhibiting analgesic compounds.

### 8.3.3 Peripheral acetylcholine source

If peripheral sites such as immune cells or afferent sensory nerves are the sites of action of α9α10-nAChR-inhibiting conotoxins, and the injured-state extracellular milieu induces expression/insertion of the receptor in the cell surface, the question still remains as to the source of ACh that induces these changes. Agranulocytes and antigen-presenting cells only express ChAT mRNA after stimulation with Con A (3mg/mL) or LPS (1ug/mL), and no ChAT mRNA has been detected in either resting or stimulated macrophages (Kawashima et al., 2007). However, ChAT-immunoreactive B-cells and other lymphocytes that impact innate immunity have been shown to release ACh upon specific stimulatory conditions (Reardon et al., 2013). These lymphocytes control the recruitment of other immune cells and mediate the local inflammatory response. It is therefore likely that ACh is produced locally at sites of injury, and this ACh could influence cells expressing nAChRs (including α9α10 subtypes) if they are functionally expressed on immune cells or nerves at sites of injury.

### 8.3.4 α9α10-nAChR expression in injured tissue

The expression of functional α9-nAChR subunit protein in immune cells and in peripheral nerves has not yet been observed. While the receptor has been detected at the mRNA level, no functional receptors that could account for acute α9α10-nAChR-mediated analgesia have been found. Although reliable antibodies for α9α10-nAChRs are not yet commercially available, western blotting of injured nerves may reveal any existing functional receptors and should be performed.
8.4 Potential Risks of α9α10-nAChR Inhibitors

To date, no negative side effects have been reported for α9α10-nAChR-inhibiting analgesic conotoxins in pre-clinical or clinical tests (Vincler and McIntosh, 2007) and abuse liability appears to be negligible (Satkunanathan et al., 2005). The absence of α9α10-nAChR expression in the brain and spinal cord limit the potential for cognitive side effects. Non-conotoxin small molecule inhibitors of the α9α10-nAChR do cause dose-dependent motor incoordination (Holtman et al., 2011), though this is likely to occur via an off-target mechanism, particularly when one considers the side effect profiles of these drugs (as discussed in Chapter 3).

The α9α10-nAChR has recently been implicated in stress responses (Colomer et al., 2010), and a more detailed exploration here has confirmed the importance of this receptor for normal coping with environmental stress. In the experiments presented here, α9-nAChR KO mice proved to have dysregulated corticosterone responses to stress and were liable to increased anxiety- and depression-like behaviour in response to relevant environmental stressors, particularly chronic stress. It is likely that the lack of α9α10-nAChRs in the HPA axis meant that the KO mice could no longer respond in the usual dynamic way to stressors. The implications of these findings with regard to affective regulation suggest that caution must be taken when pursuing α9α10-nAChR ligands as clinical treatments. α9α10-nAChR-inhibiting analgesics may cause or exacerbate mental health disorders (anxiety, depression, poor coping with stress) in vulnerable individuals. However, there are factors that suggest such risks may be temporary and reversible. Conotoxins such as Vc1.1 and RgIA are reversible antagonists of the α9α10-nAChR (Clark et al., 2006; Lang et al., 2005), and so cessation of treatment may suffice in reversing any such side effects that may occur. Physiological changes that may occur due to HPA-axis manipulation are highly dynamic and may also recover upon cessation of α9α10-nAChR-inhibitor treatment. For example stress-induced upregulation of GRs in the hippocampus and amygdala is reversible within a week of stress-cession in rats (Sapolsky et
al., 1984b), suggesting that HPA-axis responses are dynamic enough to prevent any α9α10-nAChR inhibiting medications from causing long-term harm.
8.5 AFFECTIVE DISORDERS & α9α10-nAChRs

Little research has been conducted into the role of the α9α10-nAChR in stress and affective disorders. Beyond its upregulation during cold temperature-induced stress (Colomer et al., 2010), the role of α9α10-nAChR in stress was previously unknown. The present experiments have uncovered a vital role of α9α10-nAChRs in normal behavioural and physiological stress responses.

8.5.1 DYSREGULATION OF AROUSAL STATES IN α9-nAChR KO MICE

The circadian sleep rhythmicity of naïve α9-nAChR KO mice was altered compared to WT animals. The changes in sleep-wake rhythmicity are reminiscent of sleep disturbances that typically accompany HPA-axis dysregulation and affective disorders such as depression (Antonijevic, 2008). Depressed patients often experience sleep-continuity disturbances, usually associated with increased wakefulness (Kupfer et al., 1989). The behavioural changes in patients with affective disorders have been described as “over-arousal” (Gillin et al., 1984). Such over-arousal appears to be a feature of α9-nAChR KO mice, and is a phenomenon that is sensitive to socio-environmental influences. The over-arousal observed in KO mice is presumed to result from HPA-axis dysregulation, though it was not accompanied by depression-like behaviour. The effect of persistent stress on stress-induced arousal behaviour was altered in KO mice compared to WT mice. Although persistent stress caused increased stress-induced arousal in both genotypes when tested in the FST and EPM, the KO mice exhibited a reduced degree of stress-induced arousal in the FST. Thus, the deletion of the α9-nAChR appears to cause alterations in both naïve and stress-induced arousal states and psychomotor responses to stress. However, these alterations are only manifest during certain tasks (FST, not EPM), and are manipulable by socio-environmental changes (social groups in the IntelliCage).
8.5.2 Behavioural responses of α9-nAChR KO mice after stress

α9-nAChR KO mice showed vulnerability to both stress-induced depression-like (anhedonia) and anxiety-like (increased time in closed arms of EPM) behaviour. Anhedonia is thought to correlate with increased total immobility time in the FST, whereas KO mice show decreased immobility time after stress. This may appear superficially contradictory, however depression and anxiety disorders share considerable comorbidity clinically (Aina and Susman, 2006; Hettema, 2008; Hirschfeld, 2001), with aetiology often overlapping. Historically, depression and anxiety disorders were viewed as discrete clinical entities due to the different drugs used to treat the disorders; tricyclic antidepressants and benzodiazepines respectively. However, SSRIs, which were classically thought to be antidepressants, are now the most common treatments for anxiety disorders (Cryan and Holmes, 2005), and more contemporary treatments have been found to be effective in both depression and anxiety (e.g. neuropeptide (Holmes et al., 2003; Kramer et al., 1998) and CRH1 antagonists (Zobel et al., 2000)). Furthermore a high degree of genetic correlation has been shown to exist between depression and anxiety (Hettema, 2008).

The biological significance of environmental events is highly complex and results in distinct physiological and behavioural responses. It is tempting to speculate that the distinct behavioural changes that were observed in the KO mice after a similar duration of prolonged stress is owing to the difference in the modality and quality of stressors that triggered the behaviour, and the subsequent coping styles that arose. Anhedonia was triggered by psychological stress (inability to control external environment) where mobility was unrestricted, whereas arousal and anxiety-like behaviour was triggered by prolonged inescapable stress that was predominantly physical (restraint).

Unique stressors have been described to have neurochemical “signatures” that have quantitatively and qualitatively distinct central and peripheral mechanisms (Pacak, 2000). For example, Vidal and Jacob (1986) demonstrated that psychological (novelty) versus physical (loose restraint) stressors in rats induced hyperalgesia of HPA-mediated and HPA-independent mechanisms.
respectively. This hyperalgesia was accompanied by opposing physiological manifestations (hyperthermia and hypothermia respectively), and novelty- but not restraint-induced hyperalgesia was responsive to anxiolytics and anticonvulsants.

Experimental animal models of anxiety- and depression-like behaviour have been shown to be associated with the same HPA axis manipulations (Murray et al., 2008) and genetic manipulations (Tordera et al., 2007). For example, uncontrollable chronic mild stress is generally used experimentally to trigger a generalised anxiety-like state, however, it can also induce depression-like phenotypes without exacerbating anxiety (Mineur et al., 2006). Conversely, psychopathological outcomes of the same chronic-stress trigger were shown to independently elicit anxiety- and depression-like behaviours in mice, with individual vulnerability to depression-like behaviour clearly correlated with social coping mechanisms such as submissive behaviour in the resident-intruder test (Strekalova et al., 2004; Strekalova et al., 2005). A range of commonly used experimental stressors was shown by Bowers et al. (2008) to uniquely activate physiological stress responses, potentially reflecting differential glucocorticoid activation and/or metabolic adjustments. Vulnerability to different stressor types can be influenced by environmental factors, where early social enrichment proffers resilience to social stress, but not to physical stress, in adulthood (Branchi et al., 2013). Thus, the quality of the stressor determines the physiological effects of stress, as well as the psychopathological outcome.

The phenotypic manifestation of affective disorder pathology is complex, heterogeneous and dependent on the quality of the triggers. The different stressors in the current experiments therefore triggered different coping pathways resulting in either a depression-like phenotype (possibly anhedonia), or an anxiety-like phenotype (arousal, increased FST activity) depending on the stressor. It would be of interest to see whether the anhedonic KO mice exhibit increased immobility time in the FST.
8.5.3 Potential physiological changes in α9-nAChR KO mice after stress

Although hypercortisolaemia is widely associated with depression, hypocortisolaemia alongside certain personality traits such as low self-esteem and helplessness (Scarpa and Luscher, 2002) or neurotism (McCleery and Goodwin, 2001) is linked with greater vulnerability to the development of depression. The progression of such a propensity into a mental health disorder is most often triggered by aversive environmental situations (Shumake and Gonzalez-Lima, 2003). Insufficient secretion of corticosteroids is thought to confer greater vulnerability to depression, by failing to send adequate negative feedback and thereby prolonging the stress response (Shumake and Gonzalez-Lima, 2003). The muted corticosterone response observed in acutely stressed α9-nAChR KO mice (FST; see Section 7.3.2) may be indicative of the vulnerability of this genotype to depression-like behaviour upon experiencing the appropriate trigger. When the stressor was prolonged, it was observed that inescapable physical stress (chronic restraint) induced anxiety-like behaviour accompanied by an exaggerated corticosterone response. It is of great interest to determine whether the emotional/psychological stress during IntelliCage testing, which induced a depressive phenotype (see Section 6.3.5), coincides with altered corticosterone secretion. As discussed above in Section 8.5.1, whether or not depression- or anxiety-like behaviour manifests is often stressor-dependent, though physiological markers such as corticosteroid responses may provide a useful predictor for individual vulnerability to the development of these disorders.

The suggested mechanistic changes in α9-nAChR KO mice that cause the altered corticosteroid responses are speculative at this stage. However, a number of potential mechanisms exist, including regulation of forebrain GR expression and pituitary dysfunction. The inability of KO mice to dynamically regulate α9α10-nAChR expression in response to stress may cause alterations in downstream regulation of GR expression. Mice lacking forebrain GRs show increased depression-like behaviour (Boyle et al., 2005), whereas overexpression of forebrain GRs increases emotional lability and anxiety-like behaviour (Wei et al.,
In clinical populations, dysregulation of daily cortisol rhythms is common in sufferers of affective disorders. It is suggested that disrupted HPA-axis negative feedback to the pituitary, but not the hypothalamus, may account for abnormal cortisol fluctuations (Gold et al., 2002). Whether or not α9-nAChR KO mice exhibit the altered daily glucocorticoid fluctuations that are observed clinically remains to be tested.

8.5.4 IMPLICATIONS OF STRESS DYSREGULATION FOR PAIN STATES IN α9-nAChR KO MICE

As discussed in Section 1.8, the HPA-stress-axis and the subjective pain experience are closely linked. Abnormalities of HPA-axis function have been shown in chronic pain conditions such as chronic back pain and rheumatoid arthritis (Eijsbouts et al., 2005; Lentjes et al., 1997). HPA-axis dysfunction is also associated with affective disorders such as depression (Holsboer et al., 1984). Furthermore, chronic pain and depressive disorders have a high rate of comorbidity (Bair et al., 2003), and the two conditions can be treated with medications that share overlapping mechanisms of action (Mico et al., 2006).

In light of this apparent relationship between HPA-axis dysregulation and chronic pain, taken together with my findings of altered KO mouse phenotype (both experimental (Chapter 3; pain phenotype) and observational (see Section 6.1; calmer demeanour)), and the known expression of the α9α10-nAChR in the HPA axis (Colomer et al., 2010; Elgoyhen et al., 1994), I hypothesised that HPA-axis dysregulation in KO mice may affect their pain phenotype. In particular, I predicted that the KO mice, having lesser pain reactivity (in mechanical hyperalgesia only), would display reduced stress-induced responses when compared to WT animals.

The calmer demeanour of KO mice overall, particularly during PAM testing, suggested that decreased stress responses reduced the salience of the test and/or the testing procedure. This decreased salience of the experience may have reduced the aversiveness of the procedure during future testing. The muted
corticosterone responses of KO mice to acute stress (Section 7.3.2) support this notion of decreased salience during the stressful testing procedure. If the one-week interval between PAM testing was long enough for each testing day to be perceived as an acute stress experience, then this decreased physiological stress response to acute stressors may account for the significantly higher pain threshold during mechanical hyperalgesia testing and the lack of repeated-testing-induced hyperalgesia in the KO mice (Section 3.3.4). Conversely, if the weekly PAM testing did constitute persistent stress, the decreased stress-induced arousal observed in KO mice (Section 7.3.1.1) may have decreased the motor output of subjective arousal during PAM testing, manifesting as increased withdrawal response times and higher pain threshold. However, the proposal that the HPA-stress-axis mediated the magnitude of responses to the noxious stimuli is not wholly supported, since only mechanical, and not thermal hypersensitivity was altered in the pain phenotype of KO mice (Section 3.3.2). Thus, there are likely other mechanisms involved.

No differences between WT and α9α10-nAChR KO mice were observed in baseline (pre-stress) measures of behavioural or physiological stress. Prolonged stress did, however, trigger behavioural and physiological changes in the KO mice. A prolonged-stress-induced increase in depression- (Section 6.3.5) and anxiety-like (Section 7.3.1.2) behaviour was observed, alongside an exaggerated corticosterone response. None of these changes indicate a phenotype that is less prone to pain, nor do they support the hypothesis that α9-nAChR deletion would cause decreased stress-induced responses to painful or non-painful stressors.

The α9α10-nAChR is known to be important for synapse development in the inner ear, and it is therefore conceivable that auditory deficits in α9α10-nAChR KO mice may affect the perception of environmental cues that normally act as stressors. Such altered environmental perceptions were not controlled for in the present experiments and the contribution of such differences to the changes in both pain perception and stress responses in the KO genotype is not known. However only minor deficits in auditory discrimination are present in the α9α10-nAChR KO mouse (see Section 1.5.3.2) and since none of the stimuli (pain
or stress) used here involved an auditory component, it is not likely that the differences between genotypes seen in the present experiments are due to auditory deficits.

It is not known whether the pain models or testing procedures used in the experiments presented here constitute stressors or, if they are stressors, whether they cause stress-induced changes to animal behaviour and physiology that is comparable to the stress paradigm designed in Chapter 7. Experiments examining this issue, for example by measuring the pre- and post-test corticosterone levels in WT and KO mice, may help to bridge the findings of altered pain phenotype (Chapter 3) and altered stress reactivity (Chapter 6 and Chapter 7).

**8.5.5 Broader impact of α9-nAChR subunit deletion or inhibition**

Since α9α10-nAChRs are expressed in both the adrenal and pituitary glands, it is possible that the dysfunction of both of these sites within the HPA axis contribute to the altered phenotype seen in the α9-nAChR KO mice. The intercommunication of many CNS regions and peripheral glands (Figure 8.1) likely account for the unique phenotype of α9-nAChR KO mice characterised in the present experiments, from alterations in pain thresholds and circadian patterns, to altered stress-induced arousal and susceptibility to depression- and anxiety-like behaviour. The HPA axis is highly dynamic, having continuous feedback signalling within its circuitry (Blackburn-Munro and Blackburn-Munro, 2001; Engelmann *et al.*, 2004). The light-sensitive circadian pacemaker, the SCN, which is also highly sensitive to stress, controls the rhythmicity of HPA-axis secretions including CRH, ACTH and corticosteroids (Balbo *et al.*, 2010a; Kalsbeek *et al.*, 2012). The LC-NE system contributes to arousal (Berridge and Waterhouse, 2003; Kovacs, 2013) and anxiety (Butler *et al.*, 1990; Van Gaalen *et al.*, 2002). All of these factors may be sensitive to α9α10-nAChR function in the HPA axis and may be affected by pharmacological inhibition of the receptor.
Thus increased vulnerability to affective disorders and circadian rhythm dysregulation must be considered in the context of using any α9α10-nAChR-inhibiting pharmacological agents.
Figure 8.1. Interplay between external stimuli and neuroendocrine centres. External factors (light and stress) affect multiple components of the nervous and endocrine systems that feedback onto each other, forming complex biological connections. Ant. Pit., anterior pituitary; LC, locus coeruleus; PVN, paraventricular nucleus of the hypothalamus; SCN, suprachiasmatic nucleus.
8.6 Future experiments

The experiments presented here have shed light on the role of the α9α10-nAChR in pain states, as well as in HPA-axis function and affective behaviour. However, previously unexplored functions of the receptor have been uncovered that beg further examination.

8.6.1 The α9α10-nAChR in nociception vs. pain

Pain phenotyping of α9-nAChR KO mice showed that the receptor is not necessary for the perception of pain in most modalities, with the exception of mechanical hyperalgesia in chronic pain states. This suggests specific expression of the receptor in a subtype of high-threshold mechanical sensory afferents. However, this does not account for the lack of repeated-measures-induced mechanical hyperalgesia in the KO mice. Whether or not the KO mice develop an analgesic response to the repeated testing or fail to consolidate fear memories upon previous tests is unclear. Investigating these possibilities would be interesting avenues to explore.

8.6.2 Chronic pain as a stressor in mice

As discussed in Section 7.4.4, there is no consensus as to whether mice “suffer” according to analogous measures of quality-of-life that are affected in humans. It would be of great interest to determine whether the cuff model of neuropathic pain used here elicits affective changes such as the anxiety and depression syndromes that are comorbid with human chronic pain. Furthermore, if these disturbances were evident, the involvement of the α9α10-nAChR in mediating coping style would be an intriguing avenue to explore.

Of particular interest is to determine how the pain phenotype characterised here relates to the HPA-axis disturbances and affective changes identified in the α9-nAChR KO mice. As described in Section 8.5.4 above, further experiments are needed to better understand the magnitude of stress caused by both the testing
process and the pain itself. With this greater understanding, the overall phenotype of α9-nAChR KO mice can be better understood, especially with regards to the relationship between the altered pain phenotype and the stress-response (e.g. corticosterone) dysregulation.

8.6.3 AFFECTIVE DISORDERS IN α-CONOTOXIN-TREATED MICE
Conotoxins Vc1.1 and RglA are the most potent and selective inhibitors of the α9α10-nAChR receptor. No adverse side effects have yet been reported for these conotoxins when tested as analgesics. However, the effects of α9α10-nAChR-inhibition on HPA-axis dysfunction and affective responses have not been studied after either short- or long-term drug administration. Since α9-nAChR KO mice exhibit clear alterations in naïve circadian rhythmicity and in post-stress responses, it will be important to determine whether other small molecule or conotoxin compounds that inhibit the receptor have the same effects.
8.7 CONCLUSIONS

The aim of this project, as a whole, was to determine the role of the α9α10-nAChR in pain, and to determine the validity of the receptor as a target for analgesic compounds. Potential side effects of α9α10-nAChR-inhibiting agents were explored, and the validity of a proposed mechanism of α9α10-nAChR-mediated analgesia was examined.

Results obtained from pain phenotyping of α9-nAChR KO mice have shown that while the receptor subtype does play a role in pain, this is limited to the specific modality of mechanical hyperalgesia. This previously uncharacterised dissociation of α9α10-nAChR-mediated pain has implications for rational drug design that is currently pursuing this receptor target for the acute treatment of allodynic symptoms (e.g. Holtman et al. (2011) and Wala et al. (2012)). The findings have further implications for the analgesic α-conotoxins (e.g. Vc1.1 and RgIA) that initially identified the α9α10-nAChR as a potential pain target. Although it has been known for several years that these conotoxins have alternative mechanisms of action that more adequately account for their acute analgesia than α9α10-nAChR inhibition, the present findings unequivocally rule out the necessity for α9α10-nAChR-affinity in acutely alleviating allodynia. α9α10-nAChR-inhibiting compounds may be useful for alleviating long-term mechanical hyperalgesia. This knowledge will guide further optimisation of these and other analgesic α-conotoxins as well as aid future analgesic drug design.

The findings from further behavioural phenotyping of α9-nAChR KO mice have shown for the first time the potential risks of inhibiting this receptor, which is expressed in tissues unrelated to pain and sensory perception. The expression of the α9α10-nAChR in the HPA-axis opens up multiple potential off-target side effects of α9α10-nAChR-inhibiting analgesics. Germline deletion of the α9-nAChR subunit resulted in altered circadian rhythmicity in naïve animals, altered stress-induced arousal, and a vulnerability to stress-induced anxiety- and depression-
like behaviour. Physiological stress responses were dysregulated in the absence of functional α9α10-nAChRs. Side effects related to HPA-axis dysfunction are yet to be studied in animals treated with α9α10-nAChR-inhibiting compounds, and such symptoms may only affect vulnerable subpopulations. However, in light of the effects of α9-nAChR subunit deletion described here, inhibitors of the α9α10-nAChR should be approached with caution.

Investigation of a proposed immune role of α9α10-nAChRs in α-conotoxin-mediated analgesia revealed no involvement of the receptor in the pathology of neuropathic pain. In contrast to suggestions that have been made in the literature (Mannelli et al., 2014; Vincler et al., 2006), absence of functional α9α10-nAChRs did not affect the amount of oedema produced by constriction injury in the sciatic nerve, nor did it affect the extent of infiltration of immune cells or ACh-producing cells. Once again, the present findings have demonstrated that α9α10-nAChR-affinity is not necessary for the beneficial effects of the much-studied analgesic α-conotoxins. It is apparent that alternative mechanisms are a responsible for much of the benefit associated with these conotoxins beyond α9α10-nAChR inhibition.

Based on the outcomes of this project, I hope that future drug design will be better informed about the benefits and risks of inhibiting α9α10-nAChRs and that the knowledge gained from the present experiments will help to guide the development of novel therapeutics.
REFERENCES


Bennett MI (2011). Effectiveness of antiepileptic or antidepressant drugs when added to opioids for cancer pain: systematic review. Palliative Medicine 25: 553-559.


Cuny H, de Faoite A, Huynh TG, Yasuda T, Berecki G, Adams DJ (2012). gamma-Aminobutyric Acid Type B (GABA(B)) Receptor Expression Is Needed for Inhibition of N-type (Ca(v)2.2) Calcium Channels by Analgesic alpha-Conotoxins. *Journal of Biological Chemistry* 287: 23948-23957.


ganglion neurons of the adult rat. *Autonomic Neuroscience-Basic & Clinical* 113: 32-42.


Kolosov A, Aurini L, Williams ED, Cooke I, Goodchild CS (2011). Intravenous Injection of Leconotide, an Omega Conotoxin: Synergistic Antihyperalgesic


Moore RY, Eichler VB (1972). LOSS OF A CIRCADIAN ADRENAL CORTICOSTERONE RHYTHM FOLLOWING SUPRACHiasmatic LESIONS IN RAT. *Brain Research* 42: 201-2&.


Shibasaki T, Imaki T, Hotta M, Nicholas L, Demura H (1993). Psychological stress increases arousal through brain corticotropin-releasing hormone without
significant increase in adrenocorticotropic and catecholamine secretion. *Brain Research* 618: 71-75.


Wilhelm CJ, Murphy-Crews A, Menasco DJ, Huckans MS, Loftis JM (2012). Corticotropin releasing factor-1 receptor antagonism alters the biochemical, but


APPENDIX A.

INTELLICAGE EXPERIMENTAL MODULES

Run 1 modules:
Nosepoke Adaptation
Sucrose Adaptation

Impulsivity sessions
Aversion

Run 2:
Modules were as for Run1, except for Sucrose Adaptation
APPENDIX B.

INTELLICAGE EXPERIMENTAL OPTION NOTES

Run 1 Experiment Options:

Start date: 27.09.2012

Free Adaptation (5 days; 27.09.2012-02.10.12) - Free access to water. All doors open all the time.

Nosepoke Adaptation (7 days; 02.10.12-09.10.12)- Animals must perform a nosepoke to open the doors. Doors open for 3 seconds, and only once per visit.

Drinking Session Adaptation (7 days; 09.10.12-16.10.12)- Drinking available between 04:30-06:30 and 19:30-21:30 where module switches from default to NPA.

Sucrose Adaptation I (7+1 days; 16.10.12-23.10.12)- Same as DSA but right hand side bottle in each corner is 10% sucrose. Green LEDs on RHS turn ON upon entry into the corner. These turn OFF when visit ends. Cluster settings changed so that RHS = "correct" & LHS = "incorrect". 2 NPs required to open the door (counter set to 2).
(22.10.2012)- NOTE: Replaced dirty cage with a clean cage. Scattered some old bedding around.

Impulsivity Test I (7 days; 23.10.12-31.10.12)- To access sucrose, animals must NP twice on RHS with progressively larger gap between 1st and 2nd NP. If they NP prematurely on RHS the timer is reset. If they NP on the LHS after the 1st NP the trial is aborted and they must exit the corner. If the first NP is on LHS, water door opens as normal (only once per visit) but they cannot access sucrose until they perform a new visit. Progressive delays run in 1s increments from 1-10. When they successfully perform task 10 times they progress to the next highest delay time.
(24.10.2012)- Programming was awry for impulsivity last night. Have rectified with some disturbance to animals (i.e. manually checking the programming to ensure functionality).
(26.10.2012)- Changed time increments to 500ms starting at 500ms.

Sucrose Adaptation II (11 days; 31.10.2012-12.11.2012)-Re-train with altered sucrose adaptation- Same as "Sucrose Adaptation" except only 1 NP required to open either door and LEDs go OFF after a NP.
(8.11.12) ~1645 changed boxes. Stopped-Started.

Impulsivity Test II (7 days; 12.11.12-19.11.12)- To access sucrose, animals must NP twice on RHS with progressively larger gap between 1st and 2nd NP. If they NP prematurely on RHS the timer is reset. If they NP on the LHS after the 1st
NP the trial is aborted and they must exit the corner. If the first NP is on LHS, water door opens as normal (only once per visit) but they cannot access sucrose until they perform a new visit. Progressive delays run in 500ms increments from 500ms-5sec. When they successfully perform task 10 times they progress to the next highest delay time.

**Sucrose Adaptation III** (3 days; 19.11.12-22.11.12) - Same as "Sucrose Adaptation II"

**Aversion** (5 days; 22.11.12-27.11.12) - "Correct" drinking elicits air-puff. i.e. sucrose consumption will be paired with an aversive stimulus.
Run 2 Experiment Options:


Free Adaptation (5 days; 21.12.2012-28.12.12) - Free access to water. All doors open all the time.

Nosepoke Adaptation (3 days; 28.12.12-31.12.12)- Animals must perform a nosepoke to open the doors. Doors open for 3 seconds, and only once per visit.

Drinking Session Adaptation (7 days; 31.12.12-07.01.13)- Drinking available between 04:30-06:30 and 19:30-21:30 where module switches from default to NPA.

Sucrose Adaptation I (7 days; 07.01.13-14.01.13)- Same as DSA but right hand side bottle in each corner is 10% sucrose. Green LEDs on RHS turn ON upon entry into the corner. These turn OFF when NP ON or visit ends. Cluster settings changed so that RHS = "correct" & LHS = "incorrect".

Impulsivity test (7 days; 14.01.13-21.01.13)- To access sucrose, animals must NP once on RHS with progressively larger gap between 1st and 2nd NP. If they NP prematurely on RHS the timer is reset. If they NP on the LHS after the 1st NP the trial is aborted and they must exit the corner. If the first NP is on LHS, water door opens as normal (only once per visit) but they cannot access sucrose until they perform a new visit. Progressive delays run in 0.5s increments from 0.5-5sec. When they successfully perform task 10 times they progress to the next highest delay time.

Sucrose Adaptation II (4 days; 21.01.13-25.01.13)- same as SA before.

Aversion (25.01.13-End) -" Correct" drinking elicits air-puff. i.e. sucrose consumption will be paired with an aversive stimulus.
Name: Assessment of general behaviours of KO vs. WT, and C57 vs ...

Author: Saeed Mohammed & Thomas Burton

Start date: 26.11.2013

Pre-adaptation (5 days; 26, 27, 28, 1, 2): Free access to water. All doors open all the time.

Noapseal Adaptation (3 days; 28, 29, 30): Animals must perform a nosapole to open the doors. Doors open for 2 seconds, and only once per visit.

Drinking Session Adaptation (7 days; 31, 1, 2, 3, 4, 5, 6): Drinking available between 04:30-06:30 and 18:30-20:30 where radiant switches from default to P16.

Success Adaptation I (7 days; 7, 13, 14, 15, 17, 18, 19): Same as DSA but right hand adaption in each corner is 75% success. Green LEDs an red turn ON upon entry into the corner. These turn OFF when when NP or visit ends. Quater settings changed so that RNO = Terrific & LNO = Incorrec!

Drinking test (7 days; 4, 6, 13, 15, 17, 19): To access sucrose, animals must tap once on left, with progressively longer gap between 1st and 2nd tap. If the gap is missed, a timeout occurs. If sucrose is not on the 1st tap the trial is aborted and they must restart the corner. If the 1st tap is correct, water door opens an normal (only once per visit) but they cannot access sucrose until they perform a new visit. Progression days run in 0.5 increments from 0.5-1 sec. When they successfully perform 10 times they progress to the next highest delay time.

Success Adaptation II (4 days; 21, 26, 27, 28): Same as DSA before.

Aversion (28, 11, 13, 15, 17, 19): Correct drinking elicits a puff. I.e. sucrose consumption will be paired with an aversive stimulus.
## APPENDIX C.

**INTELLICAGE MODULE SWITCH STATISTICAL ANALYSIS OUTCOMES**

### Table A1. Run1 Visits

<table>
<thead>
<tr>
<th>WT vs. KO</th>
<th>WT vs. WT</th>
<th>KO vs. KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Adaptation</td>
<td>ns</td>
<td>FA vs. NPA</td>
</tr>
<tr>
<td>NPA</td>
<td>ns</td>
<td>FA vs. DSA</td>
</tr>
<tr>
<td>DSA</td>
<td>*</td>
<td>FA vs. SAI</td>
</tr>
<tr>
<td>SAI</td>
<td>ns</td>
<td>FA vs. Impl</td>
</tr>
<tr>
<td>Imp</td>
<td>ns</td>
<td>FA vs. SAIi</td>
</tr>
<tr>
<td>SAIi</td>
<td>ns</td>
<td>FA vs. ImplII</td>
</tr>
<tr>
<td>Aversion</td>
<td>ns</td>
<td>FA vs. Aversion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FA vs. Aversion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FA vs. Aversion</td>
</tr>
<tr>
<td>NPA vs. DSA</td>
<td>ns</td>
<td>NPA vs. DSA</td>
</tr>
<tr>
<td>NPA vs. SAI</td>
<td>ns</td>
<td>NPA vs. SAI</td>
</tr>
<tr>
<td>NPA vs. Impl</td>
<td>ns</td>
<td>NPA vs. Impl</td>
</tr>
<tr>
<td>NPA vs. SAIi</td>
<td>ns</td>
<td>NPA vs. SAIi</td>
</tr>
<tr>
<td>NPA vs. ImplII</td>
<td>ns</td>
<td>NPA vs. ImplII</td>
</tr>
<tr>
<td>NPA vs. SAIIII</td>
<td>ns</td>
<td>NPA vs. SAIIII</td>
</tr>
<tr>
<td>NPA vs. Aversion</td>
<td>ns</td>
<td>NPA vs. Aversion</td>
</tr>
<tr>
<td>DSA vs. SAI</td>
<td>ns</td>
<td>DSA vs. SAI</td>
</tr>
<tr>
<td>DSA vs. Impl</td>
<td>ns</td>
<td>DSA vs. Impl</td>
</tr>
<tr>
<td>DSA vs. SAIi</td>
<td>ns</td>
<td>DSA vs. SAIi</td>
</tr>
<tr>
<td>DSA vs. ImplII</td>
<td>ns</td>
<td>DSA vs. ImplII</td>
</tr>
<tr>
<td>DSA vs. SAIIII</td>
<td>ns</td>
<td>DSA vs. SAIIII</td>
</tr>
<tr>
<td>DSA vs. Aversion</td>
<td>ns</td>
<td>DSA vs. Aversion</td>
</tr>
<tr>
<td>SAI vs. Impl</td>
<td>ns</td>
<td>SAI vs. Impl</td>
</tr>
<tr>
<td>SAI vs. SAIi</td>
<td>ns</td>
<td>SAI vs. SAIi</td>
</tr>
<tr>
<td>SAI vs. ImplII</td>
<td>ns</td>
<td>SAI vs. ImplII</td>
</tr>
<tr>
<td>SAI vs. SAIi</td>
<td>ns</td>
<td>SAI vs. SAIi</td>
</tr>
<tr>
<td>SAI vs. Aversion</td>
<td>ns</td>
<td>SAI vs. Aversion</td>
</tr>
<tr>
<td>Impl vs. SAIi</td>
<td>ns</td>
<td>Impl vs. SAIi</td>
</tr>
<tr>
<td>Impl vs. ImplII</td>
<td>ns</td>
<td>Impl vs. ImplII</td>
</tr>
<tr>
<td>Impl vs. SAIii</td>
<td>ns</td>
<td>Impl vs. SAIii</td>
</tr>
<tr>
<td>Impl vs. Aversion</td>
<td>ns</td>
<td>Impl vs. Aversion</td>
</tr>
<tr>
<td>SAIi vs. ImplII</td>
<td>ns</td>
<td>SAIi vs. ImplII</td>
</tr>
<tr>
<td>SAIi vs. SAIii</td>
<td>ns</td>
<td>SAIi vs. SAIii</td>
</tr>
<tr>
<td>SAIi vs. Aversion</td>
<td>ns</td>
<td>SAIi vs. Aversion</td>
</tr>
<tr>
<td>SAIi vs. Aversion</td>
<td>ns</td>
<td>SAIi vs. Aversion</td>
</tr>
<tr>
<td>ImplII vs. SAIi</td>
<td>ns</td>
<td>ImplII vs. SAIi</td>
</tr>
<tr>
<td>ImplII vs. Aversion</td>
<td>ns</td>
<td>ImplII vs. Aversion</td>
</tr>
<tr>
<td>SAIii vs. Aversion</td>
<td>ns</td>
<td>SAIii vs. Aversion</td>
</tr>
<tr>
<td>WT vs. KO</td>
<td>WT vs. WT</td>
<td>KO vs. KO</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Free Adaptation ns</td>
<td>FA vs. NPA ns</td>
<td>FA vs. NPA ns</td>
</tr>
<tr>
<td>NPA ns</td>
<td>FA vs. DSA ns</td>
<td>FA vs. DSA ns</td>
</tr>
<tr>
<td>DSA ns</td>
<td>FA vs. SAI ns</td>
<td>FA vs. SAI ns</td>
</tr>
<tr>
<td>SAI *</td>
<td>FA vs. ImpI *</td>
<td>FA vs. ImpI ****</td>
</tr>
<tr>
<td>Imp ns</td>
<td>FA vs. SAIII ****</td>
<td>FA vs. SAIII ****</td>
</tr>
<tr>
<td>SAIII ns</td>
<td>FA vs. ImpII ns</td>
<td>FA vs. ImpII ****</td>
</tr>
<tr>
<td>Aversion ns</td>
<td>FA vs. SAIIII ***</td>
<td>FA vs. SAIII ****</td>
</tr>
<tr>
<td>FA vs. Aversion **</td>
<td></td>
<td>FA vs. Aversion ****</td>
</tr>
<tr>
<td>NPA vs. DSA ns</td>
<td></td>
<td>NPA vs. DSA ns</td>
</tr>
<tr>
<td>NPA vs. SAI ns</td>
<td></td>
<td>NPA vs. SAI ns</td>
</tr>
<tr>
<td>NPA vs. ImpI ns</td>
<td></td>
<td>NPA vs. ImpI ns</td>
</tr>
<tr>
<td>NPA vs. SAIII ns</td>
<td></td>
<td>NPA vs. SAIII ns</td>
</tr>
<tr>
<td>NPA vs. ImpII ns</td>
<td></td>
<td>NPA vs. ImpII ns</td>
</tr>
<tr>
<td>NPA vs. SAIIII ns</td>
<td></td>
<td>NPA vs. SAIIII ns</td>
</tr>
<tr>
<td>NPA vs. Aversion ns</td>
<td></td>
<td>NPA vs. Aversion ns</td>
</tr>
<tr>
<td>DSA vs. SAI ns</td>
<td></td>
<td>DSA vs. SAI ns</td>
</tr>
<tr>
<td>DSA vs. ImpI ns</td>
<td></td>
<td>DSA vs. ImpI **</td>
</tr>
<tr>
<td>DSA vs. SAIII ns</td>
<td></td>
<td>DSA vs. SAIII **</td>
</tr>
<tr>
<td>DSA vs. ImpII ns</td>
<td></td>
<td>DSA vs. ImpII ***</td>
</tr>
<tr>
<td>DSA vs. SAIIII ns</td>
<td></td>
<td>DSA vs. SAIIII ***</td>
</tr>
<tr>
<td>DSA vs. Aversion ns</td>
<td></td>
<td>DSA vs. Aversion ****</td>
</tr>
<tr>
<td>SAI vs. ImpI ns</td>
<td></td>
<td>SAI vs. ImpI ****</td>
</tr>
<tr>
<td>SAI vs. SAIII ns</td>
<td></td>
<td>SAI vs. SAIII ****</td>
</tr>
<tr>
<td>SAI vs. ImpII ns</td>
<td></td>
<td>SAI vs. ImpII ****</td>
</tr>
<tr>
<td>SAI vs. SAIIII ns</td>
<td></td>
<td>SAI vs. SAIIII ****</td>
</tr>
<tr>
<td>SAI vs. Aversion ns</td>
<td></td>
<td>SAI vs. Aversion ****</td>
</tr>
<tr>
<td>ImpI vs. SAIII ns</td>
<td></td>
<td>ImpI vs. SAIII ns</td>
</tr>
<tr>
<td>ImpI vs. ImpII ns</td>
<td></td>
<td>ImpI vs. ImpII ns</td>
</tr>
<tr>
<td>ImpI vs. SAIIII ns</td>
<td></td>
<td>ImpI vs. SAIIII ns</td>
</tr>
<tr>
<td>ImpI vs. Aversion ns</td>
<td></td>
<td>ImpI vs. Aversion ns</td>
</tr>
<tr>
<td>SAIII vs. ImpII ns</td>
<td></td>
<td>SAIII vs. ImpII ns</td>
</tr>
<tr>
<td>SAIII vs. SAIIII ns</td>
<td></td>
<td>SAIII vs. SAIIII ns</td>
</tr>
<tr>
<td>SAIII vs. Aversion ns</td>
<td></td>
<td>SAIII vs. Aversion ns</td>
</tr>
<tr>
<td>SAIII vs. Aversion ns</td>
<td></td>
<td>SAIII vs. Aversion ns</td>
</tr>
<tr>
<td>ImpII vs. SAIIII ns</td>
<td></td>
<td>ImpII vs. SAIIII ns</td>
</tr>
<tr>
<td>ImpII vs. Aversion ns</td>
<td></td>
<td>ImpII vs. Aversion ns</td>
</tr>
<tr>
<td>SAIIII vs. Aversion ns</td>
<td></td>
<td>SAIIII vs. Aversion ns</td>
</tr>
<tr>
<td></td>
<td>WT vs. KO</td>
<td>WT vs. WT</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Free Adaptation</td>
<td>****</td>
<td>FA vs. NPA*</td>
</tr>
<tr>
<td>NPA</td>
<td>*</td>
<td>FA vs. DSA***</td>
</tr>
<tr>
<td>DSA</td>
<td>ns</td>
<td>FA vs. SAI***</td>
</tr>
<tr>
<td>SAI</td>
<td>ns</td>
<td>FA vs. ImpI****</td>
</tr>
<tr>
<td>Imp</td>
<td>ns</td>
<td>FA vs. SAII***</td>
</tr>
<tr>
<td>SAII</td>
<td>ns</td>
<td>FA vs. ImpII***</td>
</tr>
<tr>
<td>Aversion</td>
<td>ns</td>
<td>FA vs. SAIII****</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FA vs. Aversion***</td>
</tr>
<tr>
<td>NPA vs. DSA</td>
<td>ns</td>
<td>NPA vs. DSA ns</td>
</tr>
<tr>
<td>NPA vs. SAI</td>
<td>ns</td>
<td>NPA vs. SAI ns</td>
</tr>
<tr>
<td>NPA vs. ImpI</td>
<td>ns</td>
<td>NPA vs. ImpI ns</td>
</tr>
<tr>
<td>NPA vs. SAII</td>
<td>ns</td>
<td>NPA vs. SAII ns</td>
</tr>
<tr>
<td>NPA vs. ImpII</td>
<td>ns</td>
<td>NPA vs. ImpII ns</td>
</tr>
<tr>
<td>NPA vs. SAIIII</td>
<td>ns</td>
<td>NPA vs. SAIII*</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td>NPA vs. Aversion*</td>
</tr>
<tr>
<td>DSA vs. SAI</td>
<td>ns</td>
<td>DSA vs. SAI ns</td>
</tr>
<tr>
<td>DSA vs. ImpI</td>
<td>ns</td>
<td>DSA vs. ImpI ns</td>
</tr>
<tr>
<td>DSA vs. SAII</td>
<td>ns</td>
<td>DSA vs. SAII ns</td>
</tr>
<tr>
<td>DSA vs. ImpII</td>
<td>ns</td>
<td>DSA vs. ImpII ns</td>
</tr>
<tr>
<td>DSA vs. SAIII</td>
<td>ns</td>
<td>DSA vs. SAIII ns</td>
</tr>
<tr>
<td>DSA vs. Aversion</td>
<td>ns</td>
<td>DSA vs. Aversion ns</td>
</tr>
<tr>
<td>SAI vs. ImpI</td>
<td>ns</td>
<td>SAI vs. ImpI ns</td>
</tr>
<tr>
<td>SAI vs. SAII</td>
<td>ns</td>
<td>SAI vs. SAII ns</td>
</tr>
<tr>
<td>SAI vs. ImpII</td>
<td>ns</td>
<td>SAI vs. ImpII ns</td>
</tr>
<tr>
<td>SAI vs. SAIII</td>
<td>ns</td>
<td>SAI vs. SAIII ns</td>
</tr>
<tr>
<td>SAI vs. Aversion</td>
<td>ns</td>
<td>SAI vs. Aversion ns</td>
</tr>
<tr>
<td>ImpI vs. SAII</td>
<td>ns</td>
<td>ImpI vs. SAII ns</td>
</tr>
<tr>
<td>ImpI vs. ImpII</td>
<td>ns</td>
<td>ImpI vs. ImpII ns</td>
</tr>
<tr>
<td>ImpI vs. SAIII</td>
<td>ns</td>
<td>ImpI vs. SAIII ns</td>
</tr>
<tr>
<td>ImpI vs. Aversion</td>
<td>ns</td>
<td>ImpI vs. Aversion ns</td>
</tr>
<tr>
<td>SAII vs. ImpII</td>
<td>ns</td>
<td>SAII vs. ImpII ns</td>
</tr>
<tr>
<td>SAII vs. SAIII</td>
<td>ns</td>
<td>SAII vs. SAIII ns</td>
</tr>
<tr>
<td>SAII vs. Aversion</td>
<td>ns</td>
<td>SAII vs. Aversion ns</td>
</tr>
<tr>
<td>ImpII vs. SAIII</td>
<td>ns</td>
<td>ImpII vs. SAIII ns</td>
</tr>
<tr>
<td>ImpII vs Aversion</td>
<td>ns</td>
<td>ImpII vs Aversion ns</td>
</tr>
<tr>
<td>SAIII vs Aversion</td>
<td>ns</td>
<td>SAIII vs Aversion ns</td>
</tr>
</tbody>
</table>
### Table A4. Run2 Visits

<table>
<thead>
<tr>
<th>WT vs. KO</th>
<th>WT vs. WT</th>
<th>KO vs. KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Adaptation</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>NPA</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>DSA</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>SAI</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Imp</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>SAIII</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Aversion</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>FA vs. NPA</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>FA vs. DSA</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>FA vs. SAI</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>FA vs. Imp</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>FA vs. SAIII</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>FA vs. Aversion</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>NPA vs. DSA</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>NPA vs. SAI</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>NPA vs. Imp</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>NPA vs. SAIII</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>NPA vs. Aversion</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>DSA vs. SAI</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>DSA vs. Imp</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>DSA vs. SAIII</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>DSA vs. Aversion</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>SAI vs. Imp</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>SAI vs. SAIII</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>SAI vs. Aversion</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Imp vs. SAIII</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Imp vs. Aversion</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>SAIII vs. Aversion</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>
### Table A5. Run2 Nosepokes

<table>
<thead>
<tr>
<th>WT vs. KO</th>
<th>WT vs. WT</th>
<th>KO vs. KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Adaptation</td>
<td>ns</td>
<td>FA vs. NPA</td>
</tr>
<tr>
<td>NPA</td>
<td>ns</td>
<td>FA vs. DSA</td>
</tr>
<tr>
<td>DSA</td>
<td>ns</td>
<td>FA vs. SAI</td>
</tr>
<tr>
<td>SAI</td>
<td>ns</td>
<td>FA vs. Imp</td>
</tr>
<tr>
<td>Imp</td>
<td>ns</td>
<td>FA vs. SAIII</td>
</tr>
<tr>
<td>SAIi</td>
<td>ns</td>
<td>FA vs. Aversion</td>
</tr>
<tr>
<td>Aversion</td>
<td>ns</td>
<td>NPA vs. DSA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NPA vs. SAI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NPA vs. Imp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NPA vs. SAIII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NPA vs. Aversion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DSA vs. SAI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DSA vs. Imp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DSA vs. SAIII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DSA vs. Aversion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAI vs. Imp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAI vs. SAIII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAI vs. Aversion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Imp vs. SAIII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Imp vs. Aversion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAIi vs. Aversion</td>
</tr>
</tbody>
</table>
### Table A6. Run2 Licks

<table>
<thead>
<tr>
<th>WT vs. KO</th>
<th>WT vs. WT</th>
<th>KO vs. KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Adaptation</td>
<td>* FA vs. NPA ns</td>
<td>FA vs. NPA ****</td>
</tr>
<tr>
<td>NPA</td>
<td>ns FA vs. DSA ns</td>
<td>FA vs. DSA ****</td>
</tr>
<tr>
<td>DSA</td>
<td>ns FA vs. SAI ns</td>
<td>FA vs. SAI ****</td>
</tr>
<tr>
<td>SAI</td>
<td>ns FA vs. Imp ns</td>
<td>FA vs. Imp ****</td>
</tr>
<tr>
<td>Imp</td>
<td>ns FA vs. SAII ns</td>
<td>FA vs. SAII ****</td>
</tr>
<tr>
<td>SAIII</td>
<td>ns FA vs. Aversion *</td>
<td>FA vs. Aversion ****</td>
</tr>
<tr>
<td>Aversion</td>
<td>ns NPA vs. DSA ns</td>
<td>NPA vs. DSA ns</td>
</tr>
<tr>
<td></td>
<td>ns NPA vs. SAII ns</td>
<td>NPA vs. SAII ns</td>
</tr>
<tr>
<td></td>
<td>ns NPA vs. Imp ns</td>
<td>NPA vs. Imp ns</td>
</tr>
<tr>
<td></td>
<td>ns NPA vs. SAII ns</td>
<td>NPA vs. SAII ns</td>
</tr>
<tr>
<td></td>
<td>ns NPA vs. Aversion ns</td>
<td>NPA vs. Aversion ns</td>
</tr>
<tr>
<td></td>
<td>ns DSA vs. SAII ns</td>
<td>DSA vs. SAII ns</td>
</tr>
<tr>
<td></td>
<td>ns DSA vs. SAII ns</td>
<td>DSA vs. SAII ns</td>
</tr>
<tr>
<td></td>
<td>ns DSA vs. Imp ns</td>
<td>DSA vs. Imp ns</td>
</tr>
<tr>
<td></td>
<td>ns DSA vs. SAII ns</td>
<td>DSA vs. SAII ns</td>
</tr>
<tr>
<td></td>
<td>ns DSA vs. Aversion ns</td>
<td>DSA vs. Aversion ns</td>
</tr>
<tr>
<td></td>
<td>ns SAI vs. Imp ns</td>
<td>SAI vs. Imp ns</td>
</tr>
<tr>
<td></td>
<td>ns SAI vs. SAII ns</td>
<td>SAI vs. SAII ns</td>
</tr>
<tr>
<td></td>
<td>ns SAI vs. Aversion ns</td>
<td>SAI vs. Aversion ns</td>
</tr>
<tr>
<td></td>
<td>ns Imp vs. SAII ns</td>
<td>Imp vs. SAII ns</td>
</tr>
<tr>
<td></td>
<td>ns Imp vs. Aversion ns</td>
<td>Imp vs. Aversion ns</td>
</tr>
<tr>
<td></td>
<td>ns SAIII vs. Aversion ns</td>
<td>SAIII vs. Aversion ns</td>
</tr>
</tbody>
</table>
APPENDIX D.

PUBLICATIONS AND PROCEEDINGS

Publications


S. Mohammadi, M.J. Christie. α9-nicotinic acetylcholine receptors contribute to the maintenance of chronic mechanical hyperalgesia, but not thermal or mechanical allodynia. Molecular Pain. 10:64 (9 pp) DOI: 10.1186/1744-8069-10-64. Published: OCT 2 2014


Invited Speaker

Cone snails to analgesics: Is the α9-nicotinic acetylcholine receptor a good analgesic target?
(Apr 7, 2015) Hoshi University, Tokyo, Japan
Conference proceedings

Oral presentations:
Stress-induced affective dysregulation in α9-nAChR KO mice.  
*Bosch Facility User Group Meeting; Sydney, Australia* (Nov 12, 2015)

Inhibition of the α9α10-nAChR: Good pain relief of a side-effect liability?  
*Inter-University Neuroscience and Mental Health Conference; Sydney, Australia* (Sept 24, 2015)

Cone snails to analgesics: Is the α9-nicotinic acetylcholine receptor a good analgesic target?  
*Bosch Young Investigator Symposium XIV; Sydney, Australia* (Dec 9, 2014)

Limited clinical scope of selective α9-nicotinic acetylcholine receptor blocking analgesics.  
*Venoms to Drugs; Kingscliff, Australia* (Oct 21, 2014)

The Alpha-9 nACh Receptor Contributes to Mechanical Hyperalgesia, but not allodynia.  
*Bosch Young Investigators Seminar Series; Sydney, Australia* (May 14, 2014)

Using Behavioural Tests to Dissect Pain Pathways.  
*Bosch Institute Facility User Group Meeting; Sydney, Australia* (May 29, 2014)

Selected poster presentations:

α9α10-nAChR: Novel analgesic target or crucial for mental health?  
*Society for Neuroscience 2015. 152.13 (Oct 18 2015). Chicago, USA.*

α9-nicotinic acetylcholine receptors contribute to the development and maintenance of mechanical hyperalgesia, but not allodynia, in chronic inflammatory and neuropathic pain.  
*Society for Neuroscience 2013. 828.16 (November 9-13 2013). San Diego, USA.*

α9-nicotinic acetylcholine receptors contribute to maintenance of nerve injury-induced mechanical hyperalgesia but not allodynia: a dual mechanism for α-conotoxins?  
*Australian Neuroscience Society, 33rd Annual Meeting. P05-055 (February 3-6 2013). Melbourne, Australia.*

Mechanical hyperalgesia, but not allodynia, is sustained through α9 nicotinic ACh-receptor activity.  

Mechanical hyperalgesia, but not allodynia, is sustained through α9 nicotinic ACh-receptor activity.  
*Sensory Neuroscience Symposium (December 10 2012). Sydney, Australia.*

α9-nAChRs are not required for chronic neuropathic allodynia, or Vc1.1 activity in mice.  
*Annual Pain Program Grant Retreat (March 12-14 2012). Stradbroke Isle. Australia.*