

**STUDIES OF A NOVEL ANALGESIC IDENTIFIED BY ESTABLISHING A STRUCTURE-ACTIVITY RELATIONSHIP  
FOR THE ANALGESIC PROTOTYPE, ISOVALINE**

by

Timothy Fung

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

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submitted by Timothy Fung in partial fulfillment of the requirements for

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in Pharmacology and Therapeutics

**Examining Committee:**

Dr. Bernard A. MacLeod

Supervisor

Dr. Ernest Puil

Supervisory Committee Member

Dr. Michael J.A. Walker

Supervisory Committee Member

Dr. Catherine C.Y. Pang

University Examiner

Dr. Alex Scott

University Examiner

**Additional Supervisory Committee Members:**

Dr. Stephan K.W. Schwarz

Supervisory Committee Member

Dr. Brian Cairns

Supervisory Committee Member

## Abstract

Pain is a major health concern worldwide with current drugs limited by adverse effects. Isovaline is a small amino acid analgesic which produces antinociception in animal models of pain without associated toxicity supporting it as a candidate for further development. This thesis explores isovaline's structure-activity relationship for antinociception and investigates the antinociceptive properties of an analog, 1-amino-1-cyclobutane carboxylic acid (ACBC). Chapter 1 describes various aspects of pain including the epidemiology, neurobiology, and the efficacy and limitations of current pharmacotherapy. It also discusses methods of preclinical pain research and the potential of isovaline and ACBC as analgesics. Chapter 2 investigates the structure-activity relationship for the antinociceptive effects of isovaline and establishes a library of analogs to identify the pharmacophore for isovaline. We have demonstrated that formation of a cyclobutane ring is necessary for antinociception. The studies described herein identified ACBC as possessing antinociceptive effects without producing motor deficits. Chapter 3 further examines the antinociceptive properties of ACBC and its potential as a spinal analgesic. We found that ACBC produces robust and reversible antinociception following administration into the lumbar intrathecal space of mice. We demonstrate that the effects were localized to dermatomes related to the site of injection and did not ascend cephalad to produce antinociception in the forepaws or produce respiratory depression. We then investigated the antinociceptive actions of the analog, R- $\alpha$ -methylproline, and found that it did not produce antinociception, but produced behavioural abnormalities. Chapter 4 explored mechanisms that mediate the antinociceptive effects of ACBC *in vivo*. We found that ACBC does not behave like the NMDA glycine site antagonist, 7-chlorokynurenate (7-CK), and may be interacting in a competitive manner with 7-CK. In addition, we show that ACBC does not likely act at GABA<sub>B</sub> receptors as the observed antinociception was not affected by a GABA<sub>B</sub> antagonist. Chapter 5 discusses the significance and limitations of this work, and proposes future research that would further support ACBC as an analgesic candidate.

## **Lay summary**

Pain is a major health problem that affects a large number of people with currently available treatments limited by their undesired effects. Our laboratory has been studying the pain-relieving effects of a new potential treatment, isovaline, and has demonstrated that it can produce pain relief without also producing side effects. This work examined the chemical structure of isovaline and in the process identified a new compound known as ACBC that also relieves pain. We further demonstrated that ACBC produced effective pain relief after giving it directly to the spinal cord. Following this, we explored the possible mechanisms by which ACBC produces its pain relieving effects. This work shows that supports ACBC has pain relieving effects that merits further development as a pain relieving drug.

## Preface

- A version of Chapter 2 has been published and is reproduced in this thesis:
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## List of abbreviations

|                        |  |
|------------------------|--|
| <b>7-CK</b>            | 7-chlorokynurenate   |
| <b>ACBC</b>            | 1-amino-1-cyclobutane carboxylic acid                        |
| <b>ACHC</b>            | aminocyclohexane carboxylic acid                             |
| <b>aCSF</b>            | artificial cerebrospinal fluid                               |
| <b>AIB</b>             | aminoisobutyric acid   |
| <b>AMPA</b>            | $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| <b>CBD</b>             | cannabidiol  |
| <b>cGMP</b>            | cyclic guanosine monophosphate                               |
| <b>CLL</b>             | cycloleucine   |
| <b>CNS</b>             | central nervous system                                       |
| <b>CSF</b>             | cerebrospinal fluid  |
| <b>GABA</b>            | $\gamma$ -aminobutyric acid                                  |
| <b>LBD</b>             | ligand binding domain  |
| <b>mGluR</b>           | metabotropic glutamate receptor                              |
| <b>NMDA</b>            | N-methyl-D-aspartate   |
| <b>NSAID</b>           | non-steroidal anti-inflammatory drugs                        |
| <b>PGE<sub>2</sub></b> | prostaglandin E <sub>2</sub>                                 |
| <b>R-AAL</b>           | R- $\alpha$ -allylalanine                                    |
| <b>R-IVA</b>           | R-isovaline  |
| <b>R-MPA</b>           | R- $\alpha$ -methylphenylalanine                             |
| <b>R-MPR</b>           | R- $\alpha$ -methylproline                                   |
| <b>R-PPA</b>           | R- $\alpha$ -propargylalanine                                |
| <b>R-TTZ</b>           | 2-(1H-tetrazol-5-yl)butan-2-amine                            |
| <b>RNA</b>             | ribonucleic acid   |
| <b>S-IVA</b>           | S-isovaline  |
| <b>SAR</b>             | structure-activity relationship                              |
| <b>THC</b>             | $\Delta^9$ -tetrahydrocannabinol                             |

**TRPA1** transient receptor potential cation channel, subfamily A, member 1

**VFT** venus flytrap

**VPL** ventral posterolateral

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*For my parents*

# Chapter 1 - General Introduction

## Introduction

Pain is a major health concern that affects a vast number of people worldwide resulting in significant social and economic costs in addition to a poor quality of life for those who are affected (Cousins et al., 2004). Current pharmacotherapy with available analgesics is often limited by adverse effects such as sedation and dysphoria resulting in inadequate pain management, indicating a need for new analgesics. Our laboratory has identified isovaline, a small amino acid, as producing antinociception without concurrent adverse effects (MacLeod et al., 2010; Whitehead et al., 2012). However, isovaline requires large doses to produce antinociceptive effects which would be impractical in a clinical setting. Despite this, isovaline represents a candidate for development. Identification of isovaline's pharmacophore would lead to additional compounds that could be useful in pain management. The primary objective of this thesis was to conduct a structure-activity relationship study to identify the pharmacophore of isovaline and to assess analogs of isovaline for improved pharmacological properties.

The thesis is organized into five chapters as follows: the first chapter reviews the literature regarding pain and provides a review of in pain research, including the biology, efficacy and shortcomings of currently available analgesics, preclinical pain research, and receptor targets relevant to this work. The second chapter investigates a library of compounds representing diverse modifications made to isovaline's structure to determine chemical features of isovaline responsible for antinociception. The third chapter investigates possible antinociceptive properties of compounds identified in the structure-activity relationship to be active in the formalin foot assay. The compounds were administered intraperitoneally, locally, and intrathecally then tested in various murine assays of pain. This chapter identifies, 1-amino-1-cyclobutane carboxylic acid (ACBC) to be active following

systemic or intrathecal administration in animal assays of pain. The fourth chapter investigates the mechanism for the antinociceptive action of ACBC and demonstrates that it behaves differently than the NMDA antagonist, 7-chlorokynurenate (7-CK). In addition, it demonstrates that the antinociceptive effects of ACBC are unlikely to be mediated by GABA<sub>B</sub> receptors. The concluding chapter discusses the overall findings of this thesis including its significance, limitations, and future directions that can be taken.

## **Background**

### **Introduction to pain – definitions and categorization**

The International Association for the Study of Pain defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” (Merskey & Bogduk, 1994). From this definition, it is evident that pain consists of two main components: a physiological component involving sensory mechanisms and a psychological component involving emotional responses. It is important to note that pain does not necessarily have to arise from tissue damage; pain can exist independently of any stimuli (Loeser & Melzack, 1999). As such, pain is subjective and for a given stimulus, will likely produce different experiences for different individuals. Because of the subjective nature of pain, self-reporting is the most valid and reliable measure for the intensity of pain experienced. Tools for measuring pain include verbal and numerical rating scales, visual analog scales, and questionnaires (Melzack & Katz, 2013). Despite its unpleasant nature, pain serves a crucial role in maintaining the welfare of humans by signalling that the impact of factors in the external environment is not acceptable acceptance (Broom, 2001). Simply put, pain acts as a warning system to protect humans from physical damage by driving them to avoid the source of pain.

Pain is categorized as being transient, acute or chronic. Transient pain occurs in the absence of tissue damage and arises from direct activation of nociceptors by noxious stimuli. This type of pain is short-lived and terminates once the stimulus is removed (Loeser & Melzack, 1999). Acute pain occurs when tissues are damaged and release a host of pro-nociceptive and inflammatory substances. These substances activate nociceptors in the region of damage and lead to painful sensations at the site of injury (Das, 2015; Loeser & Melzack, 1999). Acute pain normally persists until the damaged tissue has healed and but is often adequately controlled with standard pharmacological treatments (McDougall, 2011). Pain is classified as chronic if it persists for longer than three months (Merskey & Bogduk, 1994). Chronic pain is more complex than both transient and acute pain and can exist regardless of tissue

damage. Chronic pain stems from maladaptive neurally mediated responses to a diverse range of underlying pathologies resulting in alterations to neuronal signalling or damage to the nervous system. However, chronic pain may be sustained by environmental or affective factors unassociated with the original cause of pain (Lynch & Watson, 2006). Currently, available analgesics such as opioids and NSAIDs provide insufficient relief for patients with chronic pain (Loeser & Melzack, 1999).

## **Epidemiology**

Pain is of a major public health concern and places a burden on both social and economic systems (“Unrelieved pain is a major global healthcare problem,” n.d.). In Canada, nearly 20% of Canadians reported living with pain resulting in costs upward of \$10 billion (Reitsma et al., 2011). Global estimates on the prevalence of pain suggest that pain affects 20% of the population worldwide with an annual cost of \$100 billion in the US alone (Cousins et al., 2004). In those, less than 50% report receiving adequate treatment indicating an urgent need for effective therapies (Cousins et al., 2004; “Declaration of Montreal: Declaration That Access to Pain Management Is a Fundamental Human Right,” 2011). It is widely recognized that patients living with pain are commonly associated with a wide range of co-morbidities including, but not limited to, neuropsychiatric disorders such as depression and anxiety and chronic conditions such as cancer, diabetes, obesity, and cardiovascular disease (Davis et al., 2011; Leo, 2005; Okifuji & Hare, 2015; Tsang et al., 2008). In addition, people living with chronic pain report experiencing significant disruptions to daily routines and subsequently have been found to have the lowest quality of life compared to other chronic diseases such as heart and lung diseases (Choinière et al., 2010; Lynch, 2011). Chronic pain patients are also at an increased risk for suicide (Hassett et al., 2014). There is also evidence demonstrating that chronic pain is associated with an increased risk of mortality (Macfarlane et al., 2017). Taken together, pain is a serious global epidemic with a significant negative impact on the well-being of patients. Although of interest, it should be emphasized that the results of this research have no direct relationship to chronic pain.

## **Neurobiology of pain perception**

The sensation of pain consists of two components, the sensory aspect involving the detection of noxious stimuli and the emotional aspect involving higher processing. On the other hand, nociception refers to the sensory aspect and the neural processing of noxious stimuli (Gebhart et al., 2009). A noxious stimulus is defined as a stimulus that damages tissue or has the potential to damage tissue (Cervero & Merskey, 1996). For the purpose of this work, the focus will be on cutaneous nociception although visceral, muscle and joint nociception exist as well. The detection of noxious stimuli begins in the peripheral nervous system with specialized sensory fibers. Nociceptive fibers relay information regarding location and intensity of noxious stimuli along the neuraxis to the brain. These fibers have a relatively high threshold of activation to respond specifically to potentially damaging stimuli (Eilers & Schumacher, 2005).

The two major groups of nociceptive fibres are C-fibers and A $\delta$ -fibers. C-fibers comprise the majority of nociceptive fibers and are unmyelinated, small diameter neurons with the slowest conduction velocity of all sensory fibers (Dubuc, n.d.; Saeed & Ribeiro-da-Silva, 2012). Activation of C-fibers are thought to produce a delayed pain sensation which lasts for a greater period of time due to a slow conduction velocity of noxious signals (Dubuc, n.d.). These nociceptive fibers are sub-divided into according to responses to combinations of mechanical, thermal, and chemical stimuli. The second group of nociceptive fibers, A $\delta$ -fibers, are larger neurons compared to C-fibers and are myelinated resulting in increased conduction velocity. Typically, A $\delta$ -fibers and C-fibers are able to perform the same functions in terms of detecting noxious mechanical, thermal, and chemical stimuli, but A $\delta$ -fibers respond with greater intensity and deliver information with greater spatial resolution (Slugg et al., 2000).

Once the noxious signal is detected in the periphery, the specialized neuronal pathways carry these signals from the periphery to the brain. These numerous pathways are complex and exist independently of tracts transmitting other sensory information. Although a detailed discussion of these

pathways is outside the scope of this thesis, a brief description on the simplified pathway of the ascending pain pathway will be described. The first order neuron, generally the nociceptive fiber, relays nociceptive signals from the point of stimulus to the dorsal horn of the spinal cord. The cell bodies of first order neurons collectively form the dorsal root ganglion. The first order neuron synapses with the second order neuron in the dorsal horn which then crosses the midline and ascends the spinal cord towards the thalamus. In the thalamus, the second order neuron synapses with a third order neuron which projects into the somatosensory cortex in a somatotopic fashion (Patestas & Gartner, 2013). The ascending pain tract provides information to the brain regarding the precise nature of the noxious stimulus and the bodily location being affected (Dubuc, n.d.).

In the ascending pain pathways, excitatory neurotransmitters are released into the dorsal horn by nociceptive fibers activated by noxious stimuli to facilitate signal transmission to the brain. These include peptides such as substance P and calcitonin gene-related peptide and excitatory amino acids such as glutamate (Dubin & Patapoutian, 2010). Glutamate is the primary excitatory neurotransmitter released by nociceptive afferents (Cheng, 2010). Glutamate activates post-synaptic  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), metabotropic glutamate receptors, kainate, and N-methyl-D-aspartate (NMDA) receptors resulting in a large influx of calcium and excitation of the neuron (Besson, 1999). These receptors are involved in long-term synaptic plasticity associated with central sensitization (Bleakman et al., 2006).

## **Analgesic pharmacology**

### **Opioids**

The opioid class of analgesics is derived from *Papaver somniferum* and its use dates back to ancient civilizations. Opioids are one of the main classes of analgesics used in the pharmacological treatments of pain. However, the use of this class of analgesics is associated with numerous adverse effects. Clinical use of opioids is associated with common adverse effects such as respiratory depression, sedation, constipation, and miosis (Bowdle, 1998). Tolerance to opioids also develops in patients with use and this results in a progressive increase in dosage to achieve the desired analgesic effect. This presents a challenge due to the different degrees of tolerance to various effects of opioids. For example, tolerance develops rapidly to therapeutic effects such as analgesia, but minimally to adverse effects such as constipation (Dumas & Pollack, 2008). In addition, the development of opioid-induced hyperalgesia further complicates the use of opioids (Ballantyne & Mao, 2003). Taken together, the adverse effects of opioids are a limiting factor to their long term use (Moore & McQuay, 2005).

The prevalence of chronic pain and the increasing prescription of opioids for chronic non-cancer pain has led to marked increase in their use (Manchikanti et al., 2012). Regardless of the lack of evidence supporting their efficacy in chronic non-cancer pain, opioids are regularly prescribed for such conditions (Kotecha & Sites, 2013). In recent years, North America has been experiencing an unprecedented opioid epidemic where their non-medical misuse has led to a doubling in emergency department visits between 2004 and 2011 and a tripling of deaths between 1999 and 2013 (Nelson et al., 2015). In patients receiving long term opioid treatment, 25% of such patients are eventually classified as nonmedical users and 10% exhibit characteristics of addiction (Nelson et al., 2015). Despite illegal opioids obtained off-prescription such as heroin being responsible for an increasing number of incidents and toxicities (Lucyk & Nelson, 2017), many patients are first exposed to opioids via a prescription (Nelson et al., 2015). Often times, users dependent on prescription opioids transition to

heroin due to its relative accessibility and affordability (Kotecha & Sites, 2013). In other instances, users seek out illicit sources of the opioid they were prescribed, but are misled and unknowingly administer a stronger, synthetic opioid instead (Lucyk & Nelson, 2017). Although health policies and educational programs attempt to reduce the prescriptions of opioids, a delicate balance must be maintained to minimize opioid abuse while ensuring access for patients in need (Murthy, 2016). To address the opioid epidemic, development of safe, non-addictive analgesics must be made a priority to provide adequate care while reducing patient harm.

### **Nonsteroidal anti-inflammatory drugs**

Nonsteroidal anti-inflammatory drugs (NSAIDs) are well-established analgesics and the most commonly used drugs for the management of pain and inflammation (Laine, 2001). NSAIDs exert their analgesic effects by preventing the synthesis of prostaglandins via the inhibition of cyclooxygenase 1 (COX-1) and/or cyclooxygenase 2 (COX-2) enzymes (Zeilhofer & Brune, 2013).

NSAIDs can produce serious toxicities, especially in susceptible populations, resulting in morbidity and mortality (Vonkeman & van de Laar, 2010). The toxicity associated with the use of this class of analgesics arises from the same mechanisms responsible for their therapeutic effects. Among the adverse effects associated with NSAID use, the development of ulcers in the gastrointestinal tract, renal dysfunction, and cardiovascular events are the most serious (Scheiman & Hindley, 2010; Zeilhofer & Brune, 2013). Non-selective COX-inhibitors inhibit COX-1 production of prostaglandins which play a role in protecting the gastrointestinal system. The depletion of protective prostaglandins in the gastrointestinal system leaves it more vulnerable to injury (Sostres et al., 2010). In addition, inhibition of COX-1 present in platelets prevents the synthesis of thromboxane necessary for clotting which further complicates gastrointestinal injuries, but can be beneficial in the cardiovascular system (Sostres et al., 2010). In an attempt to reduce the gastrointestinal adverse effects stemming from the inhibition of COX-1, COX-2 specific compounds, termed coxibs, were developed. Although the selectivity for COX-2

reduces the incidence of adverse gastrointestinal effects, coxibs are associated with increased cardiovascular risks such as myocardial infarctions, stroke, and heart failure (Solomon et al., 2005).

### **Aniline analgesics**

The aniline class of analgesics consists solely of acetaminophen. The major adverse effect associated with the use of acetaminophen is the risk of hepatic damage including the potential for serious liver failure (Blieden et al., 2014). In acetaminophen overdose, prompt treatment is crucial for detoxification otherwise toxicity to prevent liver failure and death (Blieden et al., 2014). In susceptible patients, the use of NSAIDs can be severely limited due unacceptable toxicity. This indicates that it is necessary to develop a new class of analgesics.

### **Local anesthetics**

Local anesthetics reversibly block the generation of action potentials by blocking voltage gated sodium channels and preventing  $\text{Na}^+$ -influx, effectively impeding the generation and transmission of impulses (Scholz, 2002). Local anesthetics are normally applied to a restricted area surrounding the target nerve region, thus preventing afferent transmission of nociceptive and other sensory signals from that region.

Toxicity associated with local anesthetics arise from the same membrane and excitatory mechanisms that produce their therapeutic effects. Toxicities are separated into two main categories, system effects and direct damage to the neuron. Early signs of systemic toxicity include a metallic taste in the mouth, tongue paresthesia and at higher doses muscular twitching is observed followed by seizure activity (Lagan & McLure, 2004). In addition, blockade of sodium channels by local anesthetics in excitable cardiac tissues can result in disrupted cardiovascular electrophysiology. Excessive and toxic, elevated blood levels of local anesthetics are due to misadministration into blood vessels or peripheral tissues (Mulroy, 2002). Proper localization and administration of local anesthetics to reduce the incidence of adverse effects remains one of the main drawbacks of local anesthetics usage in pain

management (Golembiewski & Dasta, 2015). This limitation currently precludes the use of local anesthetics outside of settings where trained personnel are responsible for administering the drugs. Although continuous infusion devices are being developed to allow patients to self-administer local anesthetics, issues such as the pump setup and operation, catheter dislodgement, and tissue necrosis at the point of pump entry are associated with their use (Golembiewski & Dasta, 2015). Direct damage to neurons at the site of administration also occurs at sufficiently high concentrations of local anesthetics. However, the mechanisms that mediate these effects are unclear (Becker & Reed, 2012).

### **Antidepressant drugs**

Anti-depressants have long been used in the treatment of chronic pain. In addition to their anti-depressive effects, such drugs also possess distinct analgesic effects (Peter et al., 2013). The first class of antidepressants to be used in the treatment of pain were the tricyclic antidepressants with the most effective being desipramine, amitriptyline, and nortriptyline (Peter et al., 2013). Initially they were thought to relieve pain by affecting moods or mental state whereas studies in animals suggest that these drugs behave like local anesthetics by blocking sodium channels (Sudoh et al., 2003). Serotonin-noradrenaline reuptake inhibitors are now increasingly used for the treatment of pain (Marks et al., 2009). Typically, antidepressants which inhibit both the noradrenaline and serotonin re-uptake are the more effective analgesics which is supported by the finding that selective serotonin reuptake inhibitors have little efficacy in the treatment of pain (Peter et al., 2013). The exact mechanisms in which antidepressants treat pain are unclear, but they likely exert their analgesic action by modulating the descending pain pathways by inhibiting the reuptake of norepinephrine although the inhibition of serotonin may also produce analgesia in certain types of pain (Benarroch, 2008). Adverse effects of these drugs at analgesic doses include sedation, somnolence, anticholinergic effects, and cardiovascular effects.

## **Anticonvulsants**

Anti-epileptics that are commonly used for the treatment of neuropathic pain including gabapentin, pregabalin, carbamazepine, sodium valproate, and phenytoin (Ryder & Stannard, 2005). These drugs act through diverse mechanisms of action, but are generally thought to exert their analgesic effects by blocking voltage gated ion channels, in particular, Na<sup>+</sup> and Ca<sup>2+</sup> channels (Todorovic et al., 2003). By blocking these channels, anti-epileptics inhibit the conduction of impulses, thus reducing the transmission of nociceptive signals. Due to their diverse mechanisms of action, each anti-epileptic is useful for treating specific forms of neuropathic pain (Morisset et al., 2013). Adverse effects of this class of analgesics generally affect the CNS, gastrointestinal, and cardiovascular systems (Ryder & Stannard, 2005).

## **Cannabinoids**

Cannabis has been used for thousands of years to treat a wide variety of ailments and medical conditions and in particular, for pain management. Although cannabis has long been used medicinally, it is only in recent history that scientific investigations have been undertaken to examine the efficacy of cannabis as a viable therapeutic option for the conditions it is alleged to treat. Due to large numbers of anecdotal reports of cannabis being an effective treatment for managing pain, there has been significant interest from the scientific community to investigate the potential of cannabis as an analgesic (Savage et al., 2016). Cannabis contains a multitude of molecules known collectively as cannabinoids. The concentration and presence of specific cannabinoids differ between marijuana cultivars and the cannabinoid profile is unique to each cultivar. Of these cannabinoids,  $\Delta^9$ -tetrahydrocannabinol (THC) and cannabidiol (CBD), are the most studied and are thought to be responsible for many of the effects observed with cannabis (Volkow et al., 2014). Preclinical studies using animal models have demonstrated the analgesic and anti-hyperalgesic properties of THC (Hill et al., 2017). This is paralleled in human studies which generally report a therapeutic benefit of cannabis in managing pain, specifically in neuropathic pain conditions. However, the quality of the studies and the strength of the evidence

were mixed indicating the need for high quality studies to better understand the therapeutic effects of cannabis (Hill et al., 2017b).

### **Spinal opioids**

Spinal (intrathecal) administration is a widely used route for delivering analgesic agents directly to the neuraxis (Whiteside & Wildsmith, 2005). Drugs are administered as a single bolus by puncturing the dura and administering the agent to the cerebrospinal fluid surrounding the spinal cord to produce rapid and robust analgesia (Abdelbarr et al., 2014). Spinal administration differs from epidural administration where drug solutions are delivered into the epidural space and have to diffuse across the dura to exert effects resulting in a slow onset of action. The spinal route of administration is restricted to the lumbar region so as to avoid damage to the spinal cord and adverse effects on respiration (“Spinal Anesthesia,” 2017). As a result, spinal analgesics are normally used in procedures involving structures from the lower abdomen and below (“Spinal Anesthesia,” 2017). A prominent example regarding the use of spinal analgesics is in obstetric procedures where opioids and local anesthetics are administered to produce analgesia and anesthesia (Vercauteren, 2003).

Spinal opioids produce robust analgesia following their administration into the intrathecal space. The use of spinal opioids was pioneered by Tony Yaksh who demonstrated it was possible to produce analgesia by administering opioids into the spinal subarachnoid space in rats (Yaksh & Rudy, 1976, 1977). Like systemically administered opioids, spinal opioids exert their analgesic effects predominantly by activating presynaptic opioid receptors located on the spinal cord and reducing the release of excitatory neurotransmitters from afferent nociceptive fibers (Hindle, 2008). Due to direct application to the site of action, less drug is required compared with systemic and epidural administration (Chaney, 1995). Opioid receptors in the spinal cord are present in large numbers in the dorsal horn where nociceptive C and A $\delta$ -fibers synapse with second order neurons to transmit signals along the ascending pathways (Pasternak & Abbadie, 2013). Localization of opioid receptors to primary

nociceptive afferents allows for the development of segmental analgesia following spinal administration of opioids. This allows for analgesia to be restricted to the desired region of the body without significant accompanying motor, sensory, or autonomic effects (Moulin & Coyle, 1986).

Opioids administered into the intrathecal space are generally classified according to lipophilicity. The decrease in lipophilicity is inversely correlated with the potency of the agent (Farquhar-Smith & Chapman, 2012). Lipophilicity plays a large factor in the pharmacokinetics of these drugs by dictating the volume of distribution and the onset of action. Lipophilic opioids have faster onset times and shorter durations of action compared to hydrophilic opioids which have slower onset times and longer durations of action (Hindle, 2008).

Examples of lipophilic opioids used are fentanyl and sufentanil which produce rapid analgesia of short duration (Mugabure, 2013). The lipophilicity of these compounds results in rapid clearance from the cerebrospinal fluid (CSF) after administration and a large volume of distribution (Farquhar-Smith & Chapman, 2012). Clinicians have attempted to utilize the pharmacokinetic and pharmacodynamics properties of these agents in combination with local anesthetics to achieve a faster onset of action and improved analgesia without increasing the degree of motor blockade (Hamber & Viscomi, 1999). Adverse effects of lipophilic agents are likely due to their systemic effects following rapid penetration into plasma following spinal administration. Their adverse effects are similar to those observed after systemic administration (Hindle, 2008).

At the hydrophilic end of the spectrum, morphine is the most widely used opioid. Morphine has a high spinal bioavailability due to a reduced ability to diffuse out of the CSF resulting in an extended contact time with the cord and subsequently, an extended duration of action (Mugabure, 2013). In addition, the high concentration in the CSF ascends cephalad due to bulk flow and produces wider segmental analgesia (Chaney, 1995). Slow cephalad migration also increases the incidence of delayed

adverse effects when spinal opioids activate opioid receptors located in the brain. Of these adverse effects, delayed respiratory depression caused by activation of opioid receptors in the ventral medulla poses the greatest risk to patients (Chaney, 1995). The long elimination time and potential for delayed adverse effects poses a significant challenge for the administration of morphine intrathecally (Ruan, 2007). A major complaint from patients receiving intrathecal opioids is the development of pruritus that can be generalized or localized to the face, neck, and upper thorax. The incidence of pruritus varies between 30 – 100% with parturients being more susceptible at an incidence rate between 60 – 100% (Szarvas et al., 2003). Although the exact mechanism that mediates this issue is unclear, it is thought to be due to the cephalad migration of opioids followed by interaction with opioid receptors at the trigeminal nucleus (Chaney, 1995). Because pruritus is a subjective sensation, it is difficult to treat and to develop new medications for treating pruritus. Balancing the development of pruritus due to neuraxial administration with adequate analgesia remains a challenge (Szarvas et al., 2003). These observations highlight the need for spinal analgesics that are restricted to the site of administration and exhibit limited cephalad migration.

Local anesthetics can be administered intrathecally to produce a robust nerve block to the lower half of the body (Abdelbarr et al., 2014). Local anesthetics that are administered intrathecally include bupivacaine, lidocaine, and ropivacaine. A combination of a sub-anesthetic dose of local anesthetic with an opioid produces synergistic effects which result in a greater degree of analgesia with reduced adverse effects (Bujedo, 2014). However, the risk of adverse effects associated with the use of spinal opioids remains (Bujedo, 2014). Because local anesthetics block sodium channels indiscriminately, spinal administration of these drugs may result in sensory, autonomic, and motor blockade (Moulin & Coyle, 1986). Common adverse effects experienced by patients following spinal administration are numbness, paresthesia, weakness, and neurotoxicity resulting in cell death (Cohen & Dragovich, 2007). Absorption into systemic circulation can also result in adverse CNS effects and cardiotoxicity (Moulin & Coyle, 1986).

A challenge associated with the spinal administration of local anesthetics is controlling the spread of the agent through the CSF to minimize the risk of complications (Abdelbarr et al., 2014). In addition to this, even in patients with adequate needle placement, drug concentration, and administration, approximately 3% still experience a failure to achieve block highlighting the complexity of spinal local anesthetic administration (Borgeat & Aguirre, 2010).

### **NMDA antagonists**

This class of analgesics produce pain relief by binding to the NMDA receptor and preventing its activation. NMDA receptor antagonists represent a diverse chemical group of compounds due to the multiplicity of binding sites present on the receptor. Such drugs can act as channel blockers, allosteric modulators, or competitive antagonists to inhibit activation of the receptor. NMDA receptor antagonists are normally used in the perioperative setting to reduce the incidence of hyperalgesia, allodynia, and the development chronic sensitization and neuropathic pain (Richebe et al., 2015). The most well-known drug in this class of analgesics is ketamine which exerts its effects predominantly by uncompetitively blocking the NMDA receptor channel, but also acts on several other receptors as well (Maher et al., 2017). Ketamine is used perioperatively to reduce the opioid dose required for analgesia and has been shown to produce improved outcomes compared to opioid administered alone (Gritsenko et al., 2017). In patients experiencing postoperative tissue damage or various neuropathic pain states such as fibromyalgia and postherpetic neuralgia, sub-anesthetic doses of ketamine have been found to produce pain relief (Vranken, 2015). At anesthetic doses, ketamine produces psychomimetic effects such as hallucinations and dysphoria as well as a dissociative state (Gorlin et al., 2016). Sub-anesthetic doses of ketamine are also able to produce psychomimetic effects and may reactivate psychosis in susceptible patients (Gorlin et al., 2016). Ketamine possesses addictive potential and is amongst the most abused substances worldwide (Bell, 2012). Despite the beneficial effects of ketamine, the adverse effects of ketamine limit its routine use in patients (Wallace, 2001). To improve the therapeutic index of NMDA

antagonists, it has been proposed that investigating agents, such as partial agonists, that allow for a low level of receptor activation would improve the safety of compounds that act at this receptor (Koller & Urwyler, 2010).

## **Preclinical pain research in mice**

### **Animal models for studying antinociception**

Animal models are a valuable tool for drug discovery to establish a foundation for a drug's properties in an animal. Alternative methods have been proposed to replace the use of intact and conscious animals, but these techniques do not adequately reflect the complexity of a whole animal or capture all elements of a drug's behaviour *in vivo* (Arora et al., 2011). Advances in the understanding of pain and the development of therapeutics will benefit from combining new approaches with animal models to produce optimal translatable research (Mogil et al., 2010). Despite the importance of animal models, it is important to note that any model used is a simplification compared to the disease in question (Berge, 2011).

Exposing an animal to a noxious stimulus has been shown to alter physiological processes, produce changes in behaviour, and elicit characteristic activity in response to the stimulus (Berge, 2011). The antinociceptive properties of an experimental compound can then be quantified on the basis of observed changes in pain-related behaviour and responses. These pain responses can be evoked using a variety of stimuli including chemical, thermal, or mechanical insults. Although there is a wide range of nociceptive assays available, at their core, all assays can be defined by the modality of stimulus, site of administration, and history of the site (Le Bars et al., 2001). The choice of model or modality of stimulus is crucial in appropriately testing a hypothesis and obtaining valid data.

### **Chemical stimulation**

Chemical stimulation can be used in both acute and chronic pain models. In acute models, a chemical irritant is injected locally into the skin or other organs. These chemicals induce nocifensive behaviour by directly activating nociceptive afferents, producing inflammation, and/or damaging tissue at the site of injection (Berge, 2011). Nociceptive assays using chemical irritants are generally of a longer duration and are inherently inescapable (Le Bars et al., 2001). As a result, the behaviour elicited with this

modality of stimulation are considered to be spontaneous, but not reflexive (Le Bars et al., 2001). By evoking spontaneous nocifensive behaviour, chemically-based assays have the advantage of allowing observers to observe freely moving animals without the need to be present or actively applying additional stimuli (McNamara et al., 2007). To quantify the nociception in chemical assays, often an observed behaviour such as licking and biting is measured rather than a threshold. However, changes in thermal and mechanical thresholds as a result of a chemical stimulation can also be measured to investigate sensitization (Berge, 2013). Due to its persistent and inescapable nature, chemical nociceptive assays are considered to be the most comparable to clinical pain (Le Bars et al., 2001).

The formalin foot test remains one of the most widely used assays for studying pain and analgesia. The formalin foot test was initially developed in the 1970s for use in rats and cats. The aim was to create a pain assay which avoids the need for restraint during pain testing and to produce continuous noxious stimulation to resemble pain in humans (Dubuisson & Dennis, 1977). A decade later, the assay was adapted for use in mice (Hunskar et al., 1985). In this assay, a dilute solution of formalin (referring to a solution of 37% formaldehyde w/w) is injected into the ventral surface of the paw to evoke spontaneous behaviour. A 5% formalin solution is often used to induce nociceptive activity. In mice, licking and biting the affected paw has been found to be the most reliable measure for nociception. These behaviour correlate positively with the concentration of formalin administered and negatively with the presence of an analgesic (Saddi & Abbott, 2000).

The injection of formalin produces a characteristic biphasic pattern of responses in mice which can be divided into two temporally distinct phases. The early (phase I) phase lasts from 0 – 10 minutes after the injection of formalin is marked by intense licking and biting behaviour. Phase I is thought to arise from direct activation of C, A $\delta$ , and A $\beta$  fibers by formalin (Berge, 2013). Due, at least in part, to the activation of transient receptor potential cation channel, subfamily A, member 1 (TRPA1) located on unmyelinated afferent fibers by formalin (McNamara et al., 2007). The early phase is then followed by a

quiescent period where there is little to no activity lasting between 10 – 15 minutes post-injection. The licking and biting behaviour return during the late (phase II) phase which is thought to last from 15 – 45 minutes and has been found to exhibit sustained C and A $\delta$  fiber firing (Shields et al., 2010). However, the mechanism that mediates phase II activity remains unclear, but is thought to arise from ongoing afferent input, central sensitization and/or inflammatory processes at the site of administration (Mogil et al., 2001). Interestingly, it has been found that in mice lacking the majority of C-fibers, administration of formalin still evokes characteristic licking and biting behaviour suggesting a mechanism of action secondary to nociceptor activation (Shields et al., 2010).

The formalin foot test is capable of detecting the antinociceptive effects of a wide range of clinically available analgesics with the late phase being more sensitive (Berge, 2011; Hunskaar & Hole, 1987). Opioids reduce nocifensive behaviour in both phases of the test, but produce more robust antinociception in the second phase (Le Bars et al., 2001). Local anesthetics such as lidocaine are antinociceptive in both phases (Bittencourt & Takahashi, 1997). Other analgesics such as NSAIDs, gabapentin, and NMDA antagonists reduce licking and biting behaviour only in phase II. The sensitivity of phase II to analgesics suits it for detecting the potential antinociceptive properties of experimental compounds.

Recently, our laboratory has investigated the use of an intraplantar injection of hypertonic saline as a chemical stimulus in mice (Asiri et al., 2017). Prior to such use, hypertonic saline was investigated as an algescic agent following intradermal injection into the abdominal wall of mice (Hwang & Wilcox, 1986). However, analgesic effects of opioids, but not of NSAIDs, were detected suggesting the assay was inadequate to use as a general antinociceptive screen. In addition, hypertonic saline has been used to induce muscle pain in both preclinical and clinical research indicating a high degree of relevance with respect to translatability (Dubick & Wade, 2004; Svendsen et al., 2005). When injected into the ventral surface of a mouse paw, hypertonic saline elicits immediate and intense activity characterized by licking

and biting of the site of injection. Such behaviour is short-lived and exhibit an exponential decay with all activity abolished by 30 minutes post-injection. The licking and biting behaviour were taken to reflect the degree of pain the animal was experiencing. The observed behaviour were found to be dependent on the concentration of saline used with 10% saline eliciting the maximum duration of nocifensive behaviour (Asiri et al., 2017). In addition, these behaviour were attenuated by the pre-administration of opioids, NSAIDs, and local anesthetics. Although the mechanisms of action by which hypertonic saline induces nociception have yet to be investigated, it appears unlikely to be driven primarily by inflammatory processes. This is supported by histological evidence that revealed minimal inflammation in treated tissue (Asiri et al., 2017). Regardless, the short duration of the assay coupled with the sensitivity for a wide range of analgesics makes it ideally suited for investigating the potential antinociceptive activity of experimental compounds.

### **Thermal stimulation**

Thermal stimuli, particularly the use of heat, is a commonly used modality of stimulus in both human and animal research to induce phasic nociception (Berge, 2013). In animals, application of a noxious thermal stimulus to the skin at certain sites of the body such as the paws or tail elicits flexion responses (Mogil et al., 2001). As a result, thermal nociceptive assays measure threshold limits or latency to response. Elicited responses are mediated spinally or supraspinally depending on the method used for applying the heat stimulus. For example, the radiant heat tail flick test involves a spinally-mediated response whereas licking the paw in the hot plate test is thought to require higher organization to coordinate this behaviour and is therefore thought to be mediated supraspinally (Gregory et al., 2013). Thermal assays are generally of short duration and terminate when the animal either reacts to the stimulus or when a pre-determined threshold is reached to prevent tissue damage. Heat stimuli is applied through either radiation or direct contact with the heat source. However, the use of radiant heat is a relatively more selective stimulus as it does not have a tactile component (Le Bars et

al., 2001). Different classes of C and A $\delta$  fibers are activated in the presence of a thermal stimulus depending on the rate of at which the temperature increases. A slower rate of heating primarily activates C-fibers while a faster rate of heating favours activation of A $\delta$ -fibers (Berge, 2013). Factors that can affect measurements include the initial temperature of the skin prior to testing, ambient temperature, and pigmentation of the skin (Berge, 2013). The major disadvantage of thermal based assays is the limited sensitivity for different classes of analgesics. In general, these assays are best suited for detecting the activity of agents acting at  $\mu$ -opioid receptors (Le Bars et al., 2001).

### **Mechanical stimulation**

Mechanical stimulation is applied to investigate mechanically evoked pain or mechanical sensitivity in states such as allodynia and hyperalgesia. Assays utilizing mechanical stimuli measure withdrawal thresholds and latencies and are considered to be reflexive rather than spontaneous (Gregory et al., 2013). Various application methods are used depending on the purpose of the assay. To investigate mechanically evoked pain, techniques such as the tail-clip or Randall-Selitto paw-pressure test can be used (Mogil et al., 2001). Both types of stimulation apply blunt force to either the paw or tail to elicit escape behaviour or withdrawal responses in the animal. However, the tail clip applies an instantaneous supramaximal stimulus whereas the Randall-Selitto applies gradually increasing pressure. In studies investigating allodynia and hyperalgesia, von Frey hairs are commonly used to assess withdrawal thresholds (Berge, 2013). The classical von Frey hairs are a set of filaments that apply pre-specified forces while the electronic von Frey applicator applies the stimulus in a continuous manner. Methods utilizing a constant pressure stimulus have been gradually replaced with assays that use a progressively increasing stimulus (Le Bars et al., 2001).

### **Administration of drugs to mice**

There are several routes of administration that can be used for successful delivery of analgesics to mice. Selection of an appropriate route is dependent on the aims of the study as well as the nature of

the substance administered (Morton et al., 2001). Substances can be administered to the systemic circulation, restricted locally to the site of injection, or into the central nervous system.

Routes of administration that deliver substances into the systemic circulation include intravenous administration and intraperitoneal administration. Intravenous administration is the most efficient method for delivery into the systemic circulation by bypassing the need for absorption as well as avoiding the first pass effect (Turner et al., 2011). In mice, intravenous administration of substances is delivered via the tail veins. Intraperitoneal administration is a commonly used route of administration in mice as an alternative to intravenous injection (Turner et al., 2011). Substances can be injected in a relatively large volume into the peritoneal space where the main route of absorption into the systemic circulation is via the mesenteric vessels that feed into the hepatic portal vein (Lukas et al., 1971). Because of this, substances administered intraperitoneally are subject to first pass metabolism and may affect the activity of the administered compound (Turner et al., 2011). It is important to note that intraperitoneal administration more closely resembles an oral route of administration than intravenous (Turner et al., 2011).

In circumstances where systemic effects are undesirable, localized injections are useful for delivering a high concentration of a substance directly to a physically restricted space. Due to a relatively small amount of substance that is required, systemic adverse effects are minimized even when the substance is re-distributed via the systemic circulation (Zanjani & Sabetkasaei, 2010). In mice, a common site of injection for localized administration is into the ventral surface of the hind paw. Intraplantar injections are a valuable tool for investigating the peripherally-mediated antinociceptive actions of experimental compounds because compounds can be applied directly to peripheral nociceptive fibers. In addition, even when the administered compounds are eventually absorbed into the systemic circulation, the resulting systemic dose is generally sub-effective, thus avoiding any adverse effects.

Administration into the central nervous system can be easily achieved by using a lumbar puncture to apply substances directly into the intrathecal space. This route of administration presents an advantage in that structures within the central nervous system responsible for the transmission of nociceptive signals are readily accessible for modulation by exogenous compounds (Fairbanks, 2003). The technique in mice was originally developed by Hylden and Wilcox to study the behavioural effects of drugs mediated spinally (Hylden & Wilcox, 1980). To administer compounds into the intrathecal space in mice, the needle is inserted between the L5 and L6 vertebrae to puncture the dura. In mice, the level of L5-L6 corresponds to where the spinal cord ends and the cauda equina begins (Sidman et al., 1971). This region is selected to optimize accessibility while minimizing the risk of damage to the spinal cord. Successful entry of the needle into the intrathecal space is marked by a characteristic tail flick. Despite extensive investigation into the pharmacodynamics of injected compounds, relatively less work is conducted regarding the distribution of compounds within the spinal cord and CSF after administration (Fairbanks, 2003). An early study involving tritiated morphine showed that following injection, the majority remains in the spinal cord with a small fraction reaching the brain, possibly through systemic re-distribution rather than cephalad movement (Hylden & Wilcox, 1980). However, this may not apply to other compounds with different physiochemical properties. It is possible that compounds with increased hydrophilicity remain in the CSF while lipophilic properties increase the volume of distribution (Farquhar-Smith & Chapman, 2012). A limitation of this route of administration is the inability to conduct *post hoc* analysis to verify the success of the injection (Fairbanks, 2003). However, with appropriate training, intrathecal administration represents the most rapid and efficient method for investigating the effects of experimental compounds in the central nervous system (Fairbanks, 2003). The development of analgesics that are effective spinally results in a clear clinical benefit by offering alternatives to patients for whom systemic administration is ineffective or contraindicated (Fairbanks, 2003).

## **Clinical applicability of analgesia data from mouse to humans**

The management of pain in humans remains a priority with substantial effort being focused on the development of effective treatments. To do this, animal models are often employed and play a key role in these efforts (Mogil et al., 2010). Animal models of pain have greatly contributed to the understanding of the pathophysiology of pain and have led to the development of new analgesics (Whiteside et al., 2008). Most animal models combine a method for inducing a hypersensitive state with a detectable behavioural endpoint that is believed to reflect the degree of pain the animal experiences (Whiteside et al., 2013). Pain models in animals are broadly divided into two categories: ones that are designed to mimic a clinical condition and those that use a noxious stimulus to generate a response. Animal models which use a noxious stimulus to elicit reflexive behaviour have been useful in understanding the properties of clinical and experimental analgesics (Gregory et al., 2013). Furthermore, these models have been demonstrated to be useful for screening compounds for analgesic activity (Whiteside et al., 2013). In addition, the analgesic activity of all clinically available compounds can be detected using preclinical models of pain (Mogil et al., 2010).

The challenge of applying findings from preclinical research to clinically relevant drugs remains. As such, the clinical applicability or “predictive validity” of animal models has been hotly debated due to the failure of drugs in human trials despite exhibiting efficacy in animals (Whiteside et al., 2008). However, the failure of drugs to translate into the clinical setting is not necessarily the fault of animal models; many factors such as data interpretation and the reliance on singular models may be responsible for this shortcoming (Mogil et al., 2010). Although the lack of success for compounds entering clinical testing is disappointing, there are also examples of successes in advancing lead compounds into the clinic such as the atypical analgesic, ziconotide (Mogil et al., 2010). Despite the limitations of animal models, they play a crucial role in drug research and when used appropriately, provide invaluable information towards the development of useful drugs.

## Development of novel amino acid analgesic prototypes

### Isovaline

Isovaline (2-amino-2-methylbutanoic acid) is a naturally occurring amino acid. Isovaline was initially identified on the Murchison meteorite, a large carbonaceous meteorite that fell in 1969, and puzzled researchers as to its synthesis and origins (Kvenvolden et al., 1971). A subset of the amino acids, the  $\alpha$ -methyl alkanolic acids, found on the meteorite was identified to be present in an enantiomeric excess of the L-enantiomer. Although isovaline was not initially reported to be initially present in uneven quantities (Pollock et al., 1975), a later study employing different techniques reported the L-enantiomer of isovaline to be in excess (Cronin & Pizzarello, 1999). This has led to various theories attempting to explain the imbalance with one theory suggesting that  $\alpha$ -substituted alkanolic acids may have had a significant role in biochemistry leading up to the formation of RNA (Cronin & Pizzarello, 1999). Isovaline was considered to be non-proteinogenic in large terrestrial proteins, but filamentous fungi have been found to synthesize both R- and S-isomers of isovaline (Kvenvolden et al., 1971; Brückner *et al.*, 2009). In particular, the R-enantiomer of isovaline has been identified in membrane modifying peptide antibiotics produced by fungi (Raap et al., 2005).

In our laboratory, isovaline was investigated due to its structural similarity to one of the major endogenous neurotransmitters,  $\gamma$ -aminobutyric acid (GABA). GABA is present in pathways responsible for the transmission of nociceptive signals and activates two subtypes of GABA receptors, GABA<sub>A</sub> and GABA<sub>B</sub> (Enna & McCarson, 2006). Early electrophysiological studies using brain slices demonstrated that isovaline inhibited the firing of thalamic neurons by increasing K<sup>+</sup> membrane conductance (Cooke et al., 2009). This finding was consistent with literature reports that agonism of GABA<sub>B</sub> receptors activated inward rectifying potassium currents (Sodickson & Bean, 1996). The effect of isovaline at GABA<sub>B</sub> receptors was supported by later studies that demonstrated the effects of isovaline were modulated by GABA<sub>B</sub> receptor antagonists and positive allosteric modulators, but not by antagonists for GABA<sub>A</sub>,

GABA<sub>C</sub>, glycine<sub>A</sub>,  $\mu$ -opioid, or nicotinic receptors (Cooke et al., 2012). These findings were paralleled in an *in vivo* study which demonstrated that the anti-allodynic effects of isovaline were blocked by the GABA<sub>B</sub> antagonist, CGP 52432, and enhanced by the positive GABA<sub>B</sub> modulator, CGP 7930 (Whitehead et al., 2012). However, despite the evidence supporting the activity of isovaline at GABA<sub>B</sub> receptors, it is interesting to note that isovaline behaves differently than the prototypical GABA<sub>B</sub> agonist, baclofen. In rat brain slices, isovaline induced responses in a subpopulation of neurons which were responsive to baclofen (Cooke et al., 2012). Isovaline also did not alter potassium currents in AtT-20 cells or cultured rat hippocampal neurons expressing GABA<sub>B</sub> receptors while baclofen increased potassium conductance in these preparations (Pitman et al., 2015). In guinea pig ileum, isovaline reduced the amplitude of electrically evoked contractions, but increased resting tension. On the other hand, baclofen reduced both the amplitude of contractions and resting tension (Fung et al., 2016). Taken together, these findings suggest that isovaline may be acting at a subset of GABA<sub>B</sub> receptors.

GABA<sub>B</sub> receptor agonists have been found to produce antinociception in animal models (McCarson & Enna, 2006; Sawynok, 1987). Consistent with this, isovaline produces antinociceptive effects in a wide range of rodent pain assays. In the study conducted by MacLeod et al. (2010), isovaline was demonstrated to produce antinociception in several assays of chemically induced pain. In the formalin foot assay, intravenous injection of racemic isovaline reduced nocifensive behaviour in phase II of the formalin foot assay (MacLeod et al., 2010). When administered into the lumbar intrathecal space, racemic isovaline, as well as the individual stereoisomers, reduced responses in phase I and phase II of the formalin foot assay. In a model of allodynia where intracisternal strychnine produced an allodynic state characterized by vigorous responses to light touch of the trigeminal area, isovaline co-administered intracisternally with strychnine or administered into the lumbar intrathecal space reduced the allodynia score. In another study utilizing a model of PGE<sub>2</sub> induced allodynia (Whitehead et al., 2012), local administration of isovaline was demonstrated to increase the mechanical withdrawal

thresholds as assessed with von Frey filaments. Increases in threshold were observed in the ipsilateral paw treated with PGE<sub>2</sub> and isovaline, as well as the untreated contralateral paw. This finding, coupled with the lack of observed CNS effects in the study, led the authors to suggest that isovaline exerts its antinociceptive effects in the periphery rather than in the brain or spinal cord. In a recent proof of concept study investigating the potential of combining a hypnotic agent with an analgesic to produce general anesthesia, isovaline proved to be effective in a mouse model of surgical stimulus (Whitehead et al., 2015). The authors demonstrated that animals treated with isovaline did not respond to a clip applied to the base of their tail and did not produce any signs of sedation. When combined with a hypnotic dose of propofol, isovaline produced general anesthesia as defined by a loss of consciousness and loss of response to the tail clip. In a preliminary study, isovaline produced beneficial effects in a mouse model of osteoarthritis. Osteoarthritis was induced in male mice by injecting monoiodoacetate into the right knee joint and increased the number of slips and falls on a forced exercise wheel. In osteoarthritic mice, animals receiving isovaline exhibited fewer slips and falls compared to saline treated animals and similar slips and falls compared to animals treated with the NSAID, diclofenac (Whitehead et al., 2012).

The antinociceptive effects produced by isovaline were not accompanied by acute toxicity. At antinociceptive doses, intravenously administered isovaline did not produce effects in several toxicity assays (MacLeod et al., 2010). Isovaline did not affect motor function as evidenced by comparable gait, posture, behaviour, and performance on the rotarod compared to control animals. Likewise, direct application of isovaline into the central nervous system via a lumbar intrathecal injection did not produce observable adverse effects on activity, gait, posture, or appearance. Interestingly, intrathecally administered racemic isovaline produced short-lived scratching of the lower body followed by transient muscle weakness. Animals receiving isovaline intravenously exhibited no changes in respiratory rates compared to control suggesting that it does not produce centrally-mediated effects following peripheral

administration. The favourable pharmacological profile likely arises from an inability to act on the central nervous system when administered systemically. Autoradiograms of animals injected intravenously with radiolabelled isovaline showed that the accumulation in the brain was below the limit of detection (Shiba et al., 1989). This is further exemplified by the lack of effect of isovaline on body temperature when compared with baclofen. The GABA<sub>B</sub> agonist baclofen, which is able to permeate the blood brain barrier, decreased the body temperature of treated mice while isovaline, which has also been shown to act at GABA<sub>B</sub> receptors, did not produce any changes. In support of previous findings, animals receiving intraperitoneal isovaline were comparable to saline treated animals in the behavioural hypoactivity score test which measures posture, passivity, tactile responsiveness, body sag, and gait (Whitehead et al., 2012). Furthermore, isovaline did not worsen the respiratory depression caused by propofol supporting previous reports that isovaline does not produce an adverse impact on respiration (Whitehead et al., 2015). These observations suggest that isovaline does not enter the central nervous system following peripheral administration. It is possible that isovaline is actively removed from the central nervous system via neutral amino acid transporters. Neutral amino acid transporters are present in the gastrointestinal tract and carry isovaline across the intestinal wall (Christensen, 1962). Similar transporters may be present in the blood brain barrier and would be responsible for the efflux of isovaline from the central nervous system.

In addition to antinociception, there is evidence that demonstrates isovaline also possesses anti-epileptic properties. Isovaline exhibited anti-epileptic effects in two *in vitro* hippocampal models of epilepsy (Shin et al., 2011). In the first model, slices were taken from rat hippocampus and were perfused with a low magnesium-high potassium solution to induce seizure like events and stimulus evoked primary afterdischarges in the slices. Addition of isovaline to the perfusion solution decreased both the seizure like events and the stimulus evoke primary afterdischarges. Similar observations were made in the 4-AP model where isovaline reduced seizure-like events in treated slices. The group

suggested that isovaline may be acting on interneurons to attenuate seizure activity in the hippocampal models of epilepsy. These results corroborated with *in vivo* studies that demonstrated isovaline attenuated seizure activity in a rat model (Yu et al., 2014). Administration of the epileptogenic agent, 4-AP, induced hippocampal epileptiform activity in anesthetized rats which was attenuated by intravenous administration of isovaline. In awake animals, 4-AP produced wild running and jumping behaviour with tonic-clonic seizures. However, pre-treatment with isovaline reduced seizure severity to mild symptoms and did not affect performance in the open field test. Isovaline was also effective in a pilocarpine-induced model of epilepsy. Isovaline abolished epileptiform activity in the hippocampus of rats treated with pilocarpine. In addition, the administration of isovaline prior to or after the injection of pilocarpine reduced seizure severity in awake animals (Yu et al., 2015). The anti-epileptic effects of isovaline further support its development as an analgesic as several clinically approved analgesics such as gabapentin, pregabalin, and carbamazepine are anti-epileptic agents.

Isovaline represents an ideal prototype for developing other analgesics due to its favourable therapeutic index (Martin, 2015). Despite producing antinociception without observable adverse effects, isovaline is not without its drawbacks. Large doses of isovaline are required to produce antinociceptive effects which would be impractical to use in a clinical setting. In addition, unpublished findings within our laboratory suggest that the magnitude of effect for isovaline can be inconsistent and variable suggesting a therapeutic action that may not be readily generalizable to other populations and groups. With such shortcomings, it would be logical to use isovaline as a prototype compound and identify a potential pharmacophore that can be used as a template for more potent and potentially more efficacious analgesics.

### **1-Amino-1-cyclobutane carboxylic acid**

1-Amino-1-cyclobutane carboxylic acid (ACBC) is a small unnatural amino acid with a chemical structure similar to isovaline. ACBC is naturally non-proteinogenic, but can be synthetically incorporated

into peptides to produce predictable structural constraints (Benedetti et al., 2010; Washburn et al., 1979). ACBC was originally investigated as a radiolabelled soft tissue imaging agent due to its structural similarity to 1-aminocyclopentanecarboxylic acid, the 5-carbon ring analog of ACBC. In the initial study using rats bearing hepatomas, ACBC exhibited preferential uptake into tumour tissues and had better tumour-nontumour concentration ratios in soft tissue such as muscle, kidney, and testis compared to other tested amino acids (Washburn et al., 1978). A subsequent study demonstrated that the preferential uptake of ACBC into tumour tissues extended to other soft tissue tumours as well and supported its utility as a tumour imaging agent (Washburn et al., 1979). Following animal studies, radiolabelled ACBC was advanced into the clinic where it was demonstrated to be useful as a tumour imaging agent due to its high affinity for malignant tumours and low renal excretion (Hubner et al., 1981). In patients with recurrent brain tumours, 1- $^{11}\text{C}$ ACBC correctly identified the tumours in 19 of 20 patients (Hubner et al., 1998). However, the utility of ACBC as a tumour imaging agent was limited by the short decay half-life of  $^{11}\text{C}$ . Regardless, it served as the prototype for developing other agents that are currently used in the clinic (Hong et al., 2010).

The properties of ACBC have been investigated in a number of *in vitro* and *in vivo* preparations. In membrane preparations of rat forebrain tissue, ACBC was found to inhibit [ $^3\text{H}$ ]glycine binding with no effect on the binding of [ $^3\text{H}$ ]strychnine or L- $^3\text{H}$ ]glutamate suggesting ACBC was specific for the glycine co-agonist binding site on NMDA receptors (Hood et al., 1989). These findings were paralleled in another study that demonstrated ACBC inhibited the binding of two NMDA glycine site ligands, [ $^3\text{H}$ ]ACPC and [ $^3\text{H}$ ]5,7-DCKA, in rat forebrain tissue (Popik et al., 1995). Binding of ACBC to the glycine site also enhanced the binding of antagonists specific for the NMDA binding site (Compton et al., 1990).

The functional significance of ACBC's interaction at the glycine site of NMDA receptors was investigated using *Xenopus* oocytes injected with rat brain mRNA. Using this preparation, increasing concentrations of ACBC administered with a fixed concentration of glycine produced a right shift in the

NMDA dose-response curve and reduced the maximal response of activation suggesting non-competitive inhibition of NMDA. At a fixed concentration of NMDA, ACBC produced a right shift in the dose response curve of glycine without affecting the maximal response indicating that ACBC acts as a competitive antagonist at the glycine site (Watson et al., 1989). However, further studies demonstrated that ACBC also exhibits partial agonist activity at NMDA receptors. In *Xenopus* oocytes treated with rat brain mRNA, ACBC administered without glycine produced slight potentiation of the responses to NMDA. In the presence of glycine and NMDA, ACBC produced a dose-dependent inhibition of NMDA current, but was unable to produce full inhibition of the response. Interestingly, increasing concentrations of glycine did not produce a consistent shift in the potency of ACBC as was seen for another glycine site partial agonist, HA-966. This suggests that ACBC may not be acting solely as a partial agonist at the NMDA receptor and may possess activity at targets as well (Watson & Lanthorn, 1990). Consistent with this, ACBC exhibited effects that differed from another partial agonist, D-cycloserine in rat hippocampal-entorhinal brain slices. ACBC decreased the frequency of mini excitatory postsynaptic currents while D-cycloserine had no effect. However, the authors suggest that this difference may be attributable to the low efficacy of ACBC which would essentially render ACBC a competitive antagonist against endogenous agonists and thus, explaining the reduction in frequency. This theory may also explain the differences observed in evoked NMDA receptor mediated excitatory postsynaptic currents between ACBC and D-cycloserine (Lench et al., 2015).

There are conflicting reports in the literature regarding the conformational changes of NMDA receptors that occur following the binding of ACBC. In one study, the authors used small molecule fluorescence resonance energy transfer techniques to study the conformational change induced by a series of partial agonists. They reported that the degree of closure of the glycine binding domain showed a positive correlation between the degree of cleft closure and the efficacy of the ligand. The antagonist, 5,7-dichlorokynurenic acid produced the smallest degree of closure while the full agonists,

glycine and serine, produced the greatest degree of closure. As a partial agonist, ACBC induced a degree of closure that fell between that of an antagonist and a full agonist (Dolino et al., 2015). In contrast, Inanobe et al. (2005) resolved the crystal structure of NMDA with ACBC bound (Inanobe et al., 2005) and demonstrated that although ACBC produced only partial activation of the NMDA receptor, no differences were detected in the degree of cleft closure compared with glycine.

Despite studies investigating the activity of ACBC *in vitro*, less is known regarding its activity *in vivo*. Intracerebellar administration of ACBC in mice did not affect the basal level of cGMP in brain tissues while glycine and D-serine produced marked increases in cGMP levels. This is in contrast with *in vitro* reports of ACBC blocking NMDA-induced increases in cGMP suggesting that *in vitro* findings regarding activity and mechanisms of action may not necessarily translate into *in vivo* studies (Rao et al., 1990). The authors also demonstrated that ACBC is bioavailable in the brain following systemic administration indicating that ACBC is capable of crossing the blood brain barrier. However, in both mice and rats, ACBC was rapidly cleared from the cerebrospinal fluid (Rao et al., 1990). Furthermore, the authors reported that ACBC did not reverse the increase in cGMP induced by D-serine while another partial agonist, HA-966 did suggesting that ACBC differs from other NMDA partial agonists. In rats receiving an infusion of ACBC via the external carotid artery, ACBC reduced the influx of aminocyclohexane into the brain. Aminocyclohexane is transported into the brain by neutral amino acid transporters. This finding suggests that ACBC competes with aminocyclohexane for the transporter which would then reduce the amount of aminocyclohexane that crosses the blood brain barrier (Aoyagi et al., 1988). The ability of ACBC to cross the blood brain barrier is exemplified in a clinical study that demonstrated that ACBC was capable of detecting brain tumours indicating that ACBC must be able to cross the blood brain barrier following peripheral administration (Hubner et al., 1998). Aside from being useful as a tumour imaging agent, the therapeutic potential of ACBC remains unknown. ACBC has been reported to be well tolerated in mice with an acute dose up to 2.9 g/kg producing no deaths observed

within 60 days. The same study found that ACBC was also well-tolerated with no signs of toxicity or changes in blood cell profiles in dogs and a marmoset at dose of 200 mg/kg (Washburn et al., 1979). In whole animal studies, ACBC has been screened as a potential candidate as an anti-ischemic, but failed to demonstrate activity in assays of ischemia (Rao et al., 1990). Limited pilot studies in our laboratory have suggested that ACBC produced antinociception in mouse models (Chung, 2009; Wang, 2008). However, more comprehensive studies are required to fully characterize and understand the antinociceptive effects of ACBC and its potential as an analgesic.

### **GABA<sub>B</sub> receptors**

The activity of the major inhibitory neurotransmitter, GABA, is mediated via two receptor systems, ionotropic GABA<sub>A</sub> receptors and metabotropic GABA<sub>B</sub> receptors. The response to GABA is biphasic with the early response being attributed to activation of GABA<sub>A</sub> receptors and the late response being due to GABA<sub>B</sub> receptor activation (Couve et al., 2000). Metabotropic GABA<sub>B</sub> receptors are coupled to G-proteins which modulates adenylyl cyclase and a range of ion channels including chloride, potassium and calcium channels (Gassmann & Bettler, 2012). GABA<sub>B</sub> receptors are obligate heterodimers composed of two subunits, a GABA<sub>B1</sub> (GB1) and GABA<sub>B2</sub> (GB2) subunit, each responsible for a different aspect of signalling (Galvez et al., 2001). The GB1 subunit contains a ligand binding site in the Venus Flytrap (VFT) module of its extracellular domain and is responsible for ligand recognition and binding (Geng et al., 2013). In the apo-form, the VFT module exists in an open conformation, but binding of a ligand to the recognition site induces closure of the VFT module (Geng et al., 2013). However, the presence of GB1 alone is insufficient for triggering a functional response (Galvez et al., 2001). The C-terminal of GB1 contains an endoplasmic reticulum targeting sequence which prevents trafficking to the cell surface (White et al., 1998). Although mutation of this sequence allows for transport of the subunit to the cell surface, binding of an agonist to GB1 still does not produce a functional response (Calver et al., 2001). Interaction with GB2 via the coiled-coil domains in the C-terminal domains of both subunits is

required for formation of a functional GABA<sub>B</sub> receptor (Marshall et al., 1999). The extracellular domain of GB2 is similar to GB1 including the presence of a VFT module (Geng et al., 2013). However, the VFT module of GB2 is unable to bind any known ligands and remains constitutively in the open conformation (Xu et al., 2014). Although the extracellular domain of GB2 does not have a known ligand binding site, it modulates the ligand binding affinity of the GB1 subunit (White et al., 1998). The C-terminal of the GB2 subunit masks the retention sequence and allows transport of the functional GABA<sub>B</sub> receptor to the cell surface (Pagano et al., 2001). The transmembrane domain of GB2 also interacts with the coupled G-protein messenger system to initiate the signalling cascade upon binding of an agonist (Galvez et al., 2001).

GABA<sub>B</sub> receptors are predominantly expressed within the central nervous system, but also exist in the periphery in the enteric nervous system, on free nerve endings, and in synovial tissues (Bowery, 2016; Tamura et al., 2009; Whitehead et al., 2012). In relation to pain, GABA<sub>B</sub> receptors are expressed in both the ascending and descending systems to modulate the transmission of nociceptive signals (McCarson & Enna, 2006). Along the spinal cord, GABA<sub>B</sub> receptors are expressed in high concentrations in structures that comprise the ascending pain pathway, particularly in the area around where first order primary nociceptive afferents synapse with second order transmission neurons (McCarson & Enna, 2006). Supraspinally, GABA<sub>B</sub> receptors are found in regions responsible for pain processing such as the thalamus (Ulrich & Bettler, 2007). Generally, GABA<sub>B</sub> receptors are thought to contribute to the maintenance of nociceptive thresholds and activation of these receptors result in antinociception (McCarson & Enna, 2006).

A variety of ligands modulate the activity of GABA<sub>B</sub> receptors at the binding domain of the VFT module. Baclofen is the first and best characterized agonist at GABA<sub>B</sub> receptors and served as the prototype for developing other agonists (Froestl, 2010). Baclofen was initially developed to increase the lipophilicity of GABA to improve diffusion across the blood brain barrier into the CNS (Froestl, 2010).

Clinically, baclofen is indicated for the treatment of spasticity due to its muscle relaxant effects with some evidence suggesting therapeutic potential for treating alcohol dependence (Brennan et al., 2013; Bryan, 2013). Consistent with the expression of GABA<sub>B</sub> receptors on structures responsible for nociceptive signalling, there is also evidence for the clinical use of baclofen in treating patients suffering from neuropathic pain (Yomiya et al., 2010; Zuniga et al., 2000). This is further supported by preclinical studies which have shown that baclofen produces antinociceptive effects in animal models of pain following both systemic and intrathecal administration indicating a role of GABA<sub>B</sub> receptors in the pain experience (Balerio & Rubio, 2002; Santos et al., 1999; Wilson & Yaksh, 1978). Antagonists were eventually developed to facilitate the characterization of the GABA<sub>B</sub> receptor and to investigate potential therapeutic uses for GABA<sub>B</sub> ligands (Enna, 2001). Amongst these, CGP 35348 was developed as a selective antagonist for the GABA<sub>B</sub> receptor and was found to block the anti-nociceptive and muscle relaxant effects of baclofen (Froestl, 2010; Malcangio et al., 1991; Olpe et al., 1990). In contrast to GABA<sub>B</sub> receptor activation by agonists, blockade of the receptor by CGP 35348 produces hypersensitivity to mechanical stimuli in rats (Hao et al., 1994). Although there is some evidence for pharmacological effects of GABA<sub>B</sub> antagonists, there is currently no evidence for their therapeutic effects clinically (Enna, 2001).

### **NMDA receptors**

The actions of the excitatory neurotransmitter glutamate are mediated by two major groups of receptors, metabotropic glutamate receptors and ionotropic glutamate receptors (Chaffey & Chazot, 2008). Ionotropic glutamate receptors are further divided into three sub-groups, AMPA, kainate, and NMDA. The family of NMDA receptors are ligand gated cation channels formed from a combination of three subunits, GluN1 – 3 (Chaffey & Chazot, 2008). GluN1 – 3 share a significant level of homology consisting of an extracellular amino-terminal domain, an extracellular ligand binding domain (LBD), a transmembrane domain, and an intracellular C-terminal domain (Vyklícky et al., 2014). GluN1 is further

subdivided into 8 splice variants that do not appear to produce different channel properties (Chaffey & Chazot, 2008). GluN2 subunits are encoded by 4 separate genes (GluN2A – D) and GluN3 subunits are encoded by two genes (GluN3A and B) (Vyklícky et al., 2014). NMDA receptors are heterotetramers consisting of two obligatory GluN1 subunits and a combination of GluN2 and GluN3 subunits (Vyklícky et al., 2014). The combination of subunits that form the NMDA receptor determines its functional properties (Cull-Candy et al., 2001). Binding of an agonist to the LBD induces a closure of the domain leading to conformational changes that open the channel pore (Vyklícky et al., 2014). NMDA receptors are unique in their activation requiring the binding of both glycine and glutamate and voltage dependent removal of magnesium from the pore opening (Lee et al., 2014). Each GluN1 subunit binds to a molecule of glycine while each GluN2 subunit binds a molecule of glutamate (Clements & Westbrook, 1991). Interestingly, receptors composed of GluN1/GluN3 require only glycine for activation, but little is currently known regarding this subunit combination (Chatterton et al., 2002). Numerous binding sites are present on NMDA receptors allowing for modulation of these receptors by numerous ligands (Regan et al., 2015).

NMDA receptor complexes of various subunit compositions are distributed throughout the body. In the central nervous system, NMDA receptors are located within the brain in various structures including the cerebral cortex and thalamus and spinally in the substantia gelatinosa of the dorsal horn (Rigby et al., 2002). In the peripheral nervous system, NMDA receptors are thought to be located on axonal terminals of both myelinated and unmyelinated primary afferents (Coggeshall & Carlton, 1998). NMDA receptors, particularly those containing the GluN2B subunit, have been found to be critical in nociception and for the induction and maintenance of pain states (Petrenko et al., 2003). NMDA receptors are involved in the development of persistent pain states where activation promotes development and blockade of the receptor inhibits development of these states (Vorobeychik et al., 2013). This includes important roles in the development of chronic pain through involvement in

processes such as wind up and central sensitization (Eide, 2000). Upon repeated stimulation, nociceptors continuously release glutamate and other pro-nociceptive molecules leading to the removal of  $Mg^{2+}$  from the channel pore and subsequent activation of NMDA receptors (Latremoliere & Woolf, 2009). This leads to increased synaptic efficacy and activation of pathways that initiate and maintain central sensitization (Latremoliere & Woolf, 2009). In primates, NMDA receptor activation sensitized spinothalamic tract neurons resulting in increased responses to marginally noxious mechanical stimuli (Willis et al., 1996). Administration of NMDA receptor antagonists inhibited the hyperexcitability of these neurons and the development of central sensitization (Willis et al., 1996). Similarly in wind up, application of NMDA receptor antagonists inhibited the progressive increase in neuronal firing in response to repetitive stimulation supporting a key role of NMDA receptors in this process (Eide, 2000).

NMDA receptors possess numerous competitive and allosteric ligand binding sites that ligands can interact with to modulate the receptor (Jansen & Dannhardt, 2003). Exogenous NMDA receptor agonists are specific, binding to either the glutamate binding site on GluN2 or the glycine binding site on GluN1 (Monaghan & Jane, 2009). Agonists at the glutamate site include glutamate, aspartate, and NMDA while glycine site agonists include glycine, D-serine, and D-cycloserine (Monaghan & Jane, 2009). Due to concerns regarding excitotoxicity, there have been limited investigations into developing glutamate site agonists for therapeutic use (Kemp & McKernan, 2002). However, glycine site agonists have been demonstrated to provide some benefit when used in conjunction with standard antipsychotic treatment in patients with schizophrenia (Kemp & McKernan, 2002). A wide range of antagonists are available that bind orthosteric and allosteric sites, as well as the channel pore (Monaghan & Jane, 2009). Of the orthosteric antagonists, ligands interacting with the glycine site have better therapeutic ratios compared to glutamate site ligands (Monaghan & Jane, 2009). A diverse set of compounds antagonize the NMDA receptor at various allosteric sites on both the GluN1 and GluN2 receptors. Channel blockers of NMDA include ketamine, phencyclidine, and MK-801 which occupy the pore to prevent receptor

activation (Monaghan & Jane, 2009). In terms of nociception, preclinical and clinical studies have provided evidence to support the antinociceptive properties of NMDA receptor antagonists in persistent, tonic, and phasic pain (Berrino et al., 2003; Lutfy et al., 1997; Parsons, 2001). In addition, peripheral stimulation of NMDA receptors with glutamate or NMDA produces nocifensive behaviour which are blocked by the administration of antagonists (Zhou et al., 1996). Competitive antagonists for the glycine site, such as 7-CK, reduce nocifensive responses in a preclinical pain models (Christoph et al., 2005; Leeson & Iversen, 1994). Similarly, ketamine produces antinociception in animal models and is also used clinically for its analgesic and anesthetic effects (Gao et al., 2016; Lutfy et al., 1997).

### **Structure-activity relationships**

Structure-activity relationship studies play a central role in drug discovery and development by being involved in the initial screening of compounds to establish a library and to optimize lead compounds (Guha, 2013). Structure-activity relationship studies are conducted to examine the influence of structure on a compound's physical and chemical properties and to investigate how modifications to the structure alter these properties. At its core, structure-activity relationships are based on the premise that the chemical structure of a compound determines the compound's interactions within a biological system and therefore, its pharmacological effects (McKinney, 2000). The link between a structure-activity relationship study and the mechanism of action produces a rationale that can be used to predict the activity of other, untested compounds (McKinney, 2000). Furthermore, understanding the structure-activity relationship of a particular compound allows for the development of other compounds with improved potency, toxicity profiles, and efficacy.

Structure-activity relationship studies are generally essential for drug development. In the field of pain and analgesia, a notable example of using structure-activity relationships is the development of the opioid class of analgesics. Morphine and codeine were originally isolated from opium and their structures served as the scaffold to develop numerous  $\mu$ -opioid agonists (Pasternak & Pan, 2013). As

such, many opioids display similarities to various aspects of morphine's structure. Even before the elucidation of morphine's chemical structure, derivatives were synthesized (with heroin being amongst the first) in an attempt to reduce the respiratory depression, gastrointestinal effects, addiction, and tolerance caused by the use of morphine (Jacobson, 1978). Following the identification of morphine's structure and the rise of receptor-binding studies, the structure-activity relationship for morphine is now well established.

In terms of methodology, there are two general methods for developing a structure-activity relationship for a compound: by using a statistical approach such as a quantitative structure-activity relationship or by using a physical approach such as the identification of a pharmacophore. The pharmacological actions for a given class of drugs are determined by specific structural properties that allow the drugs to interact with the site of action (Harrold & Zavod, 2013). Characterizing these structural properties allows for the identification of the pharmacophore which is defined as the minimum structural characteristics of a compound for drug activity. To explore the characteristics of the pharmacophore, modifications can be made to the lead compound to investigate their impact on desired pharmacological properties such as target specificity, efficacy, and potency (Harrold & Zavod, 2013). Functional groups are recognizable and classifiable units of bound atoms within a molecule and are important because they contribute to the overall properties of the molecule (Hardinger, 2008). A commonly used procedure for modifying the chemical structure of a compound is to vary the functional groups that are present (Harrold & Zavod, 2013). Using this approach, functional groups are added, removed, or exchanged on the lead compound. By varying the functional groups present on the lead candidate, compounds with different physiochemical and electrostatic properties and steric conformations can be studied (Harrold & Zavod, 2013). The addition of different groups varies the electrostatic size, flexibility, reactivity, and solubility of the compounds they are substituted onto.

The compounds investigated in this thesis represent a variety of modifications. The chirality at the  $\alpha$ -carbon of isovaline gives rise to the R- and S-isomers. Enantiomers can have different therapeutic effects due to potentially different binding characteristics including different fits in the binding pocket or even a different target altogether. A prominent example of differences in therapeutic activity between enantiomers is morphine. Morphine was initially synthesized as a racemic mixture comprised of the naturally occurring configuration and its enantiomer. It was found that the racemate possessed half the expected activity and eventually revealed that the unnatural enantiomer produced no analgesia, thus highlighting the importance of configuration for therapeutic effects (Patrick, 2017). Likewise, the R- and S-isomers of isovaline were studied here individually to determine if the enantiomers were both capable of producing analgesic effects. Addition of an unsaturated bond to isovaline alters the hydrophobicity of isovaline and increases the ability of isovaline to interact with any hydrophobic regions via van der Waals interactions in the target binding pocket. The less bulky nature of an alkene and alkyne allows the aliphatic R-group to approach closer to a potential hydrophobic region or possibly to allow bond formation between the alkyne and reactive side chains in the target protein (Patrick, 2017). The addition of a phenyl ring to isovaline increases the hydrophobicity and steric size. Like unsaturated aliphatic bonds, the phenyl ring is able to form van der Waals interactions with hydrophobic regions at the binding target or attraction between the electron cloud above and below the plane of the benzene ring to a centre of positive charge such as arginine or lysine side chains. Using baclofen as an example at the GABA<sub>B</sub> receptor, the phenyl ring of baclofen interacts with a tryptophan residue via ring-stacking interactions. A similar hydrophobic interaction is seen when GABA binds and activates the GABA<sub>B</sub> receptor (Geng et al., 2013). This suggests that this interaction with the tryptophan residue is critical in receptor activation and may be improved in isovaline via the addition of an aromatic or phenyl ring to the aliphatic side chain. Isosteric replacement by substituting a tetrazole in place of a carboxylic acid, retains the broader biological properties of the functional group while altering the physiochemical and

electrostatic properties of the overall molecule (Lassalas et al., 2016). Other modifications that can be made include limiting the flexibility of the compound via carbon degradation to reduce the degrees of freedom or cyclization to increase steric hindrance and restricting the number of conformations that can be adopted by isovaline. The purpose of examining different molecules is to develop a library of compounds which possess diverse characteristics and pharmacological effects.

In the structure-activity relationship conducted in this thesis, the initial library of compounds were selected, in consultation with a chemist, to produce a preliminary set of compounds with diverse electrostatic and steric properties. In the experiments described in this work, compounds arising from functional group modification were specifically studied in the context of their effects on antinociception. With this library, compounds with desired traits could then be developed further to potentially yield novel drugs.

## Summary and objectives

Pain is composed of two parts, a sensory component, termed nociception, which is mediated by structures in both the peripheral and central nervous systems and an emotional component which is mediated supraspinally. Nociceptive signals are detected by nociceptors in the periphery and are transmitted along the ascending pain pathway into the brain. Under normal conditions, pain is a defense mechanism that signals actual or potential tissue damage and protects us from injuring ourselves. However, under pathological conditions, pain becomes maladaptive. Patients living with persistent pain experience a low quality of life and are often diagnosed with multiple co-morbidities.

Currently available analgesics such as opioids and NSAIDs are limited by their adverse effects resulting in patients receiving inadequate pain management, thus prompting the need for new analgesics. A candidate is isovaline, a small amino acid that produces antinociception without respiratory depression or other apparent central nervous system effects. In chapter 2, we conduct a structure-activity relationship study for antinociception to identify the pharmacophore of isovaline and other potential compounds to investigate.

The structure-activity relationship study identified two amino acids of interest, ACBC and methylproline. Little is known regarding the antinociceptive effects of these two compounds. In chapters 3, we aim to further investigate the antinociceptive effects and potentials as spinal analgesics for ACBC and methylproline. In chapter 5, we investigate the *in vivo* receptor mechanism that mediates the antinociceptive effects of ACBC.

## Chapter 2 – Variations of Isovaline Structure Related to Activity in the Formalin Foot Assay in Mice

### Introduction

Pain therapy with currently available analgesics is constrained by their adverse effects often leaving patients with inadequate pain management. Long-term use of analgesics is often stopped due to intolerable effects such as sedation and dysphoria; effects manifesting from actions in the central nervous system (CNS). Despite a great deal of research directed towards the discovery of new analgesics, little progress has been made in the development of novel drugs for clinical pain. Our laboratory has discovered a novel prototype analgesic, isovaline - an unusual amino acid. This simple amino acid produces analgesia without respiratory depression or apparent central nervous system effects. Isovaline is structurally similar to the inhibitory neurotransmitters, glycine and  $\gamma$ -aminobutyric acid (GABA). Isovaline is antinociceptive in several pain models. For example, isovaline blocks the allodynic responses of mouse skin or foot to sensory stimuli, induced by strychnine or prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and suppresses the acute (I) and chronic (II) phases of pain in the rodent formalin-foot model (Asseri et al., 2015; MacLeod et al., 2010; Whitehead et al., 2012). In a recent study, we showed that isovaline administered alone results in a loss of painful response to tail clip applied to mice, and in combination with the hypnotic, propofol, produces general anesthesia (Whitehead et al., 2015). Isovaline at analgesic and supra-analgesic doses appears to be well tolerated, producing little or no changes in respiratory rate, body temperature, and motor coordination (MacLeod et al., 2010; Whitehead et al., 2012; Whitehead et al., 2015).

Isovaline produces analgesia by enhancing GABA<sub>B</sub> receptor modulation of nociceptive information (Whitehead et al. 2012). Isovaline does not significantly penetrate the blood-brain barrier on systemic administration (Shiba et al. 1989). However, RS-, R- and S-stereoisomers produce

analgesia when directly applied to the brainstem or spinal cord in the formalin foot model (MacLeod et al. 2010). In brain slices, application of isovaline to thalamic neurons that receive nociceptive input increases membrane conductance for potassium ( $K^+$ ) (Cooke et al., 2009, 2012). Pre-treatment with the GABA<sub>B</sub> antagonists, CGP 35348 and CGP 52432, blocks the conductance increase, suggesting mediation through GABA<sub>B</sub> receptors (Cooke et al., 2009, 2012). Likewise, the analgesia produced by peripherally administered isovaline is partly attributable to actions at GABA<sub>B</sub> receptors since CGP 52432 blocks its effects in the von Frey withdrawal assay in the PGE<sub>2</sub> model (Whitehead et al., 2012). Peripheral isovaline in mice also interacts with Group II metabotropic glutamate receptors, suggesting additional receptor mediation in nociceptive systems (Asseri et al., 2015). Isovaline hence is unique among analgesics, acting on dual G-protein coupled receptor systems in peripheral tissue with no apparent acute toxicity.

Isovaline is well tolerated at high doses, but is unfortunately not potent. A more potent analgesic derivative of isovaline could be found in structurally related analogs. These analogs were chosen as modifications commonly used to establish structure-activity relationships in drug design. The amino acids in this study represent systematic modifications to the backbone and functional groups of the isovaline structure. The effects of these compounds have not been studied on pain models. This is the first study determining the structure-activity relationship of isovaline and analgesia.

## Materials and methods

### Docking simulations

Docking simulations were performed to develop an *in silico* method to rapidly screen compounds for potential binding activity at the GABA<sub>B</sub> receptor. At the time when the docking simulations were conducted, the crystal structure of the ligand binding domain of the GABA<sub>B</sub> receptor had not yet been resolved. As a result, a chimera was used to mimic the GABA<sub>B</sub> receptor. The GABA<sub>B</sub> chimera was based on the crystal structure of the closed form of leucine-isoleucine-valine binding protein (LIVBP) (Protein Data Bank ID: 2LIV). Based on the sequence alignment and site-directed mutagenesis experiments performed by Galvez et al. (1999) (Galvez et al., 1999), the three dimensional model of GABA<sub>B</sub> was created using the UCSF Chimera modeling program (UCSF, California, USA). Amino acid residues that were identified by Galvez et al. (1999) to be important for proper ligand binding by GABA<sub>B</sub> include S246, S247, S269, S270, Q312, and Y470. These residues were swapped with the corresponding amino acid residue as shown by the sequence alignment between LIVBP and GABA<sub>B</sub>. Furthermore, amino acid residues that form the binding pocket of LIVBP were swapped with its partner in the GABA<sub>B</sub> receptor to reflect the different binding pocket composition of the GABA<sub>B</sub> receptor. The amino acid substitutions made to LIVBP to form the GABA<sub>B</sub>R binding pocket are as follows: Y18G, A100G, A101S, T102S, A103S, H145Q, F276E, G227W, A282D, and T279L. The resulting chimeric model was subjected to a “minimize structure” calculation in UCSF Chimera to account for energy disturbances that were introduced with the substituted amino acid residues. A steepest descent and conjugate gradient steps of 100 with step size 0.02 Å for both were used in the calculation. Hydrogens and charges were added in using AutoDockTools (Scripps Research Institute, California, USA). Ligand files were prepared using the SMILES string for GABA and  $\gamma$ -hydroxybutyric acid (GHB) and the isomeric SMILES string were used for R-baclofen, S-baclofen, R-isovaline (R-IVA), and S-isovaline (S-IVA) (Table 1). All subsequent

amino acid positions that are listed for the GABA<sub>B</sub>R chimera will be referring to its position in LIVBP as shown by sequence alignment done Galvez et al. (1999).

Once the receptor macromolecule for the GABA<sub>B</sub>R chimera was prepared using AutoDockTools, amino acid residues thought to participate in ligand binding and recognition were made to be flexible to allow for movement during the AutoDock calculations. The residues selected to be flexible were Q17, Q21, Y202, E226, W227, N230, and E276 yielding a total of 12 rotatable bonds while the rest of the molecule was left in a rigid state. Each ligand that was tested in the docking simulations had all rotatable bonds able to freely rotate. Since the binding affinities of the different ligands were being explored, the binding pocket was designated to be the area for docking calculations to be done. The binding pocket was defined by a grid box centered at (46.042, -32.883, 94.111) with 42 points in the x-direction and 40 points in the y- and z-directions. Each point is separated by 0.375 Å and yields a grid box with 72283 grid points per map. AutoGrid calculated the energy of interaction between each atom type in the ligand with the macromolecule at each point that is defined by the grid box. The binding energy of each ligand was then calculated using AutoDock. Default search and docking parameters set by AutoDockTools were used with binding energies being calculated using the Lamarckian Genetic Algorithm. The results of the docking simulation were then analyzed to find the ligand conformation that has the lowest binding energy.

The protocol for the docking simulations was validated by comparing binding energies calculated by AutoDockTools and binding energies found in the literature. This was done using 2LIV as the receptor molecule and isoleucine, leucine, and valine as ligands. All residues in the LIVBP protein were held rigid while the ligands were free to rotate. The binding energies calculated for both isoforms of baclofen were used to validate the calculations done for the GABA<sub>B</sub> chimera.

## **Animals**

Female CD-1 mice weighing 25–30 g were group housed at 21°C and 55% relative humidity on a 12 h light-dark cycle with lights on at 07:00 AM. Food and water were available *ad libitum*. All experiments were performed between 08:00 AM and 06:00 PM and all procedures were approved by the Animal Care Committee at The University of British Columbia.

## **Formalin foot assay**

Animals were numbered and randomly assigned using an online randomization tool ([www.random.org](http://www.random.org)) to treatment or control groups and habituated to the testing apparatus for 1 h on the day prior to experiment with each animal tested only once. The testing apparatus was composed of a ventilated Plexiglas chamber with a clear glass bottom (10.2 x 10.2 x 10.2 cm). A video camera located underneath the chamber recorded the animals' behavior throughout the assay. On the experiment day, animals were injected intraperitoneally with 500 mg/kg treatment or vehicle in a volume of 0.2 ml. Except for R-isovaline, this treatment was given at 15 min prior to a subcutaneous injection of a 5% formalin solution (20 µl) using a 50 µl syringe and 30 gauge needle through the plantar surface of the right hindpaw. Based on a previous study (MacLeod et al., 2010), 500 mg/kg of R-isovaline (0.2 ml) was injected intraperitoneally at 45 min prior to the formalin injection. Cycloleucine was tested to examine the necessity of the cyclobutane ring and was given at a lower dose of 150 mg/kg. A preliminary study using 150, 300, and 500 mg/kg showed that a single administration of the higher doses produced toxicity which is consistent with previous reports (Greco et al., 1980; Nixon, 1976). Immediately after a formalin injection, animals were placed into the testing apparatus for 1 h. The video record was analyzed by a blinded observer who recorded the length of time that the animals licked or bit the affected paw in 3 min intervals (bins).

## **Rotarod**

A modified protocol by Rustay et al. (2003) was used to determine if compounds that attenuated nocifensive behaviour in the formalin foot assay also produced sedation or motor incoordination. The rotarod apparatus was custom made with a 3.5 cm diameter rod and 48.5 cm fall height. The rod surface was covered in a layer of duct tape to prevent slipping and care was taken to minimize the seam to prevent forming a lip that the animals could hang onto. On the first day, animals were exposed to the rotarod rotating at 3 rpm for 30 s with a 30 s inter-trial interval. If the animal fell before 30 s, it was placed in the holding area below the rod for 30 s. Animals were then placed on the rod rotating at 10 rpm with a 30 s inter-trial interval until they were able to remain on the rod for a minimum of 60 s (maximum cutoff, 180 s) for 2 consecutive trials or 10 trials, whichever occurred first. The procedure at 10 rpm was repeated on the second day and animals unable to remain on the rod for 2 consecutive trials within 10 trials were excluded from the study. Animals included into the study were injected with vehicle or treatment intraperitoneally in a volume of 0.2 ml on day 3, 30 min before testing on the rotarod, with the exception of R-isovaline which was injected 60 min prior to rotarod testing. Each animal was tested in triplicate and the latency to fall was the mean time of the 3 trials.

## **Drugs and chemicals**

R-isovaline (R-IVA), S-isovaline (S-IVA), R- $\alpha$ -methylphenylalanine, R- $\alpha$ -propargylalanine, and R- $\alpha$ -allylalanine were generous gifts from Nagase America Co. (New York, New York, USA). ACBC, aminoisobutyric acid (AIB), cycloleucine, aminocyclohexane carboxylic acid (ACHC), R- $\alpha$ -methylproline, and paraformaldehyde were purchased from Sigma-Aldrich Canada Co. (Oakville, Ontario, Canada). 2-(1H-tetrazol-5-yl)butan-2-amine was a generous gift from Biofine International (Vancouver, British Columbia, Canada). A 5% formalin solution was produced by saturating distilled water with paraformaldehyde and diluting with saline to the appropriate concentration. All other compounds were dissolved in saline.

## Data analysis

GraphPad Prism 5 (GraphPad, CA, USA) was used for statistical analyses. Student's unpaired *t*-test was used to compare effects of treatment on phase II nocifensive behaviour. Phase II was defined as 15–45 min after the injection of formalin. Dose response data were first normalized to baseline values and analyzed using a one-sample *t*-test compared to a hypothetical mean. Half-maximal effective doses (ED<sub>50</sub>s) were then calculated by fitting the data using a log[inhibitor] vs. response model according to the equation below:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{[x - \log(\text{IC}_{50})]})$$

One-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test was used to compare the effects of treatments on the latency to fall in the rotarod assay. Data were first analyzed with Grubb's test for the presence of outliers which were removed prior to analysis. Statistical significance was taken to be  $P < 0.05$ . Group sizes were determined based on pilot experiments that showed  $n = 6$  was capable of detecting a statistically significant difference for R-isovaline. Data for total duration of nocifensive behaviour exhibited in phase II, rotarod performance, and effect sizes are presented as means with 95% confidence interval (95% CI) and formalin foot time course data are presented as mean  $\pm$  standard error, with  $n$  as the number of animals.

## Results

### Docking simulations

The validation for the docking protocol was done using 2LIV as the receptor macromolecule and isoleucine, leucine, and valine as ligand molecules. In the docking simulation, the residues forming the binding pocket were given zero degrees of freedom (i.e. all residues were held rigid), but rotatable bonds in each ligand molecule were free to rotate. The results obtained from the docking simulations show that isoleucine has a mean binding energy of  $-6.92 \text{ kcal}\cdot\text{mol}^{-1}$  and standard deviation of 0.012 (n=3), leucine with a mean binding energy of  $-7.16 \text{ kcal}\cdot\text{mol}^{-1}$  and standard deviation of 0.040 (n=3), and valine to have a mean binding energy of  $-6.25 \text{ kcal}\cdot\text{mol}^{-1}$  and standard deviation of 0.004 (n=3). The rank order of binding energies is consistent with binding studies reported in the literature (Gaudin et al., 1977; Adams et al., 1991).

The first sets of docking simulations were performed using both enantiomers baclofen, a well-documented GABA<sub>B</sub>R agonist. Initially, only Tyr202 and Phe227 were made to be flexible while the remainder of the receptor model was held rigid. These conditions yielded a binding energy of  $-6.34 \text{ kcal}\cdot\text{mol}^{-1}$ . However, with only Tyr202 and Phe227 allowed to rotate, the remaining residues of the binding site may not reflect the possible rotations that could occur when baclofen is bound. This was not taken into account during validation of the assay because isoleucine, leucine, and valine are much more similar to each other in terms of size and structure. In addition to Tyr202 and Phe227, the next set of docking simulations allowed rotation of the side chains of Ser79 and Ser102 and yielded a binding energy of  $-4.70 \text{ kcal}\cdot\text{mol}^{-1}$  and standard deviation 1.201 (n=3). Ser79 and Ser102 were made to be flexible because they were suspected to hydrogen bond with the carboxyl and amino moieties of baclofen. When all residues comprising the binding site (as listed above) were made to be flexible, the calculated binding energy was  $-2.23 \text{ kcal}\cdot\text{mol}^{-1}$ .

The results obtained from the docking simulations found that R-baclofen has a binding energy of  $-1.27 \text{ kcal}\cdot\text{mol}^{-1}$  with a standard deviation of 0.425 and S-baclofen has a binding energy of  $-1.72 \text{ kcal}\cdot\text{mol}^{-1}$  with a standard deviation of 0.519. These results were in opposition to previous findings in the literature demonstrating the S-enantiomer binds with significantly less potency than the R-enantiomer (Bowery, Hill, & Hudson, 1983). The inability of AutoDockTools to differentiate between the binding affinities of R-baclofen and S-baclofen precluded docking simulations with other ligands.

### **Chemical structures for isovaline and isovaline analogs**

Figure 1 shows the chemical structures of the compounds which were tested in this study. Starting with R- and S-isovaline, various chemical moieties were introduced to the structure to produce a set of compounds with a range of electronic and steric properties (Harrold & Zavod, 2013).

### **Validation of the formalin foot assay**

The ability of the formalin foot assay to detect analgesia was verified using morphine and acetylsalicylic acid (ASA) as positive controls. Intravenous morphine reduced time exhibiting nocifensive behaviour in a dose-dependent manner with an  $\text{IC}_{50}$  of  $1.8 \text{ mg/kg}$  (95% CI, very wide) and Hill slope of  $-11.9$  (95% CI, very wide) ( $n = 8$  per dose) (Fig. 2a). Animals treated with ASA intraperitoneal exhibited a reduction in time exhibiting nocifensive behaviour compared to control ( $23 \text{ s}$  [95% CI,  $-19, 65$ ;  $n = 5$ ] vs  $230 \text{ s}$  [95% CI,  $83, 377$ ;  $n = 5$ ];  $P = 0.0055$ ) (Fig. 2b).

### **Effects of R- and S-isovaline and cyclization on nocifensive behaviour in formalin assay**

Administration of R-isovaline ( $500 \text{ mg/kg}$ ) decreased the amount of time that mice licked and bit their hindpaw in the Phase II response to formalin injection. Figure 3 shows that animals administered R-isovaline reduced time exhibiting nocifensive behaviour by  $108 \text{ s}$  [95% CI,  $18, 197$ ] compared to animals administered saline (total mean,  $211 \text{ s}$  [95% CI,  $137, 284$ ;  $n = 6$ ] vs.  $318 \text{ s}$  [95% CI,  $245, 391$ ;  $n = 6$ ];  $P = 0.028$ ). Figure 4 shows a similar effect of its enantiomer, S-isovaline, which reduced total nocifensive

time 85 s [95% CI, 7, 163] compared to its saline control (209 s [95% CI, 131, 286;  $n = 6$  with 1 outlier excluded] vs. 293 s [95% CI, 239, 348;  $n = 6$ ];  $P = 0.036$ ). The effect of cyclization and conformational restriction on activity in the formalin assay is depicted in Figure 5. Animals administered ACBC exhibited a decrease of 148 s [95% CI, 12, 285] compared to the duration of nocifensive behaviour exhibited by animals administered saline (195 s [95% CI, 75, 315;  $n = 6$  with 1 outlier excluded] vs. 344 s [95% CI, 232, 456;  $n = 6$  with 1 outlier excluded];  $P = 0.036$ ).

### **Effects of substitution in aliphatic side chain and acidic functionality**

Four derivatives with changes in the aliphatic side chain and acidic functionality of isovaline did not produce detectable effects on nocifensive behaviour in the formalin assay (Figure 6). Multiple bond alterations in the aliphatic chain did not result in detectable activity of the derivatives. Time course data for the compounds are shown in Fig. 6a, c, e, and g. Animals administered R- $\alpha$ -propargylalanine (307 s [95% CI, 190, 425;  $n = 5$ ]) and R- $\alpha$ -allylalanine (375 s [95% CI, 168, 583;  $n = 5$ ]) did not show more or less time at nocifensive behaviour than the saline group (344 s [95% CI, 232, 456;  $n = 5$ ];  $P = 0.54$  and  $0.72$ , respectively) (Fig. 6b, f). The preceding control values for propargylalanine are the same as for allylalanine. The introduction of a phenyl moiety in R- $\alpha$ -methylphenylalanine resulted in no activity (278 s [95% CI, 50, 506;  $n = 5$ ] vs. 230 s [95% CI, 83, 377;  $n = 5$ ];  $P = 0.64$ ) (Fig. 6d). Alteration in the acidic functionality as well as steric size of isovaline as seen in 2-(1H-tetrazol-5-yl)butan-2-amine did not result in significant activity in the formalin assay (212 s [95% CI, 97, 327;  $n = 6$ ] vs. 224 s [95% CI, 91, 358;  $n = 6$ ];  $P = 0.86$ ) (Fig. 6h).

### **Importance of cyclobutane ring formation as determined from effects of aminoisobutyric acid, R- $\alpha$ -methylproline, cycloleucine, and aminocyclohexane carboxylic acid**

Following the demonstration that R-isovaline, S-isovaline, and ACBC administered intraperitoneally were capable of reducing phase II nocifensive behaviour, additional compounds were

tested to explore the importance of cyclobutane ring formation that can be adopted by R-isovaline and S-isovaline and is present in ACBC. Time course data for the compounds are shown in Fig. 7a, c, e, and g. Aminoisobutyric acid (Fig. 7b) administered at a dose of 500 mg/kg did not produce a detectable difference in phase II nocifensive behaviour compared with a saline-treated group (259 s [95% CI, 181, 338;  $n = 6$ ] vs. 304 s [95% CI, 164, 443;  $n = 6$ ];  $P = 0.45$ ). Similarly, R- $\alpha$ -methylproline (Fig. 7d) did not produce a significant change from control group in total nocifensive behaviour time (283 s [95% CI, 139, 427;  $n = 6$ ] vs. 426 s [95% CI, 276, 576;  $n = 6$ ];  $P = 0.11$ ). Cycloleucine (Fig. 7f) administered at a dose of 150 mg/kg did not produce a detectable difference (326 s [95% CI, 163, 490;  $n = 6$ ] vs. 171 s [95% CI, 86, 256;  $n = 6$  with 1 outlier excluded];  $P = 0.07$ ). A significant difference was not detected between groups administered aminocyclohexane carboxylic acid (Fig. 7h; 326 s [95% CI, 237, 416;  $n = 6$ ] and saline (248 s [95% CI, 123, 372;  $n = 6$ ];  $P = 0.22$ ).

### Effects on rotarod performance

The possibility that R-isovaline, S-isovaline, and ACBC produce sedation or motor incoordination was tested using the rotarod assay. At 500 mg/kg doses in 6 animals per group, R-isovaline, S-isovaline, and ACBC all produced falls from the rotating rod with a latency of 144 s (95% CI, 115, 173), 124 s (95% CI, 90, 159), and 120 s (95% CI, 75, 165), respectively (Fig. 8b). ANOVA followed by Dunnett's test revealed no significant differences in the latency to fall when comparing R-isovaline, S-isovaline, and ACBC to saline (123 s [95% CI, 73, 173;  $n = 6$ ]). The positive control, diazepam, given at a 2 mg/kg dose decreased the latency to fall from the rotarod in all mice when compared to saline (Fig. 8a).

### Dose-response relationships

Dose-response relationships were constructed for R-isovaline, S-isovaline, and ACBC using the formalin foot assay. Doses tested were 0, 10, 30, 100, 300, and 500 mg/kg, and an additional dose of 1000 mg/kg was tested for ACBC ( $n = 6$  for each dose). One outlier was excluded from each of the

following groups: 10 mg/kg and 300 mg/kg R-isovaline, 500 mg/kg S-isovaline, and 500 mg/kg ACBC.

Total time exhibiting nocifensive behaviour during phase II was recorded and data were normalized to their respective 0 mg/kg (vehicle) controls. Control values were taken as 100% and the normalized data were plotted as a percent of vehicle.

As shown in Figure 9a, R-isovaline had an ED<sub>50</sub> of 97 mg/kg (95% CI, 5, 2088) and a calculated maximum effect of 97% (95% CI, 40, 155). From the dose-response curve for S-isovaline, the calculated ED<sub>50</sub> was 47 mg/kg (95% CI, 3, 849) and maximum effect was 40% (95% CI, 14, 67) (Fig. 9b). From the dose-response curve for ACBC (Fig. 9c), the calculated ED<sub>50</sub> was 323 mg/kg (95% CI, 21, 4873) and maximum effect was 82% (95% CI, 15, 149).

## Discussion

This study for the first time, demonstrated structural characteristics of the non-proteinogenic amino acid, isovaline, which contribute to analgesia. In the course of the present work, a previously little studied amino acid analgesic was identified, ACBC. A set of compounds was chosen to represent modified structures of isovaline to investigate the possibility of using small, non-standard amino acids for the treatment of pain. We demonstrated that system administration of both R and S isomers of isovaline at 500 mg/kg doses reduced nocifensive behaviour in phase II of the formalin assay in mice. Docking simulations were conducted to investigate binding interactions between ligands and a GABA<sub>B</sub> chimera, but the procedure was unable to be validated. We investigated the impact of structural modifications made to the isovaline molecule, examining the effects of intraperitoneal administration of the analogs in this assay. The cyclized analog, ACBC, formed by a bond between aliphatic and  $\alpha$ -methyl groups, also reduced the total time of phase II behaviour. Administration of R-isovaline, S-isovaline, or ACBC to animals did not affect their ability to maintain balance or latency to fall off a rotarod rotating at a constant speed, indicating that the compounds do not significantly affect conscious control of motor coordination. This suggests that the reduction in phase II nocifensive behaviour is a result of an analgesic effect and not due to sedation or lack of motor coordination. The above findings are consistent with the previous demonstrations that racemic isovaline produce antinociception in the formalin foot, tail clip, and glutamate-induced nociceptive assays, as well as prostaglandin E<sub>2</sub> and strychnine induced allodynia (Asseri et al., 2015; Chung, 2009; Lee et al., 2013; MacLeod et al., 2010; Whitehead et al., 2015). No analgesic effects were detected for any of the other compounds in the first series of molecules, examining the consequences of changes made to the aliphatic and carboxylic acid groups or in the second series of compounds, investigating the necessity of the cyclobutane ring.

We first verified the ability of the formalin foot assay in our laboratory to detect analgesia using the positive controls, morphine and ASA. We demonstrated that intravenous morphine produced

robust, dose-dependent analgesia in phase II of the formalin foot assay. Likewise, ASA also produced analgesic effects at a dose comparable to literature values for phase II (Hunskar et al., 1985). These data verify that in our laboratory, the formalin foot assay is capable of detecting the effects of established analgesics.

Docking simulations were attempted using AutoDockTools to investigate binding interactions with a receptor of interest, GABA<sub>B</sub>, by using a chimera of the receptor modelled off the bacterial leucine-isoleucine-valine binding protein. However, the technique could not be validated as the software was unable to correctly rank order the binding potency of R-baclofen and S-baclofen. S-baclofen was calculated as having a greater affinity to the receptor compared with R-baclofen which contradicts *in vitro* binding studies that demonstrated R-baclofen is approximately 1000 fold more potent at the GABA<sub>B</sub> receptor (Bowery et al., 1983). The difference may be due in part to the inability of AutoDockTools to include ionic species in its calculations which would preclude proper binding simulations of baclofen to the receptor. The absence of divalent ions in the binding calculations may explain why S-baclofen and R-baclofen appear to have similar binding affinities. It may be possible that the binding of R-baclofen is dependent on calcium while S-baclofen has a low affinity for GABA<sub>B</sub> regardless of the presence or absence of calcium. Interestingly, there has been work published by Galvez et al. (2000) that contradict the need for calcium in baclofen binding to GABA<sub>B</sub>. Their results show that addition of 1 mM EGTA to remove Ca<sup>2+</sup> ions did not affect the binding affinity of baclofen, but did decrease the activity of baclofen in a function assay (Galvez et al., 2000). At the time these simulations were performed, the crystal structure of the GABA<sub>B</sub> receptor had not yet been resolved. Since then, the ligand binding domain of the GABA<sub>B</sub> receptor has been resolved (Geng et al., 2013). Renewed studies using the GABA<sub>B</sub> receptor rather than a chimera would yield improved results and complement the structure-activity relationship studies conducted in this work. It would provide a quick and efficient

method of identifying potential ligands at this receptor without the need for performing a large number of screening assays.

The first series of compounds tested, R-isovaline, S-isovaline, R- $\alpha$ -methylphenylalanine, R- $\alpha$ -propargylalanine, R- $\alpha$ -allylalanine, 2-(1H-tetrazol-5-yl)butan-2-amine, and ACBC covered a diverse range of electrostatic properties; the series is representative of common modifications made to compounds in structure-activity relationship studies. Due to the structural rigidity of isovaline, modifications to its backbone were not expected to drastically change its conformation. Addition of a phenyl ring produced R- $\alpha$ -methylphenylalanine which shares structural similarities to the GABA<sub>B</sub> agonist, baclofen (Kerr et al., 1990). To our surprise, although baclofen has significant analgesic effects (Balerio & Rubio, 2002; Gaillard et al., 2014), R- $\alpha$ -methylphenylalanine was devoid of antinociceptive activity. The addition of unsaturated bonds to produce R- $\alpha$ -allylalanine and R- $\alpha$ -propargylalanine increased the size and length of the ethyl group. However, these modifications reduced the analgesic effect of the compound below detectable levels. Substituting the carboxylic acid with a tetrazolic group maintained the acidic functionality while increasing the steric size. The lack of an analgesic effect suggests that the increase in steric size of the acidic group precludes receptor interaction. Interestingly, cyclization of the ethyl and methyl groups of isovaline to form a cyclobutane ring (ACBC) retained antinociceptive activity. These observations suggest that the binding pocket of R-isovaline is relatively small as any increase in size decreases the analgesic effect.

The second series of compounds including aminoisobutyric acid, aminocyclohexane carboxylic acid, and R- $\alpha$ -methylproline tested the necessity of the cyclobutane ring in producing analgesia. In aminoisobutyric acid, the ethyl side chain is shortened to a methyl group, thus preventing the formation of the cyclobutane ring; this resulted in a molecule that lacked analgesic effects. Likewise, increasing the 4-carbon ring to a 5-carbon or 6-carbon ring reduced the analgesic effect below detectable limits. In R- $\alpha$ -methylproline, cyclization between the ethyl R-group and the amino group produced a compound that

did not produce analgesia. These data suggest that either isovaline's conformational stability or ACBC's cyclobutane conformational restriction is necessary for analgesia.

Dose-response curves were generated for R-isovaline, S-isovaline, and ACBC. The ED<sub>50</sub> of R-isovaline determined in this study was consistent with the lower range in the literature (75–740 mg/kg), determined by work on the same assay system, with differences likely attributed to differing routes of administration (Chung, 2009; MacLeod et al., 2010). The estimated maximum reduction in duration of nocifensive behaviour in phase II was comparable to previous findings (MacLeod et al., 2010). Despite the large confidence intervals of the estimates, the dose-response curves for R-isovaline, S-isovaline, and ACBC suggest a small, but consistent decrease in nocifensive behaviour with increasing doses. Given the variability of analgesic drug effects in different assays, it would be prudent for future studies to examine these compounds in other nociceptive assays.

To assess the potential confounding factors of sedation and motor incoordination, the animals were tested on a rotarod following administration of R-isovaline, S-isovaline, and ACBC (500 mg/kg). This surrogate measurement detected "sedation" caused by 2 mg/kg diazepam and is comparable to sedative doses of diazepam found in previous studies (Capacio et al., 1992; Cavalcante Melo et al., 2012). No change was detected in the latency to fall for R-isovaline, S-isovaline, and ACBC suggesting that the reduction in nocifensive behaviour is not a result of sedation or motor incoordination.

Interestingly, ACBC has been shown to act as a partial agonist at NMDA receptors, producing less receptor activation compared with full agonists (Inanobe et al., 2005; Watson et al., 1989). NMDA receptors play a significant role in pain signal transmission. Antagonism of NMDA receptor activation is well known to produce analgesia (Bennett, 2000; Chizh et al., 2000; Petrenko et al., 2003; Vorobeychik et al., 2013); however, complete antagonism of NMDA receptor activity is also associated with adverse effects (Cvrček, 2008; Petrenko et al., 2003). It is possible that the application of partial agonists at

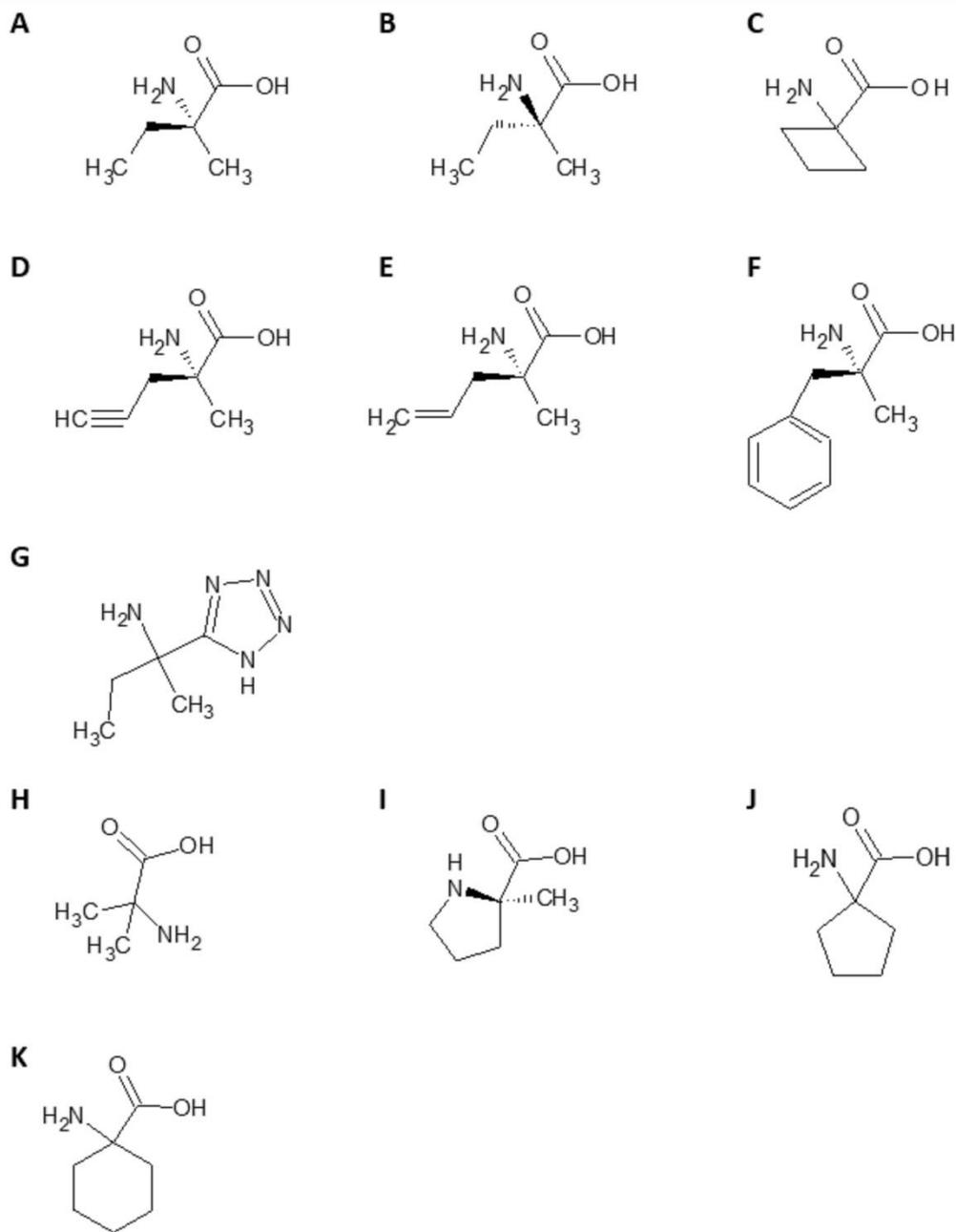
NMDA receptors may avoid the toxicity associated with full receptor antagonism. Our findings suggest that ACBC is well-tolerated at an analgesic dose because treated animals exhibited behaviour and rotarod performance comparable to vehicle treated animals. Although isovaline produces analgesia through activation of GABA<sub>B</sub> and group II mGlu receptors (Asseri et al., 2015; Whitehead et al., 2012), its structural similarity to ACBC raises the possibility that isovaline also may act at NMDA receptors.

Despite identifying key features of isovaline responsible for antinociceptive effects, some shortcomings must be addressed. For example, in this study aimed to establish a preliminary structure-activity relationship for isovaline derivatives, we cannot rule out the presence of type II errors for compounds found to produce no antinociception as they may have small antinociceptive effects which were not detectable using our experimental design. In addition, the other compounds may exhibit antinociceptive effects at doses not tested in study. However, we were interested in identifying compounds that produce comparable antinociception to isovaline at the dose selected. As well, this study only investigated the effects of these compounds on spontaneous behaviour produced by formalin. Most studies utilizing the formalin assay have investigated drug activity within the phase I and phase II windows. The existence of a phase III (e.g. beyond 60 minutes) has been suggested, but has not been established due to uncertainties such as the necrotizing effects of formalin (Kim et al., 1999; Tjølsen et al., 1992). It is possible that the compounds may be effective in other pain modalities such as mechanical or thermally induced pain. Although isovaline appears to undergo minimal metabolism *in vivo* (Butts & Sinnhuber, 1941), less is known regarding the other compounds. It is possible that due to the route of administration, the other compounds were subjected to immediate hepatic metabolism. While promising antinociceptive effects were detected in several compounds using the formalin test, the results may not readily translate into man as this study was conducted in mice (Mao, 2012). Nonetheless, this study established a preliminary library of compounds to investigate the impact of structural modifications on the antinociceptive properties of isovaline.

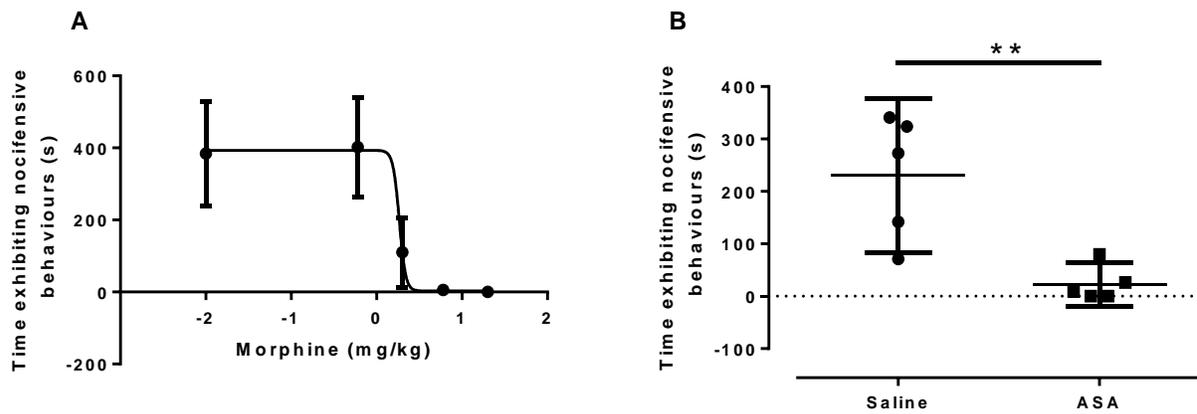
In summary, we developed a preliminary structure-activity relationship based on the unusual amino acid, isovaline. Common structural modifications to this molecule led to changes in antinociceptive effects. In this paper, we demonstrated that, like the R-stereoisomer and its cyclized isomer (ACBC), S-isovaline had antinociceptive effects following systemic administration. Both R-isovaline and ACBC decreased responses in phase II of the formalin foot assay without compromising performance on the rotarod in mice. These new data may lead to drug molecules with superior pharmacological profiles for the treatment of pain.

**Table 1 – Ligand SMILES Strings**

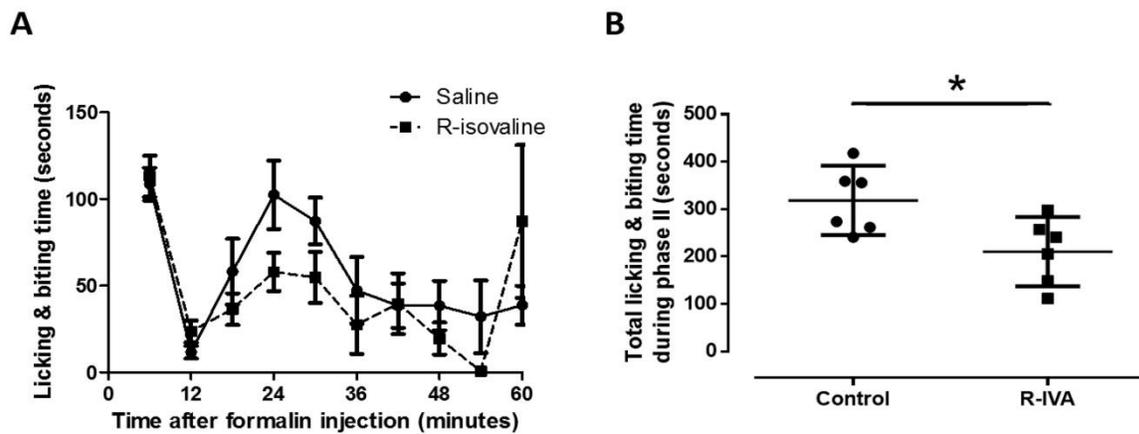
| <b>Ligand</b>                       | <b>SMILES</b>                                  |
|-------------------------------------|--|
| GABA                                | <chem>C(CC(=O)O)CN</chem>                      |
| $\gamma$ -hydroxybutyric acid (GHB) | <chem>C(CC(=O)[O-])CO</chem>                   |
| R-baclofen                          | <chem>C1=CC(=CC=C1[C@@H])(CC(=O)O)CN)Cl</chem> |
| S-baclofen                          | <chem>C1=CC(=CC=C1[C@H])(CC(=O)O)CN)Cl</chem>  |
| R-isovaline                         | <chem>CC[C@](C)(C(=O)O)N</chem>                |
| S-isovaline                         | <chem>CC[C@@](C)(C(=O)O)N</chem>               |



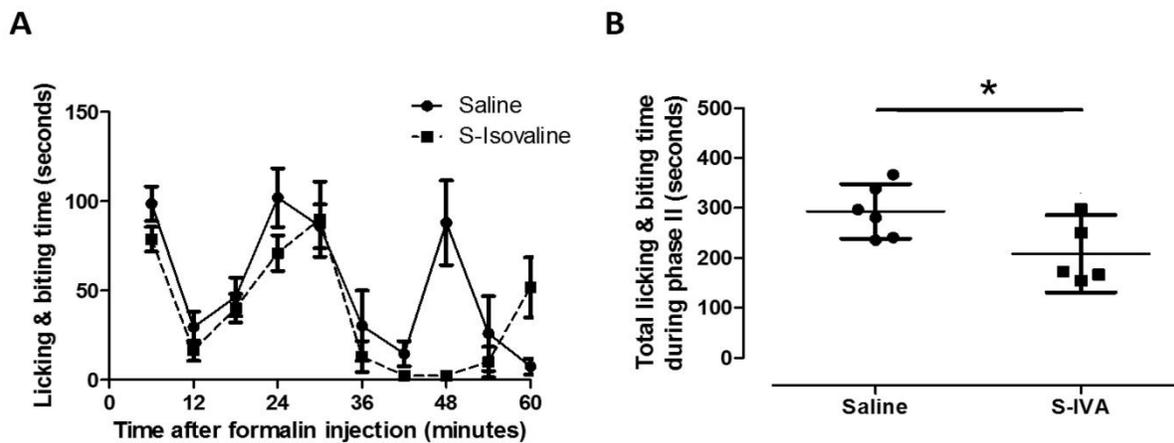
**Fig. 1 – Chemical structures of isovaline and structurally-related compounds.** A) R-isovaline B) S-isovaline C) ACBC D) R- $\alpha$ -propargylalanine E) R- $\alpha$ -allylalanine F) R- $\alpha$ -methylphenylalanine G) 2-(1H-tetrazol-5-yl)butan-2-amine H) aminoisobutyric acid I) R- $\alpha$ -methylproline J) Cycloleucine K) Aminocyclohexane carboxylic acid.



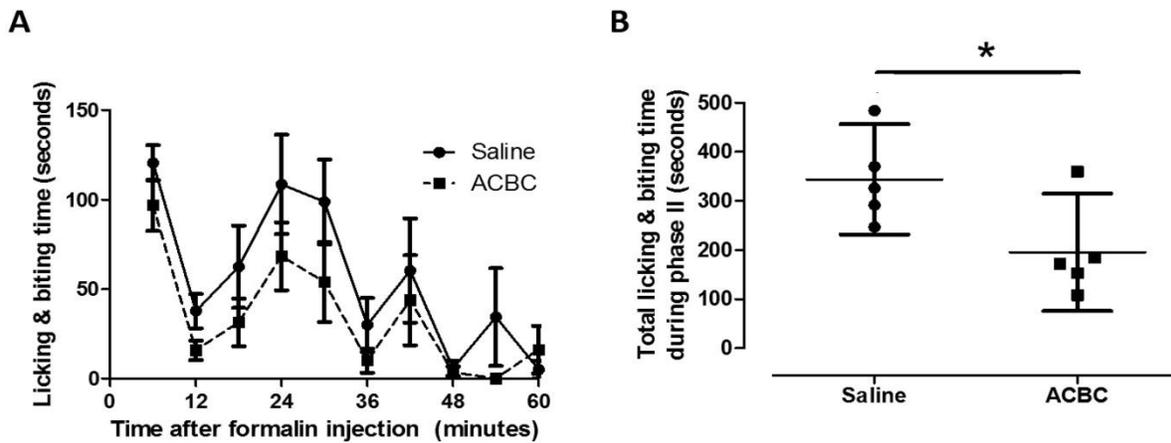
**Fig. 2 – Validation of formalin foot assay.** A) Morphine intravenous dose response curve on phase II of the formalin foot assay.  $n = 8$  per dose. Data presented as mean with 95% CI B) 300 mg/kg ASA intraperitoneal reduces the total licking and biting time in phase II of the formalin foot assay.  $n = 5$  animals per group. \*\* indicates  $P < 0.01$ . Each point represents an individual animal with summary bars presented as mean  $\pm$  95% CI.



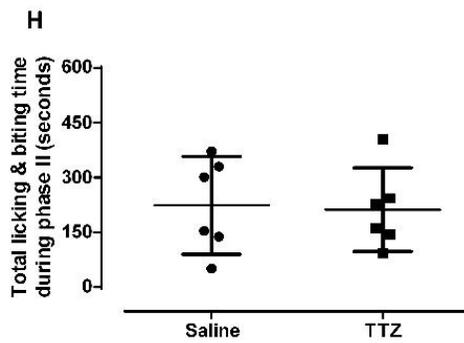
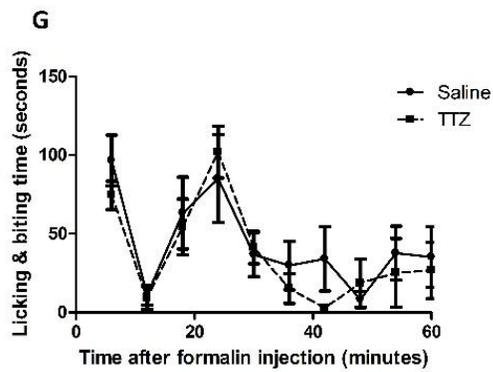
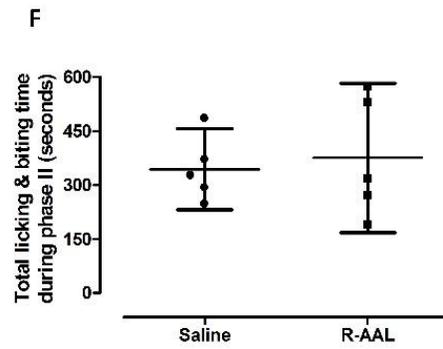
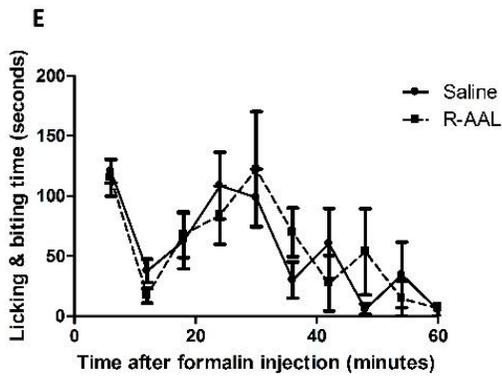
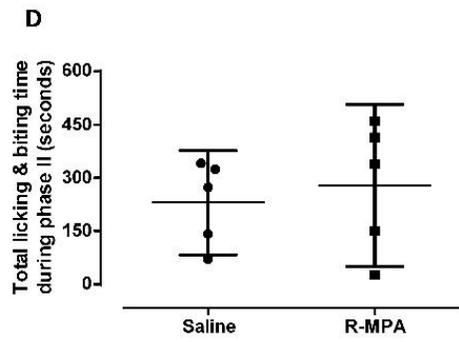
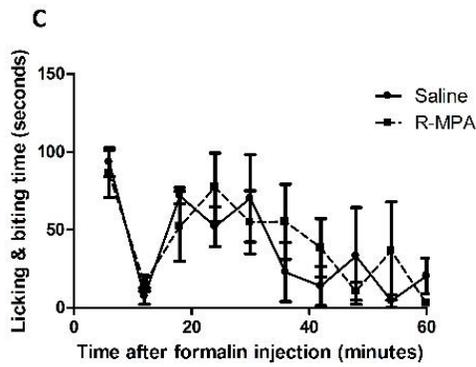
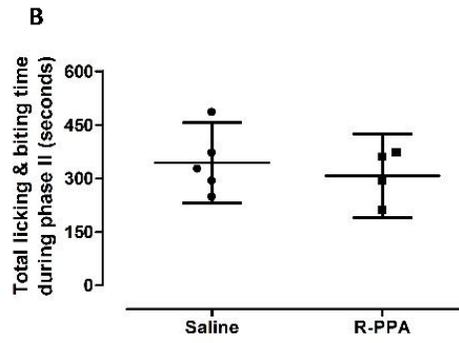
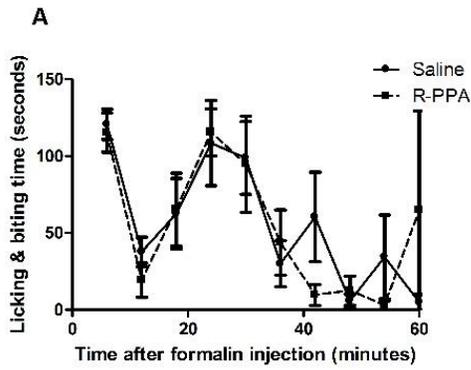
**Fig. 3 – R-isovaline reduces nocifensive behaviour in phase II of the formalin assay.** A) Time course of observed licking and biting behaviour recorded for an hour after the intraplantar injection of 5% formalin for animals injected with R-IVA (500 mg/kg) or saline (0.9% NaCl).  $n = 6$  animals per group. Data is presented as mean  $\pm$  SEM. B) R-IVA (500 mg/kg) intraperitoneal reduces the total licking and biting time in phase II of the formalin foot assay.  $n = 6$  animals per group. \* Indicates a statistically significant difference ( $P = 0.028$ ). Each point represents an individual animal with summary bars presented as mean  $\pm$  95% CI.



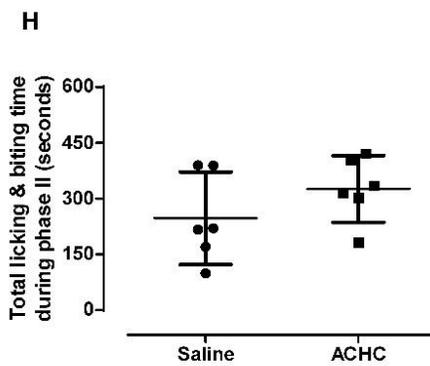
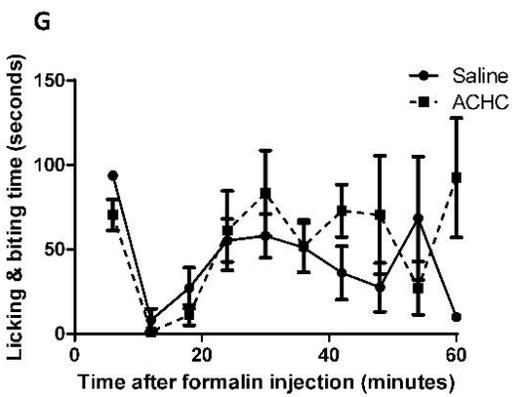
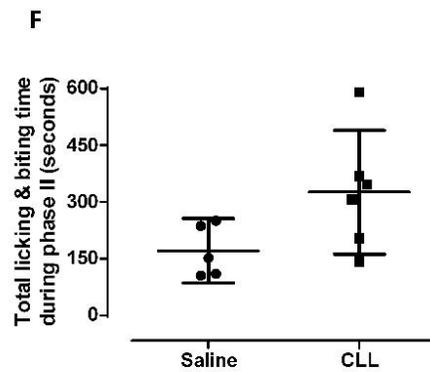
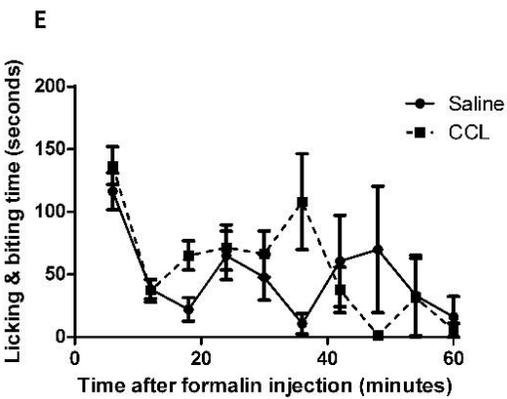
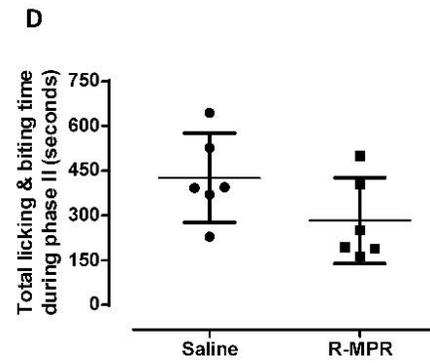
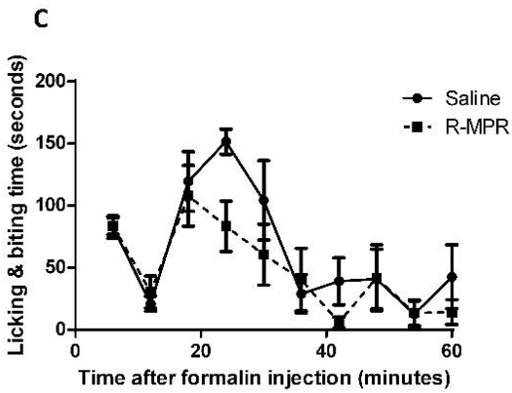
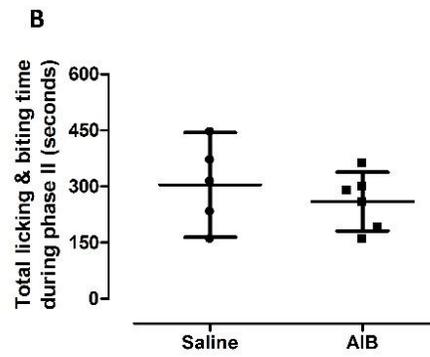
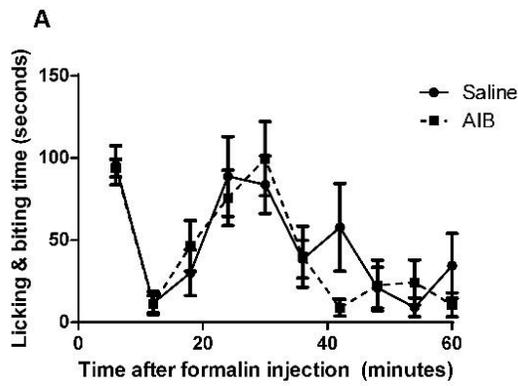
**Fig. 4 – S-isovaline reduces nocifensive behaviour in phase II of the formalin assay.** A) Time course of observed licking and biting behaviour recorded for an hour after the intraplantar injection of 5% formalin for animals injected with S-IVA (500 mg/kg) or saline (0.9% NaCl).  $n = 6$  animals per group with 1 outlier excluded from the S-IVA group. Data are presented as mean  $\pm$  SEM. B) S-IVA (500 mg/kg) intraperitoneal reduces the total licking and biting time in phase II of the formalin foot assay.  $n = 5 - 6$  animals per group with 1 outlier excluded from the S-IVA group. \* Indicates a statistically significant difference ( $P = 0.036$ ). Each point represents an individual animal with summary bars presented as mean  $\pm$  95% CI.



**Fig. 5 – ACBC reduces nocifensive behaviour in phase II of the formalin assay.** A) Time course of observed licking and biting behaviour recorded for an hour after the intraplantar injection of 5% formalin for animals injected with ACBC (500 mg/kg) or saline.  $n = 5$  animals per group with 1 outlier excluded from the ACBC and saline group. Data are presented as mean  $\pm$  SEM. B) ACBC (500 mg/kg) intraperitoneal reduces the total licking and biting time in phase II of the formalin foot assay.  $n = 5$  animals per group with 1 outlier excluded from the ACBC and saline group. \* indicates a statistically significant difference ( $P = 0.036$ ). Each point represents an individual animal with summary bars presented as mean  $\pm$  95% CI.

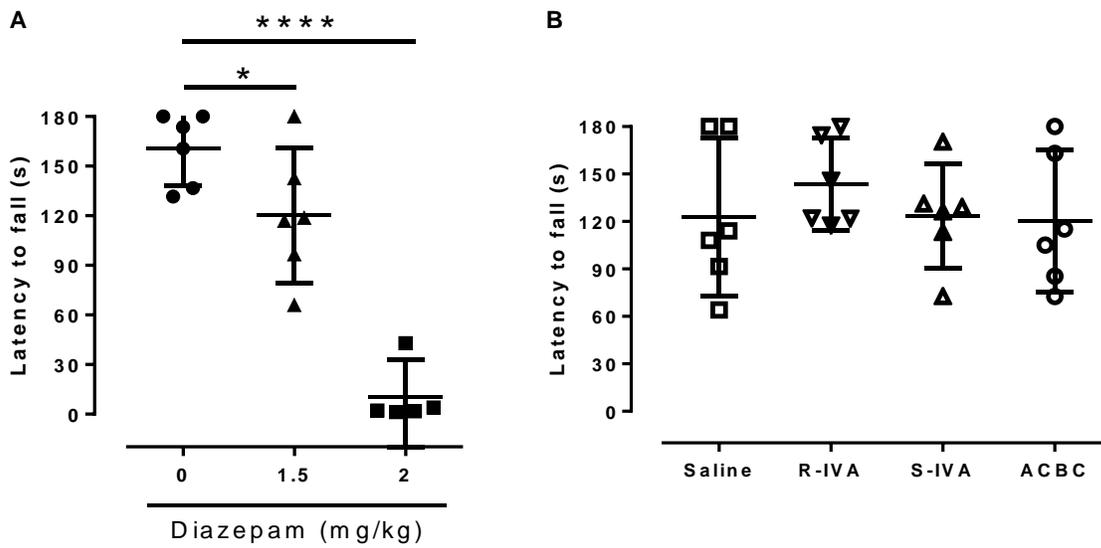


**Fig. 6 – Modifications affecting steric size eliminates the analgesic effect.** A, B) Time course and phase II summary for R- $\alpha$ -propargylalanine (R-PPA).  $n = 5$  animals with 1 outlier excluded from the R-PPA group. C, D) Time course and phase II summary for R- $\alpha$ -methylphenylalanine (R-MPA).  $n = 5$  per group. E, F) Time course and phase II summary for R- $\alpha$ -allylalanine (R-AAL).  $n = 5$  per group. G, H) Time course and phase II summary for 2-(1H-tetrazol-5-yl)butan-2-amine (R-TTZ).  $n = 6$  per group. Time course data are presented as mean  $\pm$  SEM. Phase II summaries are presented as individual animals with summary bars presented as mean  $\pm$  95% CI. All drugs were tested at 500 mg/kg intraperitoneal.

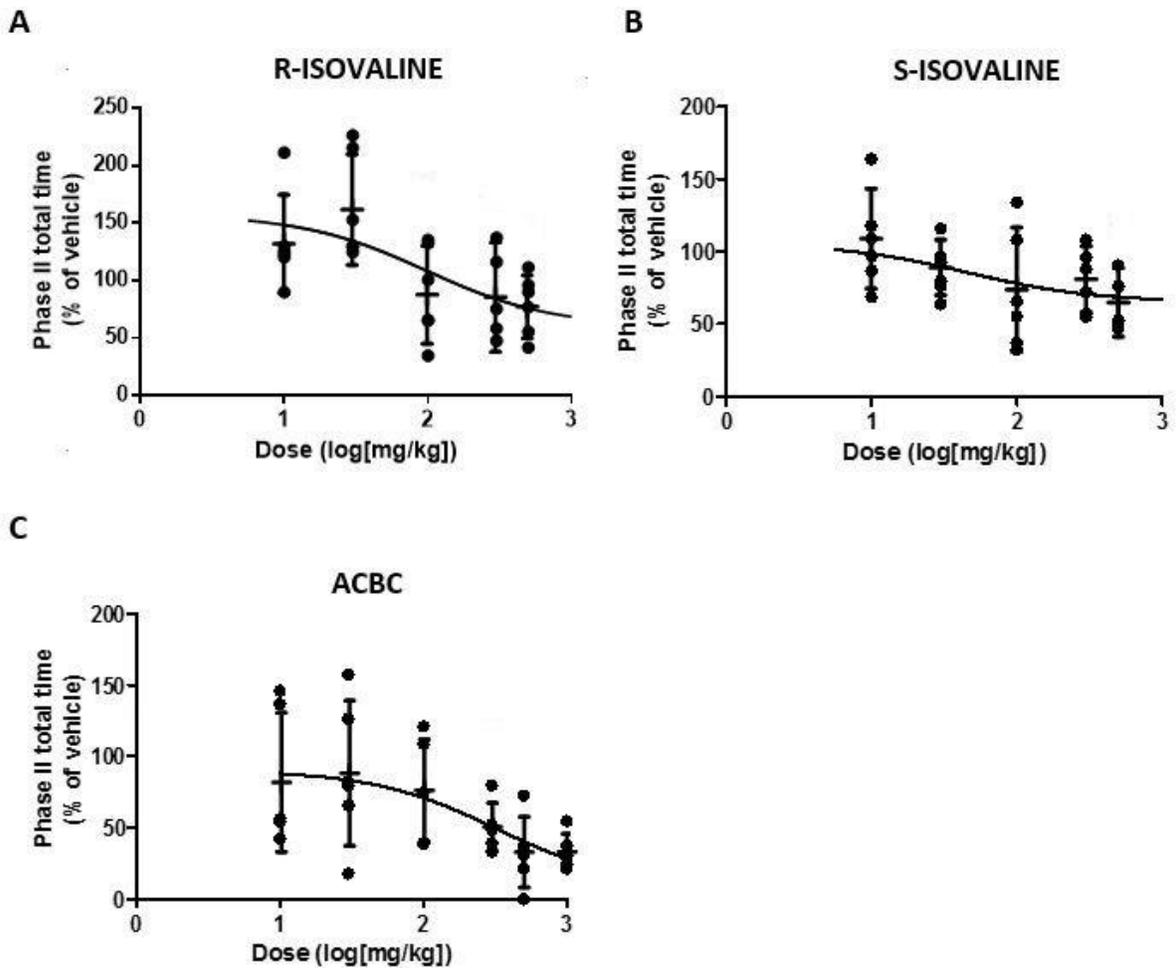


**Fig. 7 – Modifications preventing the formation of a cyclobutane ring eliminates the analgesic effect.**

A, B) Time course and phase II summary for aminoisobutyric acid (AIB).  $n = 6$  per group with 1 outlier excluded from the saline group. C, D) Time course and phase II summary for R- $\alpha$ -methylproline (R-MPR).  $n = 6$  per group. E, F) Time course and phase II summary for cycloleucine (CLL).  $n = 6$  per group with 1 outlier excluded from the saline group. G, H) Time course and phase II summary for aminocyclohexane carboxylic acid (ACHC).  $n = 6$  per group. Time course data are presented as mean  $\pm$  SEM. Phase II summaries are presented as individual animals with summary bars presented as mean  $\pm$  95% CI. All drugs were tested at 500 mg/kg administered intraperitoneally with the exception of cycloleucine which was tested at 150 mg/kg.



**Fig. 8 – ACBC, S-IVA, and R-IVA do not impair rotarod performance at an analgesic dose.** A) Diazepam intraperitoneal reduced the latency to fall. B) Pre-treatment of animals with a 500 mg/kg of R-IVA, S-IVA, or ACBC does not produce a detectable change in the latency to fall from a fixed-speed rotarod. Each point represents an individual animal with summary bars presented as mean  $\pm$  95% CI. \* indicates  $P < 0.05$ , \*\*\*\* indicates  $P < 0.0001$ .



**Fig. 9 – Dose-response curves for R-IVA, S-IVA, and ACBC.** Dose-response curves for A) R-isovaline, B) S-isovaline, and C) ACBC.  $n = 5 - 6$  animals per dose. Data were normalized to the phase II total time of the vehicle control for each compound and presented as individual recordings with summary bars representing mean with 95% CI.

## Chapter 3 – Investigating the Potential of Isovaline and Isovaline Analogs as Spinal Analgesics

### Introduction

Neuraxial administration of opioids has been a popular and efficacious option for providing intraoperative and postoperative analgesia since the discovery of opioid receptors along the central neuraxis (Szarvas et al., 2003). However, their use by such routes is associated with adverse effects such as respiratory depression which is potentially lethal, particularly in susceptible or respiratory-compromised individuals (Chaney, 1995; Ruan, 2007). This severe complication arises from cephalad spread and activation of brainstem opioid receptors, thus warranting a search for non-opioid analgesic alternatives which are effective when administered intrathecally.

Our laboratory has focused on a novel non-opioid analgesic prototype, isovaline, a small amino acid that produces antinociception without apparent adverse effects in various murine pain models following systemic or central administration. These include the formalin foot test, skin or paw allodynia induced by strychnine or prostaglandin E<sub>2</sub>, and responses to tail clip application (Asseri et al., 2015; Lim & Lee, 2010; MacLeod et al., 2010; Whitehead et al., 2015; Whitehead et al., 2012). To define the molecular basis of isovaline's analgesic action and develop agents with increased potency, we conducted a preliminary structure-activity relationship (SAR) study of compounds related to isovaline. These revealed that a cyclized amino acid, 1-amino-1-cyclobutane carboxylic acid (ACBC) produces antinociceptive effects in the formalin foot test (Fung et al., 2017).

ACBC is an amino acid analog of isovaline in which the ethyl and methyl groups on the  $\alpha$ -carbon of isovaline are joined to form a cyclobutane ring. *In vitro* studies have demonstrated that ACBC interacts with the glycine co-agonist binding site on N-methyl-D-aspartate (NMDA) receptors; for example, ACBC acts as a partial agonist at this site inhibiting glycine binding to NMDA receptors and

glycine-induced potentiation of NMDA receptor currents (Hood et al., 1989; Inanobe et al., 2005; Watson et al., 1989). Initial studies using mice in our laboratory showed that systemically administered ACBC produces antinociceptive effects in both phases of the formalin foot test and increases withdrawal latencies in the hot water tail immersion assay. Both systemic and local administration of ACBC blocks glutamate-induced allodynia (Chung, 2009). These antinociceptive effects were not accompanied by adverse effects on heart rate, respiratory rate, behaviour, or rotarod performance (Chung, 2009; Fung et al., 2017).

In contrast to the experiments with the systemic and local routes of administration, the potential of isovaline and its analogs, ACBC and R- $\alpha$ -methylproline, as central neuraxial (intrathecal) analgesics have not been thoroughly investigated. Preliminary evidence has suggested that both isovaline and ACBC produces antinociception in the murine formalin foot test following intrathecal administration (Wang, 2008). To further investigate these effects, we first assessed the antinociceptive effects of isovaline and the analogs in another assay for pain, the hypertonic saline assay, following systemic (intraperitoneal) or after local administration. We then investigated if lumbar intrathecal administration of isovaline and its analogs block nociception and if such effects were limited to the dermatomal distribution corresponding to the site of administration or consistent with effects within the brain. We assessed the adverse effects on respiratory function and motor coordination from the ability of animals to rear and walk. We used the formalin foot test as well as the hypertonic saline foot assay to determine antinociceptive effects.

## Materials and methods

### Animals

Female CD-1 mice weighing 25 – 30 g were group housed at 21°C and 55% relative humidity on a 12 h light-dark cycle with lights on at 07:00 AM. Food and water were available *ad libitum*. All experiments were performed between 08:00 AM and 06:00 PM with all procedures approved by the Animal Care Committee at The University of British Columbia. Animals were numbered and randomly assigned to treatment group using an online randomization tool ([www.random.org](http://www.random.org)) with each animal tested only once. The experimenter administering the drug was unaware of the type or concentration of the drug. The evaluation of effect was also performed from video records by a blinded observer. This study is reported according to ARRIVE guidelines (Kilkenny et al., 2010).

### Hypertonic saline assay

The hypertonic saline assay has recently been shown by our laboratory to quickly and reliably detect antinociceptive properties of a range of analgesic compounds (Asiri et al., 2017). On the day of testing, animals were habituated to the testing apparatus for 2 h prior to experiments. The testing apparatus was composed of darkened Plexiglas chambers (diameter, 9 cm; height, 19 cm) on a clear glass bottom. Video cameras placed under the chambers recorded the animals' behaviour for the duration of the assay. Following habituation, animals were injected with treatment or vehicle at 15 minutes prior (with the exception of the reversibility study where treatment was administered 30 minutes prior) to the injection of 10% hypertonic saline (10 µl) using a 0.3 ml syringe attached to a ½" 30 G needle into the plantar surface of the right hindpaw. Immediately after the injection of hypertonic saline, animals were placed into the testing apparatus for 30 minutes. Recorded videos were analyzed in 5 minute bins by a blinded observer who measured the length of time the animals licked or bit the affected paw.

In the experiment investigating the retention of ACBC to the site of injection, 10  $\mu$ l of 10% hypertonic saline was injected into the right forepaw 15 min after the administration of ACBC to induce nocifensive responses.

### **Formalin foot assay**

The formalin foot assay followed a similar protocol as described above for the hypertonic saline assay. Modifications made to the protocol were the use of 5% formalin (20  $\mu$ l) and extending the duration of the test to 1 hr. The early phase of formalin was defined as 0–5 min and the late phase as 20–40 minutes.

### **Radiant heat tail flick assay**

Animals were placed into a small restraining chamber with the tail exposed. The tail was held straight, parallel to the floor of the chamber, by a small weight attached to the tail which allowed for movement, but ensured the tail remained under the light source between trials. Intensity of the light source was empirically adjusted to produce withdrawal latencies between 1 – 2 seconds. After a 3 min habituation period to the Plantar Test Apparatus (IITC, California, USA), the middle portion of the tail was exposed to radiant heat. The stimulus was stopped when the animal flicked its tail. Each animal was tested in triplicate with a 3 min inter-trial interval prior to drug administration to establish a baseline and re-tested in triplicate following the administration of ACBC. The mean of the three trials was taken to be the withdrawal latency of the animal.

### **Ramped hot plate assay and rectal temperature**

Animals were placed in clear, well-ventilated chambers and habituated to the Hot/Cold Plate Analgesia Meter apparatus (IITC, California, USA) for 1 h prior to testing. Following habituation, animals were administered 0, 62.5, 125, 250, or 500 mM ACBC intrathecally and allowed 15 min to recover. Animals were then placed individually onto the surface of the ramped hot plate set at 32.1 °C. The surface of the testing apparatus was then heated at a rate of 5.3 °C by a blinded experimenter until the

animal withdrew or licked one of the hind paws or until the maximum cut off of 52.1 °C was reached. The withdrawal latency of each animal was recorded.

### **Assessment of motor blockade**

A blinded observer rated animals in hypertonic saline and formalin foot assays to grade any motor deficits that occurred within 15 minutes after the intrathecal administration of ACBC. Animals' motor deficits were classified into three categories: Normal, Moderate, or Severe. Normal animals were defined as those without any observable motor deficits and were indistinguishable from treated animals in terms of mobility. Moderate motor deficits were defined as minor effects on gait and mobility. Severe motor deficits were defined as major effects on gait and mobility or paralysis of the hind limbs.

### **Assessment of effects on respiratory rate**

At 5 minutes post-injection, mice were placed individually into a well-ventilated 50 ml Falcon tube with a darkened tip. The tube was secured onto a stand facing lengthwise to a video camera. Animals were recorded for 10 mins and the number of breaths taken during the longest uninterrupted segment during the 9<sup>th</sup> – 10<sup>th</sup> min of video recording (14<sup>th</sup> – 15<sup>th</sup> min post-injection) were counted and extrapolated to breaths per minute.

### **Intrathecal administration of isovaline and isovaline analogs**

Intrathecal injections of isovaline, isovaline analogs, or artificial cerebrospinal fluid (aCSF) were made according to a modified protocol of Njoo et al. (2014). Briefly, animals were anesthetized with 2.2% isoflurane in an induction chamber for 5 minutes before being maintained at 2.2% with a nose cone. The abdomen of the animal was placed over a rounded surface to induce a curvature into the spine. A small patch of fur was shaved over the site of injection and wiped with ethanol. Using the iliac crest as a landmark, the spinous processes of the 5<sup>th</sup> and 6<sup>th</sup> lumbar vertebrae (L5 and L6) were identified and the needle was carefully inserted in-between. Injections were made in a volume of 5 µl using an insulin 0.3 ml syringe attached to a ½" 29 G needle (BD, New Jersey, USA). Successful insertion

of the needle into the intrathecal space resulted in a characteristic tail flick. Upon observation of the tail flick, the needle was held in place and the solution was injected. The animal was then returned to a cage and allowed to recover.

Intrathecal injections were made according to a modified protocol of Wang (2008) and the modification is briefly described. The needle was then slowly inserted at a 20° angle to the right of the spinal column. The needle was slowly advanced into the intrathecal space while decreasing the needle angle to approximately 10°.

Strychnine was first used to compare the intrathecal injection techniques described by Njoo et al., (2014) and Wang, (2008). Five minutes after injection of 200 µM strychnine, mice were lightly brushed with a smooth plastic tube along the flanks against the grain. The presence of dynamic allodynia was assessed by a blinded observer. Dynamic allodynia was considered to be present if animals flinched, scratched, or showed signs of agitation such as running and biting behaviour.

### **Peripheral administration of isovaline, ACBC, and R- $\alpha$ -methylproline**

Animals received either intraperitoneal (intraperitoneal) or intraplantar injections of treatment or vehicle. ACBC was injected intraperitoneally at a dose of 0, 250, 500, 1000, or 2000 mg/kg in a volume of 0.2 ml using a 1 ml syringe (BD, New Jersey, USA) and ½" 27 G regular bevel needle (Terumo, New Jersey, USA). ACBC was injected intraplantarly at a concentration of 1.4 and 2.3 M (10 µl) into the ventral surface of the right hindpaw using a 0.3 ml syringe attached to a ½" 30 G needle. Doses were chosen based on pilot experiments.

### **Drugs and chemicals**

ACBC, R- $\alpha$ -methylproline, bupivacaine, strychnine, formalin, and salts used for producing aCSF were purchased from Sigma-Aldrich Canada Co. (Oakville, Ontario, Canada). Morphine was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Naloxone hydrochloride was purchased from Endo Laboratories Inc. (Garden City, New York, USA). The aCSF contained in mM: NaCl 148, KCl 3,

CaCl<sub>2</sub> 1.4, MgCl<sub>2</sub>·6H<sub>2</sub>O 0.8, Na<sub>2</sub>PO<sub>4</sub>·7H<sub>2</sub>O 0.8, and NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 0.2 dissolved in distilled water. A 10% hypertonic saline solution was produced by dissolving 100 g/L in distilled water. Formalin was diluted to the appropriate concentration using normal saline. R-isovaline, S-isovaline, ACBC, and R- $\alpha$ -methylproline were dissolved in normal saline for intraperitoneal and intraplantar injections and in aCSF for intrathecal administration. Drug doses tested were based on previous studies that demonstrated the chosen dose was effective.

### **Data analysis**

GraphPad Prism 6 (GraphPad, CA, USA) was used for statistical analyses. Nocifensive behaviour were defined as licking and biting of the affected paw. Student's unpaired *t*-test was used to analyze effects of intraperitoneal and intraplantar administration of ACBC with its respective controls. One-way analysis of variance (ANOVA) followed by *post-hoc* Dunnett's multiple comparisons test was used to compare treatment groups for the total time exhibiting nocifensive behaviour over the 30 minutes period after the injection of hypertonic saline. Data obtained following systemic administration of ACBC were fitted using a log[Inhibitor] vs response four parameter model. Data from the ramped hot plate assay were fitted using a log[Agonist] vs response four parameter model. Withdrawal latency data from the radiant heat tail flick assay were analyzed using a paired *t*-test. Grubb's outlier test was used to detect the presence of outliers which were removed prior to statistical analysis. Sample sizes were based on pilot experiments to establish *n*-values necessary for detecting a significant difference. Data for time exhibiting nocifensive behaviour exhibited over the 30 minute period are presented as mean with 95% confidence interval (95% CI) and hypertonic saline time course data are presented as mean  $\pm$  SEM. A *P* < 0.05 was taken to be statistically significant.

## Results

### **R- and S-isovaline do not reduce nocifensive behaviour in the hypertonic saline assay**

R-isovaline administered intraperitoneally did not reduce the amount of time animals exhibited nocifensive behaviour elicited by an intraplantar injection of hypertonic saline. Figure 10a shows the time course for nocifensive behaviour for R-isovaline administered at 1000 mg/kg. The total time spent exhibiting nocifensive behaviour over the 30 minute period for both groups are shown in Figure 10b. No significant difference was found between saline (87 s [95% CI, 54, 121]; n = 10) and R-isovaline (107 s [95% CI, 75, 140]; n = 10). Following intraplantar administration of 14  $\mu$ mol R-isovaline, the time course for the duration animals exhibited nocifensive behaviour is shown in Figure 10c. No significant difference was found between animals receiving an intraplantar injection of saline (96 s [95% CI, 69, 124]; n = 10) and R-isovaline (106 s [95% CI, 64, 147]; n = 10) (Fig. 10d).

S-isovaline administered intraperitoneally did not reduce the amount of time animals exhibited nocifensive behaviour induced by hypertonic saline. Figure 11a shows the time course for nocifensive behaviour for S-isovaline administered at 1000 mg/kg. The total time spent exhibiting nocifensive behaviour over the 30 minute period for both groups are shown in Figure 11b. No significant difference was found between animals treated with saline (305 s [95% CI, 238, 372]); n = 10). The time course for the length of time animals exhibited nocifensive behaviour following intraplantar administration of S-isovaline is shown in Figure 11c. No significant difference was found between animals receiving an intraplantar injection of saline (175 s [95% CI, 114, 236]; n = 10) and 14  $\mu$ mol S-isovaline (227 s [95% CI, 131, 324]; n = 10) (Fig. 11d).

### **Systemic, but not localized, administration of ACBC reduces nocifensive behaviour in the hypertonic saline assay**

ACBC administered intraperitoneally reduced the amount of time that animals exhibited nocifensive behaviour following the injection of hypertonic saline. Figure 12a shows the time course for

nocifensive behaviour for ACBC administered intraperitoneally at 0, 250, 500, 1000, and 2000 mg/kg with data for the four groups receiving vehicle pooled. The total time spent exhibiting nocifensive behaviour over the 30 minute period for each treatment group was normalized to their respective controls and presented as percent of control. The pooled normalized control value was 96% (95% CI, 85, 108; n = 39 with one outlier excluded). ACBC produced a dose-dependent reduction in the time animals exhibited nocifensive behaviour (Fig. 12b) with an  $IC_{50}$  of 441 mg/kg (95% CI, very wide) and calculated maximum reduction of 49.8% (95% CI, -69.3, 168.9).

ACBC administered intraplantarly did not reduce the amount of time animals exhibited nocifensive behaviour elicited by hypertonic saline. Figure 12c shows the time course for time exhibiting nocifensive behaviour for ACBC administered at 14 and 23  $\mu$ mol. Data for groups receiving 0  $\mu$ mol were pooled together. The total time exhibiting nocifensive behaviour over the 30 minute period for each treatment was normalized to their respective controls and presented as percent control. ACBC did not affect the time exhibiting nocifensive behaviour (Fig. 12d). Animals administered 14  $\mu$ mol and 23  $\mu$ mol exhibited 84% (95% CI, 52, 116; n = 10) and 108% (95% CI, 80, 136; n = 10) of responses compared to control, respectively.

### **Comparison of two methods for administering intrathecal injections**

Administration of 200  $\mu$ M strychnine into the intrathecal space following the procedure described by Wang (2008) or Njoo et al. (2014) both produced bilateral dynamic allodynia in 7 of 8 animals. Animals treated with aCSF vehicle did not exhibit any signs of dynamic allodynia (Table 2). These observations suggest there are no observable differences between the intrathecal injection protocols. All subsequent intrathecal injections in these studies were performed according to the protocol described by Njoo et al. (2014).

### **Validation of intrathecal injection technique**

Intrathecal administration of 0.5% bupivacaine reduced the amount of time in phase I that the animals exhibited nocifensive behaviour induced by formalin compared to aCSF control (bupivacaine: 5 s [95% CI, -4, 14] vs aCSF: 81 s [95% CI, 44, 117];  $P < 0.001$ ;  $n = 8$  per group) (Fig. 13a). Bupivacaine did not produce a statistically significant difference in phase II of the formalin foot assay (bupivacaine: 82 s [95% CI, 12, 152] vs aCSF: 41 s [95% CI, -9, 91];  $n = 8$ ) (Fig. 13b). Intrathecal administration of 5 mM morphine reduced nocifensive behaviour in both phase I (morphine: 14 s [95% CI, 7, 21] vs aCSF: 73 s [95% CI, 55, 91];  $P < 0.0001$ ;  $n = 10$ ) (Fig. 13c) and phase II (morphine: 2 s [95% CI, -2, 5] vs aCSF: 101 s [95% CI, 50, 152];  $P < 0.001$ ;  $n = 10$ ) (Fig. 13d). These data indicate that our laboratory is capable of detecting analgesia produced by intrathecally administered drugs.

### **Intrathecal R- and S-isovaline do not produce antinociceptive effects in the formalin foot test**

Intrathecal administration of 500 mM R-isovaline did not reduce the amount of time animals exhibited nocifensive behaviour elicited by intraplantar formalin. No significant differences were detected in phase I (Fig. 5a) between vehicle (81 s [95% CI, 38, 124];  $n = 8$ ) and R-isovaline (57 s [95% CI, 42, 73];  $n = 8$ ) or in phase II (Fig. 14b) between vehicle (86 s [95% CI, -13, 185];  $n = 8$ ) and R-isovaline (137 s [95% CI, 62, 211];  $n = 8$ ).

Intrathecal administration of 500 mM S-isovaline did not reduce the time animals exhibited nocifensive behaviour in phase I of the formalin foot test. No significant differences were detected in phase I (Fig. 14c) between vehicle (73 s [95% CI, 55, 91];  $n = 10$ ) and S-isovaline (89 s [95% CI, 71, 107];  $n = 10$ ). Interestingly, intrathecal administration of S-isovaline increased the time animals exhibited nocifensive behaviour in phase II (Fig. 14d) (vehicle: [101 s {95% CI, 50, 152};  $n = 10$ ] vs S-isovaline [226 s {95% CI, 139, 312};  $n = 10$ ];  $P < 0.05$ ) suggesting S-isovaline produces a nociceptive effect.

### **Intrathecal ACBC produces antinociception in the formalin foot assay and hypertonic saline assay**

Lumbar intrathecal ACBC at 500 mM in a volume of 5  $\mu$ l produced significant reductions in nocifensive behaviour elicited by 5% formalin in both phase I (control, 87 s [95% CI, 62, 112; n = 8]; 500 mM ACBC: 1.4 s [95% CI, -2.1, 4.9; n = 7 with one outlier excluded];  $P < 0.0001$ ) and phase II (control, 93 s [95% CI, 45, 107; n = 8]; 500 mM ACBC, 28 s [95% CI, -15, 72; n = 7 with one outlier excluded];  $P < 0.05$ ). However, 5  $\mu$ l 250 mM ACBC did not affect either phase I (control, 87 s [95% CI, 62, 112; n = 8]; 250 mM ACBC, 57 s [95% CI, 23, 91; n = 8]) or phase II (control, 93 s [95% CI, 45, 107; n = 8]; 250 mM ACBC, 88 s [95% CI, 24, 152; n = 8]) (Fig. 15a, b).

Lumbar intrathecal administration of ACBC dose-dependently reduced the amount of time that the animals exhibited nocifensive behaviour following injection of hypertonic saline into the hindpaw. Figure 15c shows the time course of nocifensive behaviour in animals administered 5  $\mu$ l of 0, 250, or 500 mM ACBC. The total time spent exhibiting nocifensive behaviour over the 30 minute period was summed and shown in Figure 15d. Both ACBC doses markedly reduced the times that animals exhibited nocifensive behaviour: control group had a mean of 62 s (95% CI, 49, 75; n = 10) and animals treated with 250 mM having a mean of 34 s (95% CI, 7, 61; n = 9 with one outlier excluded;  $P < 0.05$ ); 500 mM ACBC having a mean of 14 s (95% CI, 8, 20; n = 10;  $P < 0.0001$ ).

### **Intrathecal ACBC produces reversible dermatomal antinociception in the hypertonic saline assay**

To investigate the reversibility of ACBC's antinociceptive effects, we tested separate groups of animals in the hypertonic saline assay at 30 minutes rather than 15 minutes after intrathecal injections. Time courses of nocifensive behaviour for these groups are shown in Figure 16a. The time spent exhibiting nocifensive behaviour after the 30 minute test period was summed and shown in Figure 16b. No significant differences were detected between the control group (70 s [95% CI, 38, 101], n = 10) and animals treated with either 250 mM (36 s [95% CI, 25, 48], n = 9 with one outlier excluded) or 500 mM

ACBC (51 s [95% CI, 14, 87],  $n = 10$ ) indicating that the antinociceptive effects observed following lumbar intrathecal administration are due to the effects of ACBC.

Lumbar intrathecal ACBC did not affect nocifensive behaviour induced by hypertonic saline injected into the forepaw, i.e., rostral to the lumbar dermatomal distribution. Time courses of the forepaw nocifensive responses are shown in Figure 17a. The total time exhibiting nocifensive behaviour over the test period was totalled for each treatment group and shown in Figure 17b. No significant differences were detected between the control group (248 s [95% CI, 139, 357],  $n = 10$ ) and animals treated with either 250 mM (297 s [95% CI, 183, 411],  $n = 10$ ) or 500 mM (286 s [95% CI, 187, 384],  $n = 10$ ).

### **Intrathecal ACBC produces differing effects in two assays of thermal nociception**

Intrathecal ACBC produced a dose-dependent increase in withdrawal latencies in the ramped hot plate assay. The doses of ACBC tested were 0 ( $n = 8$ ), 62.5 ( $n = 7$  with 1 outlier removed), 125 ( $n = 7$  with 1 outlier removed), 250 ( $n = 7$  with 1 outlier removed), and 500 mM ( $n = 8$ ). Intrathecal ACBC increased withdrawal latency with an  $EC_{50}$  of 278 mM (95% CI, very wide) and Hill slope of 24 (95% CI, very wide) (Fig. 18a).

At a dose of 250 mM, intrathecal ACBC did not affect withdrawal latencies in the radiant heat tail flick assay. Compared with a pre-treatment withdrawal latency of 1.7 s (95% CI, 1.3, 2.2), animals treated with 250 mM ACBC intrathecally exhibited a withdrawal latency of 1.9 s (95% CI, 1.3, 2.4) ( $n = 5$ ) (Fig. 19b). No statistically significant difference was detected between pre-treatment and post-treatment values. Animals treated with vehicle exhibited no differences in withdrawal latency comparing pre-treatment (1.6 s [95% CI, 0.7, 2.5]) and post-treatment values (3.6 s [95% CI, 0, 7.3];  $n = 4$ ) (Fig. 18b).

### **The effects of intrathecal ACBC are not antagonized by naloxone and does not produce respiratory depression**

The administration of 2 mg/kg naloxone subcutaneously 10 minutes prior to the administration of 500 mM ACBC into the lumbar intrathecal space did not alter the time animals exhibited nocifensive responses in the hypertonic saline assay. Time courses of the hindpaw nocifensive responses are shown in Figure 19a. The total time exhibiting nocifensive behaviour over the test period was totalled for each treatment group and shown in Figure 19b. No significant differences were detected between vehicle (69 s [95% CI, -7, 146], n = 8) and animals pre-treated with 2 mg/kg naloxone (41 s [95% CI, -35, 117], n = 6 with 2 animals excluded). Animals administered 500 mM ACBC into the lumbar intrathecal space did not exhibit lower respiratory rates compared to vehicle control (500 mM ACBC, 208 min<sup>-1</sup> [95% CI, 156, 261; n = 7 with one outlier excluded]; vehicle, 228 min<sup>-1</sup> [95% CI, 199, 256; n = 8]) (Fig. 189).

### **Intrathecal ACBC produces mild motor blockade**

Lumbar intrathecal ACBC did not produce any evidence of motor blockade as demonstrated by the ability of the mice to rear up and move normally. The behaviour were assessed by a blinded observer. This was in marked contrast of the inability to rear or move normally after 5 µl of 70 mM lidocaine (2%) (Lim et al., 2007). No motor deficits were detected for any of the animals tested in the hypertonic saline assay at 0, 250, or 500 mM ACBC. In the formalin foot test, no motor deficits were observed for animals treated with 0 or 250 mM ACBC, but 3 out of 10 animals treated with 500 mM ACBC were rated as having moderate motor deficits (Table 3).

### **Intrathecal R-α-methylproline does not produce antinociception, but produces behavioural abnormalities**

Lumbar intrathecal injection of R-α-methylproline at both the 1.25 and 2.5 µmol concentrations produced abnormal behavioural effects in 6 of 8 animals. The effects commenced shortly after intrathecal administration and terminated within 15 minutes after the injection. Animals were conscious during this time and capable of spontaneous breathing. Figure 20 shows an example of the characteristic

appearance of an animal after intrathecal administration of R- $\alpha$ -methylproline. The observed behaviour included hindlimb and forelimb tonic flexion, licking and biting of the injection site, flexion of hindlimbs when walking, circling behaviour, absence of a righting reflex, and supine writhing against the cage floor. In contrast, 116  $\mu$ mol R- $\alpha$ -methylproline administered intraperitoneally did not produce any behavioural abnormalities (cf. Table 4).

The behavioural effects observed on intrathecal injection were reversible and not associated with antinociception in the hypertonic saline assay. Table 4 shows that animals administered R- $\alpha$ -methylproline at 1.25 or 2.5  $\mu$ mol did not show differences in the time of observed nocifensive behaviour induced by hypertonic saline (70 s [95% CI, 40, 100;  $n = 8$ ] and 84 s [95% CI, 21, 146;  $n = 8$ ]) compared to control (70 s [95% CI, 40, 100;  $n = 7$  with one outlier excluded]).

## Discussion

In this study, we investigated the antinociceptive effects of the experimental analgesics, R-isovaline, S-isovaline, ACBC, and R- $\alpha$ -methylproline administered by different routes in murine pain models. R-isovaline and S-isovaline did not produce antinociceptive effects in the hypertonic saline assay following systemic or local administration. Likewise, lumbar intrathecal administration of R-isovaline and S-isovaline did not produce antinociceptive effects in the formalin foot assay. Intraperitoneal administration of ACBC dose-dependently reduced nocifensive responses in the hypertonic saline assay, whereas no effects were observed following its intraplantar administration. Lumbar intrathecal ACBC also reduced nociceptive responses in both phase I and phase II of the formalin foot test. Lumbar intrathecal administration of ACBC produced a robust reduction in nocifensive responses to intraplantar hypertonic saline injection. The antinociceptive effects, which lasted less than 30 minutes were restricted to the dermatomal distribution corresponding to the lumbar intrathecal route of administration and hence likely mediated by mechanisms localized to the central neuraxial site. Intrathecal ACBC did not affect tail withdrawal responses induced by a thermal stimulus in the radiant heat tail flick assay, but produced antinociception in the ramped hot plate. The observed analgesic effects were not due to an ACBC-induced motor blockade since the animals displayed normal rearing and movement. The antinociceptive effects of ACBC are not mediated by  $\mu$ -opioid receptors as naloxone did not affect responses in the hypertonic saline assay and did not produce respiratory depression. These findings suggest that ACBC, but not R-isovaline, S-isovaline, and R- $\alpha$ -methylproline, has the potential to be a non-opioid analgesic that acts via a central neuraxial mechanism to produce antinociception.

R-isovaline and S-isovaline were demonstrated in previous studies to produce antinociceptive effects following systemic administration in the formalin foot assay (Fung et al., 2017). However, the

antinociception did not extend to the hypertonic saline assay which is another assay employing a chemical stimulus. Intraperitoneal and intraplantar administration of R-isovaline and S-isovaline did not produce antinociceptive effects in the hypertonic saline assay. This suggests that these compounds may be acting on mechanisms that are specific to the formalin foot assay and absent following the administration of hypertonic saline. The formalin foot assay produces inflammatory responses and tissue damage while intraplantar administration of hypertonic saline is devoid of such effects (Asiri et al., 2017). This is further supported by the report that isovaline produces anti-allodynic effects when co-administered locally with the inflammatory mediator, PGE<sub>2</sub>, suggesting that isovaline may be acting on an inflammatory mechanism (Whitehead et al., 2012). This is consistent with a lack of effect in the formalin foot assay observed in this study following intrathecal administration. R-isovaline and S-isovaline would be unable to reach the site of action in formalin-mediated inflammation in sufficient concentrations to ameliorate the inflammatory effects. Interestingly, these findings contradict MacLeod et al. (2010) that reported lumbar intrathecal administration of R-isovaline and S-isovaline reduced nocifensive responses in phase I and II of the formalin foot assay (MacLeod et al., 2010). Further studies are required to better understand the therapeutic potential of isovaline for producing spinally-mediated antinociception.

Intraperitoneal administration of ACBC decreased nocifensive responses in the hypertonic saline assay which are consistent with the observed analgesic effects in the chemical assay using formalin (Chung, 2009; Fung et al., 2017). In the present study, the effects were not attributable to peripheral actions because intraplantar pre-treatment of ACBC, even at maximum soluble doses, did not produce detectable analgesia in the same hindpaw receiving hypertonic saline. The lack of activity following intraplantar, but not intrathecal administration, suggests that ACBC is unlikely to be producing antinociception via a nerve block. Although a previous study demonstrated that intraplantar ACBC reversed mechanical allodynia induced by locally administered glutamate (Chung, 2009), the difference

in results may be attributed to differences in the assay used and mechanism of algescic responses. In the present study, the lack of response is not likely due to an insufficient amount of ACBC at the area of stimulus since ACBC does not apparently act through a peripheral mechanism to produce its analgesic effects. In support of this, the antinociceptive effects of ACBC are not as robust after intraperitoneal administration compared to the antinociception after intrathecal administration. Intrathecally applied ACBC resulted in a fast onset within 5 minutes and produced a greater reduction in nociceptive responses (74%) than systemically administered ACBC (23%). These findings suggest that systemically administered ACBC needs to cross the blood-brain barrier into the central nervous system for producing analgesia whereas intrathecally applied ACBC results in a faster onset of analgesia.

When administered into the lumbar intrathecal space, ACBC greatly reduced nocifensive behaviour when tested at 15 minutes post-injection. However, the responses for treated animals returned to control values when tested 30 minutes after injection. This suggests that the analgesic effects of intrathecal ACBC are of short duration. A previous study found that following intravenous injection, ACBC had a half-life of four minutes in mouse brain (Rao et al., 1990). Our finding that intrathecal ACBC has a short duration of action is consistent with a rapid inactivation or removal from the central nervous system. The short duration of intrathecal ACBC could account for the observations made in phase II of the formalin foot test. ACBC produced a robust decrease in phase I, but the analgesia appeared to diminish in phase II. The occurrence of phase II is conventionally thought to occur 20 minutes after the injection of formalin (Tjølsen et al., 1992) which would be well after the peak effect of ACBC, thus, resulting in the reduced analgesic effects observed in phase II. Comparatively, the analgesic action of intrathecal lidocaine in mice lasts approximately 10 minutes compared to over 100 minutes in humans indicating that clinical use may be equivalent to 10 times the duration in mice (Imbelloni, 2002; Schwarz et al., 2010).

The analgesic response to intrathecal ACBC depended on the site of hypertonic saline injection. Intrathecal ACBC did not decrease nocifensive responses induced by hypertonic saline injected into the fore paw of mice. This absence of activity suggests that ACBC is unlikely to diffuse to contiguous spinal segments or to be acting supraspinally to produce analgesia. Previous studies have found that following intrathecal injection, the concentration of administered compounds rapidly decreases with increasing distance from the site of injection (Hylden & Wilcox, 1980; Kroin et al., 1993; Ummenhofer et al., 2000). Consistent with these studies, ACBC appears to be localized to the dermatomal distribution of the site of injection and does not distribute rostrally in effective amounts. Hence, the analgesic effects observed after lumbar intrathecal injection of ACBC are likely mediated by mechanisms in the lumbar region.

While direct application of ACBC to the central nervous system produced consistent analgesia in chemically-based pain assays, it produced differing effects in the two models of thermal nociception used in this study: the ramped hot plate assay and the radiant heat tail flick assay. The effects observed with the ramped hot plate are consistent with previous observations that demonstrated ACBC was analgesic in the hot plate assay (Chung, 2009). However, the efficacy of ACBC in the ramped hot plate assay remains unclear as the plateau for maximal effect has not been shown using the dose range selected in this study. Interestingly, Chung (2009) noted that ACBC produced a bell-shaped dose response curve in the hot plate assay where doses in the extremities did not produce analgesia, but the middle doses did. This is in contrast with the findings in this work where the highest dose of ACBC administered was effective, but the lower doses were not. The observed difference may be due to differences in the route of administration. Chung (2009) administered ACBC via a subcutaneous route while ACBC was administered intrathecally in these experiments. In the radiant heat tail flick assay, intrathecal ACBC did not alter the tail withdrawal latency. However, it is quite possible that the administered dose of ACBC was too low to produce a detectable effect in the tail flick assay. Further studies are required to elucidate the effects of ACBC in assays utilizing thermal stimuli.

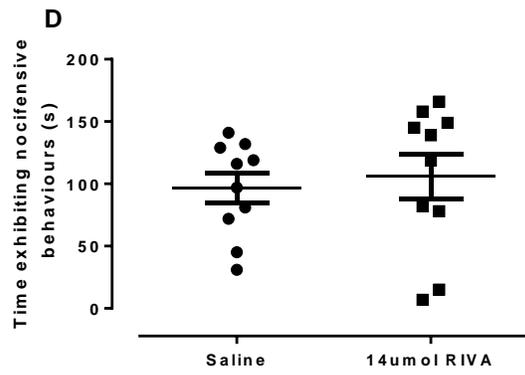
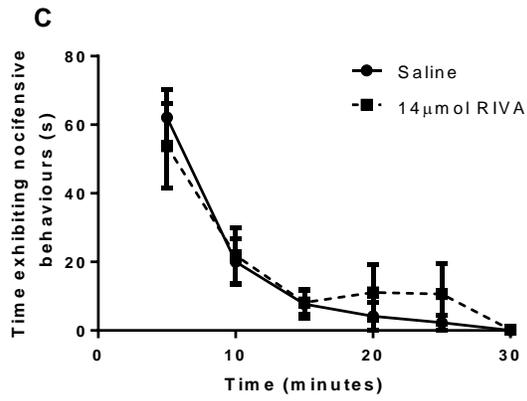
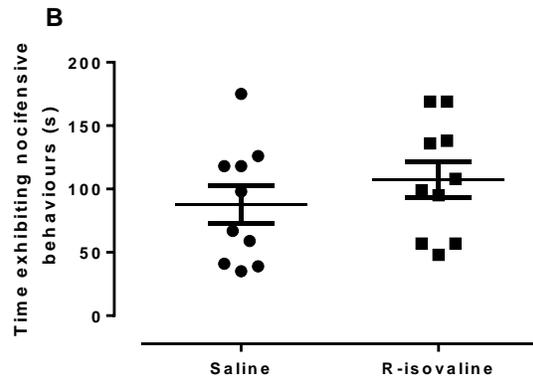
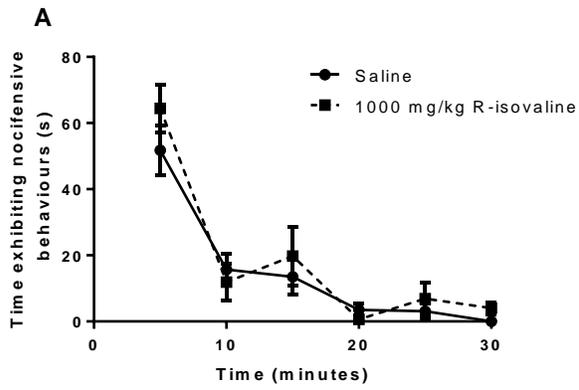
Direct application of ACBC to the central nervous system produced analgesia in chemically-based pain assays, however, it did not affect responses induced by a thermal stimulus. In the radiant heat tail flick assay, intrathecal ACBC did not alter the tail withdrawal latency. This finding is in contrast to previous observations that demonstrated ACBC was analgesic in the hot plate assay (Chung, 2009), another thermally-based assay. The observed difference in analgesia may be due to the nature of the two assays. The radiant heat tail flick assay is considered to elicit a spinally-mediated reflex response while the response on the hot plate is thought to be mediated supraspinally suggesting ACBC may not be acting on this reflex (Gardmark et al., 1998). An interesting observation by Chung (2009) noted that ACBC produced a bell-shaped dose response curve in the hot plate assay where doses in the extremities did not produce analgesia, but the middle doses did (Chung, 2009). It is possible that the doses tested in this study may have been outside the effective dose range, but highest dose tested here was effective while the lower doses were not.

Although the analgesic effects of ACBC were demonstrated, the mechanisms mediating these effects are not clear. Pre-administration of the opioid antagonist, naloxone, did not block the antinociceptive effects of lumbar intrathecal ACBC in the hypertonic saline assay. The dose of naloxone administered blocked the antinociception of an effective dose of morphine suggesting that ACBC is not a competitive agonist at  $\mu$ -opioid receptors (Hylden & Wilcox, 1980). Consistent with this, agonists at  $\mu$ -opioid receptors reduce respiratory rate (Stott & Pleuvry, 1991), however, ACBC did not decrease the respiratory rate of mice at antinociceptive doses. ACBC has been shown to reduce NMDA receptor activation *in vitro* (Hood et al., 1989; Inanobe et al., 2005; Watson et al., 1989), but the contribution of NMDA receptors to the effects of ACBC *in vivo* was not investigated in this study. Interestingly, compounds that inhibit NMDA receptor activation produce ataxia in animal models (Boyce & Rupniak, 2002). Ataxia did not occur in most animals at the highest dose of ACBC administered systemically or intrathecally, although 3 of 20 animals exhibited transient ataxia which abated by the start of testing.

Lower systemic doses of ACBC systemically produces analgesia without concurrent ataxic effects. These observations raise the possibility that ACBC may be acting through NMDA receptors to produce analgesia *in vivo*, but further studies are required to better understand the role of NMDA receptors in mediating the analgesic effects of ACBC.

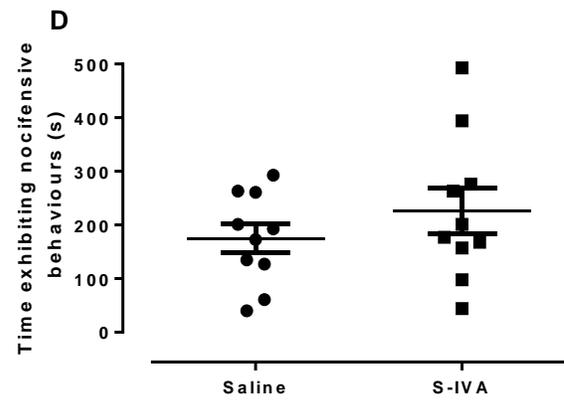
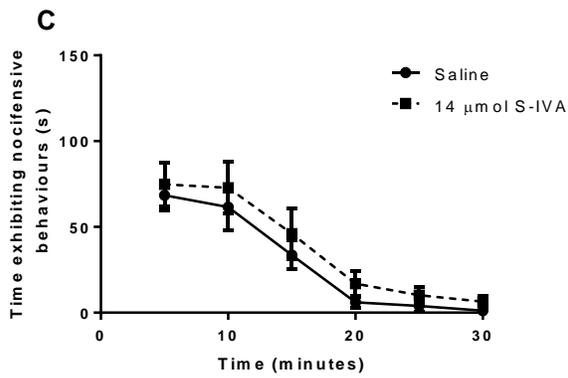
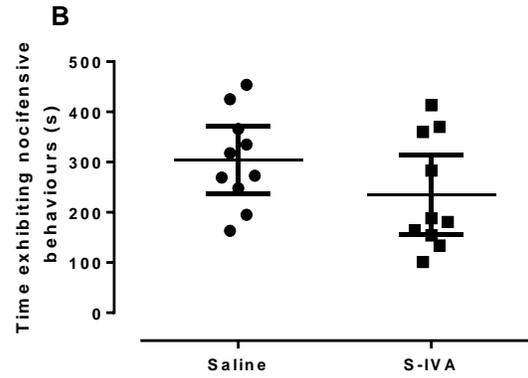
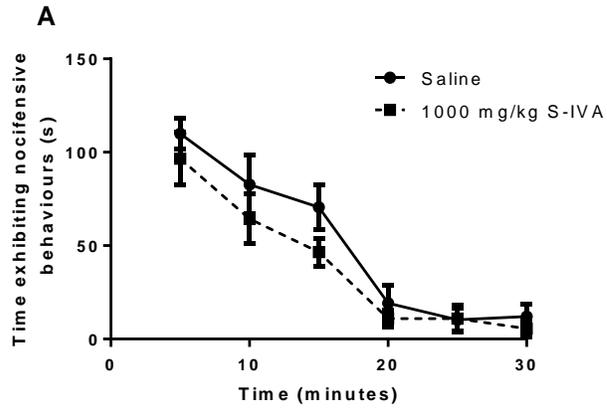
We report that intrathecal R- $\alpha$ -methylproline produced behavioural abnormalities that were not observed following intraperitoneal injection. Of note, the doses administered intraperitoneally were nearly an order of magnitude higher than those administered intrathecally, but were still not associated with behavioural abnormalities. This suggests that systemically administered R- $\alpha$ -methylproline does not cross the blood-brain barrier in appreciable amounts and is mostly restricted to the periphery. Proline, a structurally similar amino acid, applied directly to the spinal cord has excitant actions through NMDA receptor activation (Ault et al., 1987). NMDA receptor activation elicits nocifensive behaviour which are similar to those in the present study (Sakurada et al., 1990). This suggests that the observed behavioural effects of R- $\alpha$ -methylproline may result from actions at NMDA receptors. Further study of R- $\alpha$ -methylproline that elucidates its mechanism of action may yield information useful for developing an analgesic or other therapeutic agent that is restricted to the periphery. In the interim, the present observations indicate that R- $\alpha$ -methylproline is unlikely to be suitable for central neuraxial administration.

In summary, the present studies demonstrate that lumbar intrathecal administration of the non-opioid, ACBC, produces reversible antinociceptive effects of short duration in mice without evidence of motor blockade. The robust effects following intrathecal, but not intraplantar administration, suggests that ACBC is acting via a central neuraxial mechanism rather than in the periphery. These findings support the utility of ACBC as a novel non-opioid neuraxial analgesic and provide evidence supporting its further exploration. Areas for future study include the use of ACBC as an alternative to opioid adjuvants to neuraxial local anesthetics to reduce adverse effects.



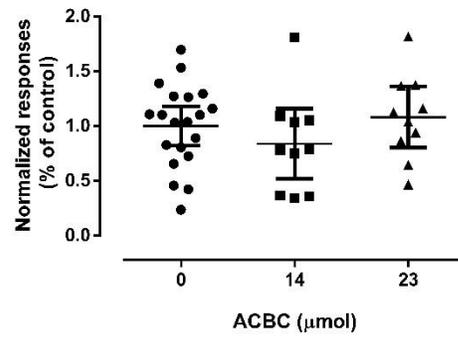
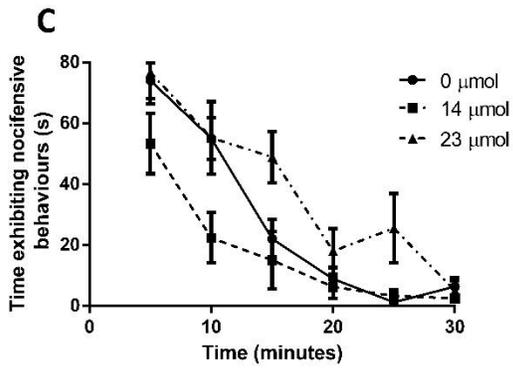
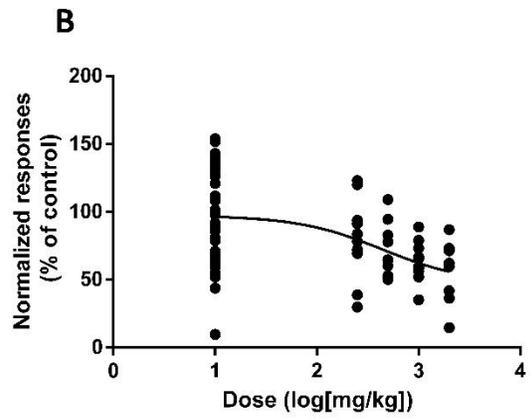
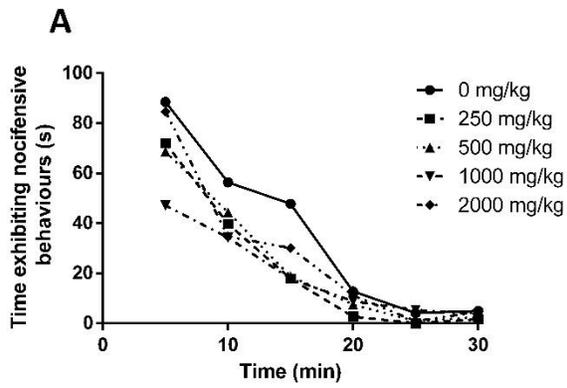
**Figure 10 – R-isovaline does not reduce nocifensive behaviour following systemic or local**

**administration.** A) Time course of observed nocifensive behaviour after the intraplantar injection of 10% hypertonic saline for animals injected intraperitoneally with 0 or 1000 mg/kg R-IVA;  $n = 10$  animals per group. Data is presented as mean  $\pm$  SEM. B) Total time of animals exhibiting nocifensive behaviour from 0 – 30 minutes after the injection of hypertonic saline. No significant difference was detected between animals administered vehicle or R-IVA. Each point represents an individual animal with summary bars presented as mean with 95% CI.  $n = 10$  per group. C) Time course of observed nocifensive behaviour elicited by 10% hypertonic saline after intraplantar injection of 0 or 14  $\mu$ mol R-IVA;  $n = 10$  per group. Data are presented as mean  $\pm$  SEM. D) Total time of animals exhibiting nocifensive behaviour from 0 – 30 minutes after the injection of hypertonic saline. No significant difference was detected between animals administered vehicle or R-IVA. Each point represents an individual animal with summary bars presented as mean with 95% CI.  $n = 10$ .



**Figure 11 – S-isovaline does not reduce nocifensive behaviour following systemic or local**

**administration.** A) Time course of observed nocifensive behaviour after the intraplantar injection of 10% hypertonic saline for animals injected intraperitoneally with 0 or 1000 mg/kg S-IVA;  $n = 10$  animals per group. Data is presented as mean  $\pm$  SEM. B) Total time of animals exhibiting nocifensive behaviour from 0 – 30 minutes after the injection of hypertonic saline. No significant difference was detected between animals administered vehicle or S-IVA. Each point represents an individual animal with summary bars presented as mean with 95% CI.  $n = 10$  per group. C) Time course of observed nocifensive behaviour elicited by 10% hypertonic saline after intraplantar injection of 0 or 14  $\mu$ mol S-IVA;  $n = 10$  per group. Data are presented as mean  $\pm$  SEM. D) Total time of animals exhibiting nocifensive behaviour from 0 – 30 minutes after the injection of hypertonic saline. No significant difference was detected between animals administered vehicle or S-IVA. Each point represents an individual animal with summary bars presented as mean with 95% CI.  $n = 10$ .

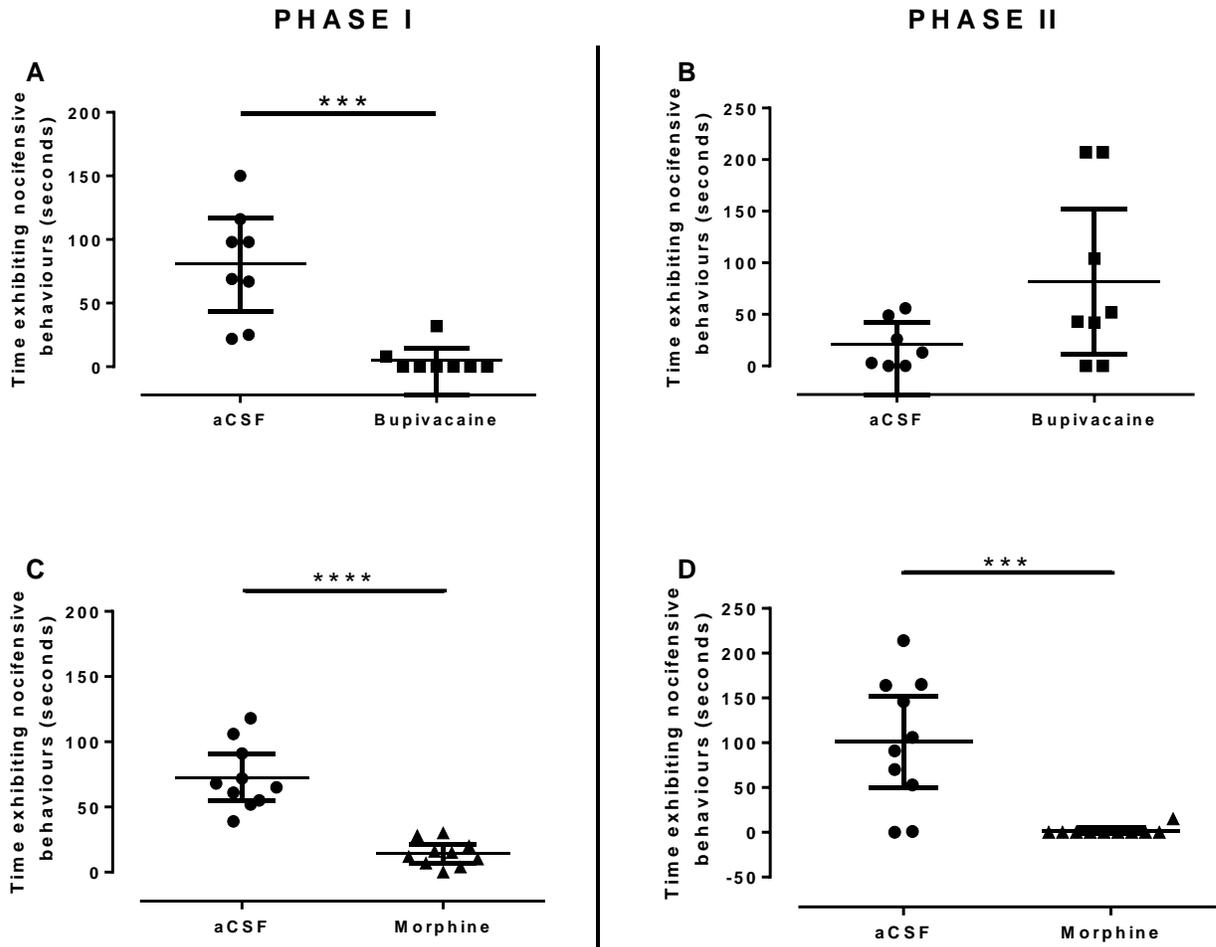


**Figure 12 – Systemic, but not local, ACBC reduces nocifensive behaviour in the hypertonic saline assay.**

A) Time course of observed nocifensive behaviour after the intraplantar injection of 10% hypertonic saline for animals injected intraperitoneally with 0, 250, 500, 1000, or 2000 mg/kg ACBC;  $n = 10$  animals per group. Animals receiving 0 mg/kg ACBC were grouped together for analysis with  $n = 40$ . Data is presented as mean. B) Total time of animals exhibiting nocifensive behaviour is expressed as percent of control from 0 – 30 minutes after the injection of hypertonic saline. Animals were normalized to their respective controls;  $n = 10$  animals per group with 0 mg/kg grouped together for analysis with  $n = 39$  (1 outlier excluded). C) Time course of observed nocifensive behaviour after the injection of 10% hypertonic saline for animals receiving 0, 14, or 23  $\mu\text{mol}$  ACBC intraplantarly.  $n = 10$  per group. Animals receiving 0  $\mu\text{mol}$  ACBC were grouped together for analysis with  $n = 20$ . Data are presented as mean  $\pm$  SEM. D) Intraplantar 14  $\mu\text{mol}$  ACBC did not reduce the time animals exhibited nocifensive behaviour in the hypertonic saline assay. Animals were normalized to their respective controls.  $n = 10$  per group with 0  $\mu\text{mol}$  grouped together for analysis with  $n = 20$ . Each point represents an individual animal with summary bars presented as mean with 95% CI.

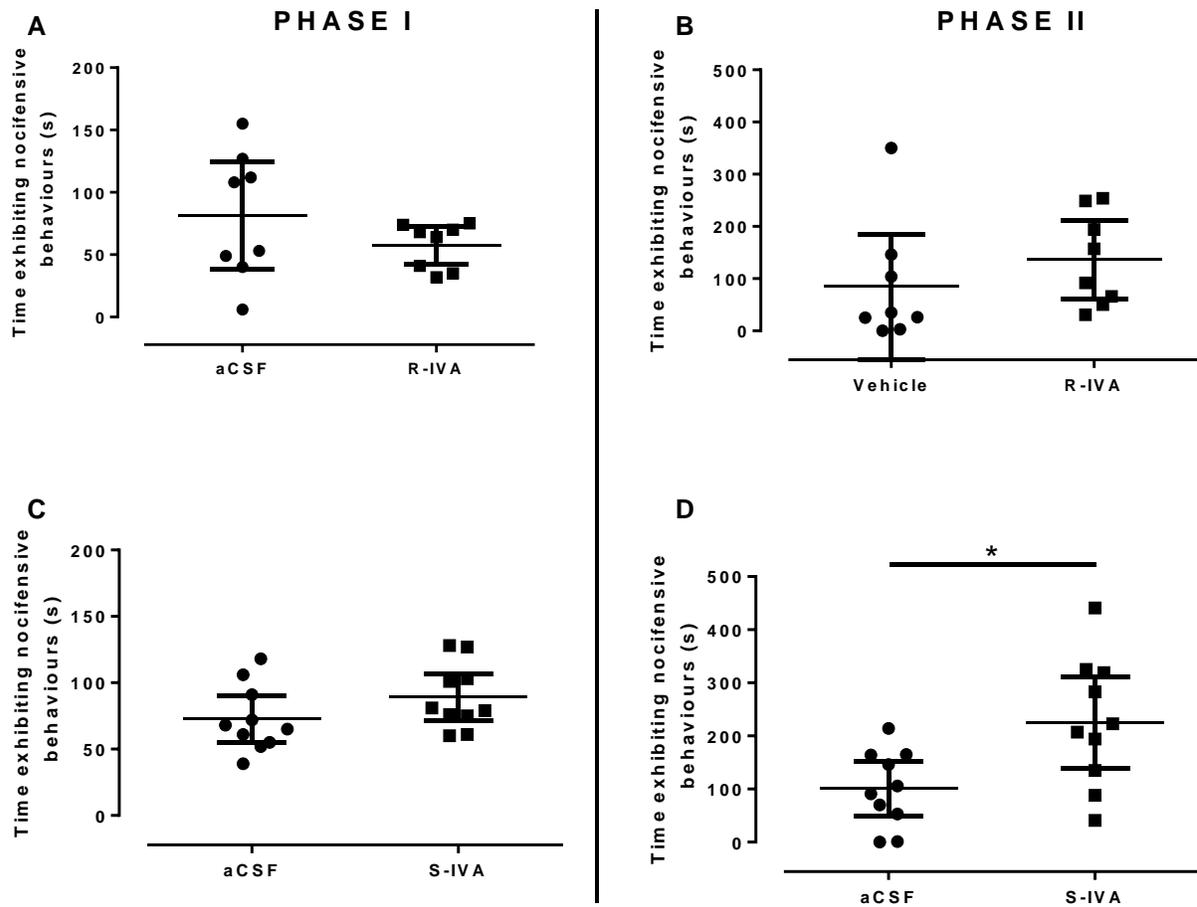
**Table 2 – Intrathecal strychnine produces dynamic allodynia regardless of injection method**

| <b>Method</b>                      | <b>Dynamic allodynia</b> |
|------------------------------------|--------------------------|
| Hylden and Wilcox (Vehicle) (1980) | 0/8                      |
| Hylden and Wilcox (1980)           | 7/8                      |
| Njoo et al (2014)                  | 7/8                      |



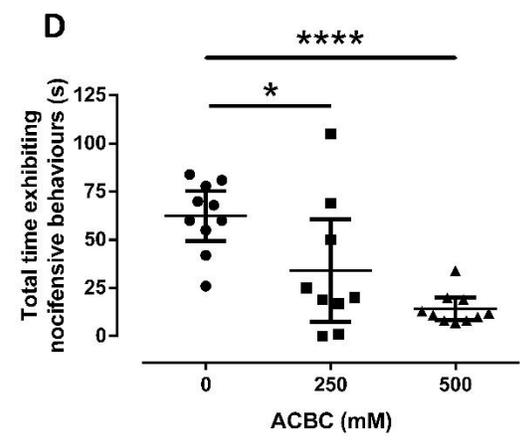
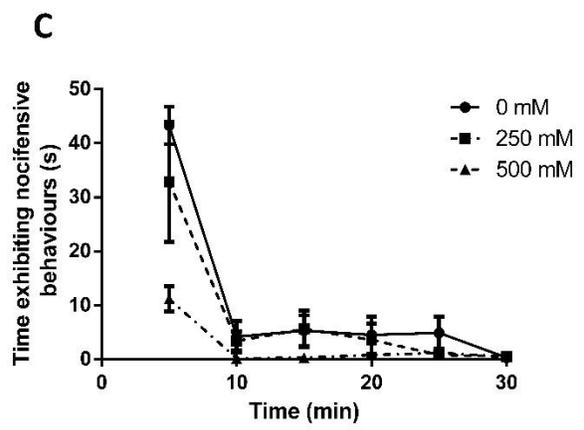
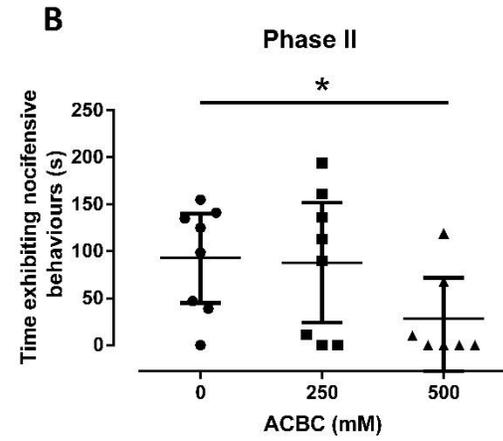
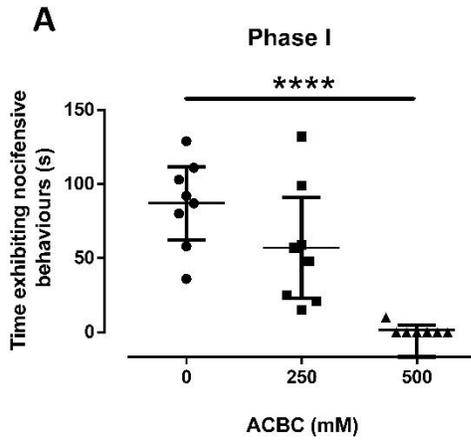
**Figure 13 – Validation of intrathecal injection technique using bupivacaine and morphine –**

Bupivacaine (0.5% in 5  $\mu$ l) reduced the total nocifensive behaviour in phase I (A), but not phase II (B) of the formalin foot assay (n = 8 per group). Morphine (5 mM) reduced the total nocifensive behaviour induced by formalin in both phase I (C) and phase II (D) of the assay (n = 10 per group). Each point represents an individual animal with summary bars representing mean with 95% CI. \* indicates  $P < 0.05$ , \*\*\* indicates  $P < 0.001$ , and \*\*\*\* indicates  $P < 0.0001$  compared to control.

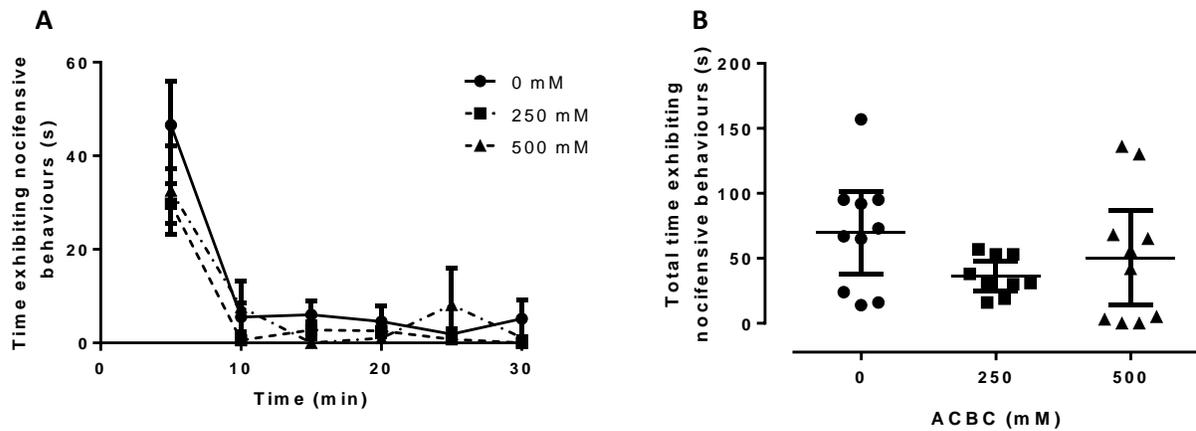


**Figure 14 – Intrathecal R- or S-isovaline does not produce antinociception in the formalin foot test.**

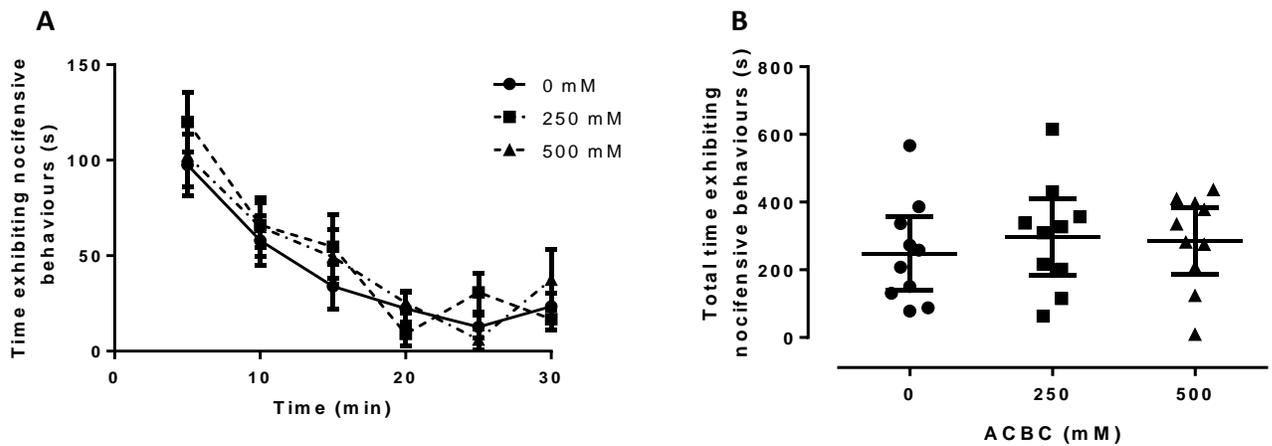
Intrathecal injection of R-IVA does not affect nocifensive behaviour in phase I (A) or phase II (B) of the formalin foot test. No significant difference was detected between aCSF and R-IVA in either phase. Each point represents an individual animal with summary bars presented as mean with 95% CI.  $n = 10$  per group. Intrathecal injection of S-IVA did not affect the time exhibiting nocifensive behaviour in phase I (C) of the formalin foot test. No significant differences were detected between aCSF and S-IVA in phase I. In phase II, S-IVA produced a significant increase in the time exhibiting nocifensive behaviour compared to animals treated with aCSF. Each point represents an individual animal with summary bars presented as mean with 95% CI.  $n = 10$ . \* indicates  $P < 0.05$ .



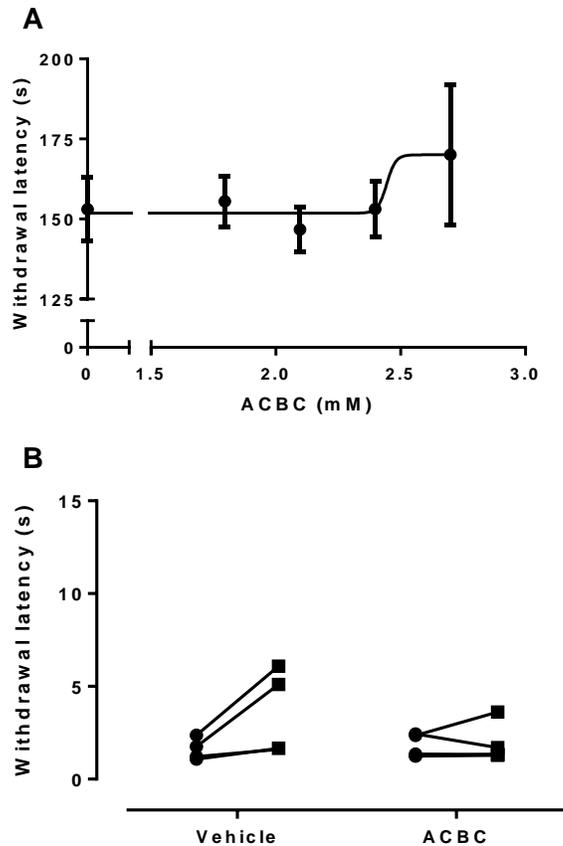
**Figure 15 – Intrathecal ACBC reduces nocifensive behaviour in the formalin foot assay and hypertonic saline assay.** ACBC administered intrathecally reduced the times of animals exhibiting nocifensive behaviour in both phase I (A) and II (B) of the formalin foot assay. Animals were treated with 0, 250, or 500 mM ACBC. Each point represents an individual animal with summary bars presented as mean with 95% CI;  $n = 8$  per group. C) Time course of observed nocifensive behaviour after the intraplantar injection of 10% hypertonic saline. Animals received 0, 250, or 500 mM ACBC.  $n = 10$  animals per group. Data are presented as mean  $\pm$  SEM. D) Intrathecal ACBC reduced the times of animals exhibiting nocifensive behaviour in a dose-dependent manner.  $n = 10$  per group. Each point represents an individual animal with summary bars presented as mean with 95% CI. \* indicates  $P < 0.05$ ; \*\*\*\* indicates  $P < 0.0001$ .



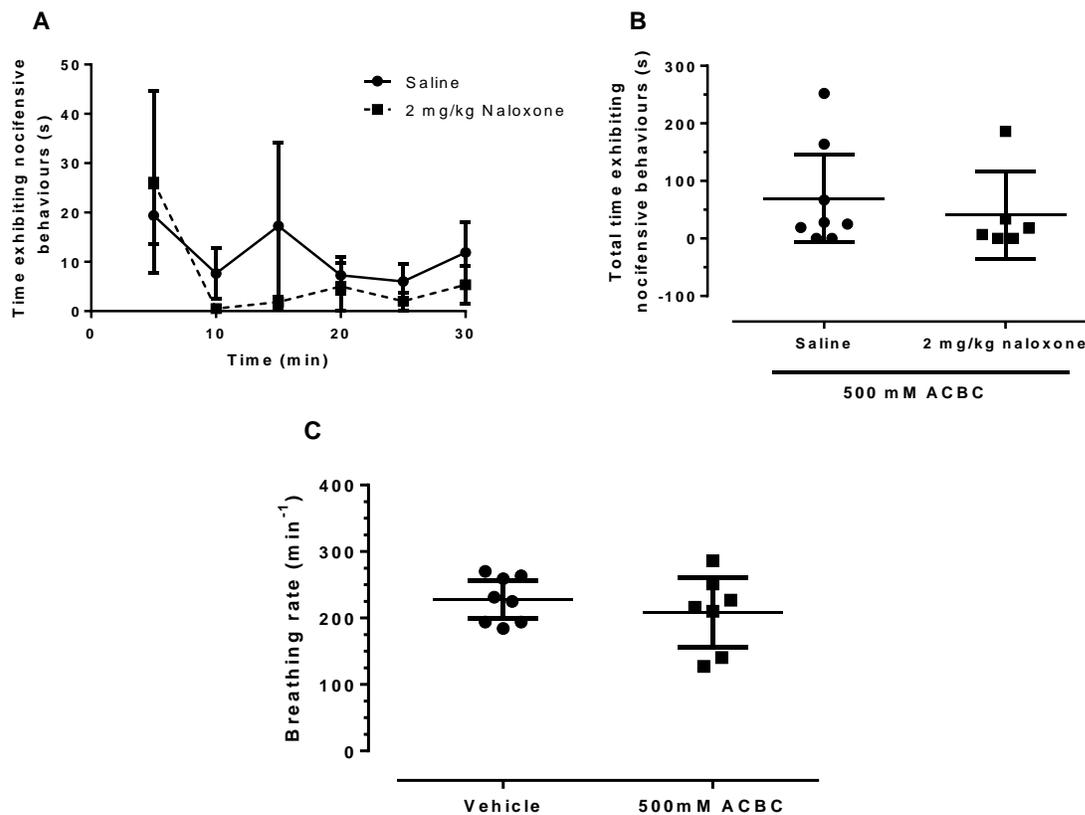
**Figure 16 – Intrathecal ACBC produces reversible antinociception.** A) Time course of observed nocifensive behaviour induced by hypertonic saline injection into the plantar surface of the right hindpaw 30 minutes after the intrathecal injection of 0, 250, or 500 mM ACBC.  $n = 10$  animals per group with 1 excluded from the 250 mM group. Data are presented as mean  $\pm$  SEM. B) Intrathecal ACBC did not reduce the times of animals exhibiting nocifensive behaviour 30 minutes after injection. Compared to the robust antinociception demonstrated in Fig 15, the lack of antinociception here demonstrated the reversibility of ACBC. No statistical differences were detected between the groups. Each point represents an individual animal with summary bars presented as mean with 95% CI.



**Figure 17 – Lumbar intrathecal administration of ACBC does not affect responses of the forepaws. A)** Time course of observed nocifensive behaviour induced by 10% hypertonic saline injected into the right forepaw. Animals received 0, 250, or 500 mM ACBC with  $n = 10$  animals per group. Data are presented as mean  $\pm$  SEM. **B)** Lumbar intrathecal ACBC did not reduce the times of animals exhibiting nocifensive behaviour when hypertonic saline was administered to the forepaw. No statistical differences were detected between the groups. Each point represents an individual animal with summary bars presented as mean with 95% CI.



**Figure 18 – Analgesic effects of ACBC increases withdrawal latency in the ramped hot plate, but not radiant heat tail flick assay.** A) ACBC administered at 0 ( $n = 8$ ), 62.5 ( $n = 7$  with 1 outlier removed), 125 ( $n = 7$  with 1 outlier removed), 250 ( $n = 7$  with 1 outlier removed), and 500 mM ( $n = 8$ ) intrathecally increased the withdrawal latency in the ramped hot plate assay. ACBC increased withdrawal latency with a calculated  $EC_{50}$  of 278 mM (95% CI, very wide) and Hill slope of 24 (95% CI, very wide). Data were fit using a  $\log[\text{Agonist}]$  vs response 4 parameter model and presented as mean with 95% CI. B) Intrathecal administration of 250 mM ACBC did not produce a statistically significant change in withdrawal latency. Each point represents the average of 3 trials for a given animal. (•) and (▪) represent pre- and post-treatment values, respectively.



**Figure 19 – ACBC is not antagonized by naloxone and does not produce respiratory depression – A)**

Time course of observed nocifensive behaviour induced by 10% hypertonic saline injected into the right hindpaw. Animals received either saline ( $n = 8$ ) or 2 mg/kg naloxone subcutaneous ( $n = 6$  with two animals excluded) prior to 500 mM ACBC administered intrathecally. Data are presented as mean  $\pm$  SEM.

B) Pre-administration of subcutaneous naloxone did not increase the times of animals exhibiting nocifensive behaviour when hypertonic saline was administered to the hindpaw. No statistical differences were detected between the groups. C) 500 mM ACBC administered intrathecally did not reduce respiratory rate compared to vehicle. Animals were injected with either vehicle ( $n = 8$ ) or 500 mM ACBC ( $n = 7$  with one outlier excluded). No statistical differences were detected between groups.

Each point represents an individual animal with summary bars presented as mean with 95% CI.

**Table 3 – Motor deficits following lumbar intrathecal ACBC in the hypertonic saline foot assay and formalin foot test**

**Hypertonic saline assay**

| <b>Dose (mM)</b> | <b>Normal</b> | <b>Moderate</b> | <b>Severe</b> |
|------------------|---------------|-----------------|---------------|
| 0                | 10            | 0               | 0             |
| 250              | 10            | 0               | 0             |
| 500              | 10            | 0               | 0             |

**Formalin foot assay**

| <b>Dose (mM)</b> | <b>Normal</b> | <b>Moderate</b> | <b>Severe</b> |
|------------------|---------------|-----------------|---------------|
| 0                | 10            | 0               | 0             |
| 250              | 10            | 0               | 0             |
| 500              | 7             | 3               | 0             |

**Table 4 – Antinociceptive activity in the hypertonic saline assay and behavioural abnormalities in animals after intraperitoneal and intrathecal administration of R- $\alpha$ -methylproline.**

| <b>Intraperitoneal Injection (<math>\mu</math>mol)</b> | <b>Time exhibiting nocifensive behaviour (s) [95% CI]</b> | <b>Observed Behavioural Abnormalities</b> |
|--|---|---|
| 0  |   | 0/6                                       |
| 116  |   | 0/6                                       |
| <b>Intrathecal Injection (<math>\mu</math>mol)</b>     |   |   |
| 0  | 70 (40, 100)  | 0/8                                       |
| 1.25   | 70 (24, 116)  | 6/8                                       |
| 2.5  | 84 (21, 146)  | 6/8                                       |



**Figure 20 – Characteristic appearance shortly after intrathecal injection of R- $\alpha$ -methylproline**

## Chapter 4 – Investigating the Role of NMDA and GABA<sub>B</sub> Receptors in Mediating the Analgesic Effects of 1-Amino-1-Cyclobutane Carboxylic Acid

### Introduction

1-Amino-1-cyclobutane carboxylic acid (ACBC) is an unnatural, cyclized amino acid with a chemical structure similar to the novel analgesic isovaline. ACBC was originally developed as a tumour imaging agent and formed the foundation for the development of other compounds that are currently used in clinic for tumour imaging (Hong et al., 2010; Washburn et al., 1979). Our laboratory identified ACBC as having analgesic effects following a structure-activity relationship study for the pain-relieving effects of isovaline (Fung et al., 2017). A recent *in vivo* investigation demonstrated that ACBC produced spinal analgesia in the hypertonic saline assay. *In vitro* studies of ACBC have found that it acts as a partial agonist at the glycine co-agonist site of NMDA receptors and inhibits the binding of glycine as well as the potentiation of NMDA receptor currents by glycine (Hood et al., 1989; Inanobe et al., 2005; Watson et al., 1989). Although the mechanism of action of ACBC *in vitro* have been investigated, it is unclear what mechanisms mediate its effects *in vivo*.

NMDA receptors play an important role in nociception where activation of the receptor facilitates nociceptive signaling and sensitization and conversely, antagonism produces antinociceptive effects (Chizh et al., 2001; Ma & Woolf, 2002; Vorobeychik et al., 2013). The contribution of NMDA receptors to the analgesic effects of ACBC will be investigated using 7-CK. 7-CK binds selectively to the glycine co-agonist site on NMDA receptors and non-competitively antagonizes NMDA-induced currents in rat cortical neurons (Kemp et al., 1988). Isolated tissue experiments using guinea pig ileum demonstrated 7-CK inhibits the response induced by NMDA and was reversed in the presence of D-serine, an NMDA glycine site agonist (Reggiani et al., 1989). In animal models, 7-CK reduces spinal

nociception following repeated electrical stimulation of C-fibres in anesthetized rats and reduces formalin-induced responses by antagonizing the glycine binding site on NMDA receptors (Chapman & Dickenson, 1995; Dickenson & Aydar, 1991). Because ACBC and 7-CK act at the glycine site to reduce NMDA receptor activation, it was assumed that both compounds acted through a similar mechanism to produce antinociceptive effects. It was hypothesized that a minimal analgesic dose of ACBC in the presence of 7-CK would produce greater analgesia than when administered alone.

Aside from NMDA receptors, another potential target for ACBC are GABA<sub>B</sub> receptors. GABA<sub>B</sub> receptors are distributed in both the peripheral and central nervous system and have been implicated in the modulation of nociceptive signaling (McCarson & Enna, 2006; Whitehead et al., 2012). Activation of GABA<sub>B</sub> receptors with agonists such as baclofen produces antinociception (Dirig & Yaksh, 1995; Froestl, 2010; Wilson & Yaksh, 1978). CGP 35348 antagonizes the antinociceptive effects of the specific GABA<sub>B</sub> agonist, baclofen, in several pain assays (Hammond, 1991; Malcangio et al., 1991; Thomas, 1996). In previous studies, isovaline, the prototype for the prior structure-activity relationship study, has been shown to act as an agonist at GABA<sub>B</sub> receptors in rat thalamocortical neurons (Cooke et al., 2012). These findings were paralleled in mouse studies that demonstrated the antinociceptive effects of isovaline were blocked by GABA<sub>B</sub> receptor antagonists and enhanced with positive allosteric modulators (Whitehead et al., 2012). Given the structural similarity of ACBC to isovaline, it would be prudent to explore the possibility of GABA<sub>B</sub> activation for the antinociceptive effects of ACBC. The selective GABA<sub>B</sub> antagonist, CGP 35348 (Olpe et al., 1990), will be used to investigate the possibility of agonism by ACBC at GABA<sub>B</sub> receptors.

In the present study, the hypertonic saline assay will be used to investigate the antinociceptive effects of ACBC. The hypertonic saline assay is able to rapidly and reliably assess the antinociception produced by tested compounds (Asiri et al., 2017). The objective of this study was to investigate the antinociceptive effects of ACBC in the presence of 7-CK and CGP 35348.

## Materials and methods

### Animals

Female CD-1 mice weighing 25 – 30g were housed in groups at 21°C and 55% relative humidity on a 12 h light-dark cycle with lights on at 07:00 AM. All experiments were performed between 08:00 AM and 06:00 PM. Food and water were available *ad libitum* when animals were not being tested. All procedures conducted in this study were approved by the Animal Care Committee at The University of British Columbia. All animals were tested only once.

### Experimental procedure

Animals were habituated in darkened Plexiglas chambers (diameter, 9 cm; height, 19 cm) on a clear glass pane for 2 h prior to testing on the day of experiments. Following habituation, animals were injected intrathecally with treatment or vehicle 15 minutes prior to the intraplantar injection of 10 µl 10% hypertonic saline using a 0.3 ml syringe with a ½" 30 G needle into the plantar surface of the right hindpaw. Drug doses selected were based on previous studies. Animals were then placed into their respective chambers and recorded with a video camera placed underneath the chambers. Animals were recorded for 30 min and the videos were analyzed in 5 min bins by a blinded observer who measured the length of time the animals exhibited nocifensive behaviour.

### Intrathecal administration

Intrathecal injections were made according to a modified protocol by Njoo et al. (2014). Briefly, animals were placed in an induction chamber and exposed to 2.2% isoflurane for 5 min before being transferred to a nose cone. The abdomen of the animal was placed over a rounded surface to induce a curvature of the spine. A small patch of fur was shaved over the site of injection and wiped with ethanol. Intrathecal injections were made using a 0.3 ml syringe with a ½" 30 G needle delivering a volume of 5 µl. With the iliac crest as a landmark, the spinous processes of L5 and L6 were identified and the needle was carefully inserted in between. Successful entry of the needle into the intrathecal space elicits a tail

flick. The needle would then be held in place and the solution was injected. The animal was returned to a cage and allowed to recover before testing.

### **Drugs and chemicals**

ACBC, CGP 35348, and salts used for producing artificial cerebrospinal fluid (aCSF) were purchased from Sigma-Aldrich Canada Co. (Oakville, Ontario, Canada). 7-CK was purchased from Ascent Scientific (Cambridge, Massachusetts, USA). Baclofen was purchased from Tocris Bioscience (Burlington, Ontario, Canada). ACSF contained in mM: NaCl 148, KCl 3, CaCl<sub>2</sub> 1.4, MgCl<sub>2</sub>·6H<sub>2</sub>O 0.8, Na<sub>2</sub>PO<sub>4</sub>·7H<sub>2</sub>O 0.8, and NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 0.2 dissolved in distilled water. 10% hypertonic saline solution was produced by dissolving 100 g/L NaCl in distilled water. All drugs were dissolved in aCSF.

### **Data analysis**

Data was analyzed using GraphPad Prism 6 (GraphPad, CA, USA). Effects of treatment on time exhibiting nocifensive behaviour of the 30 min test period were compared. Nocifensive behaviour was defined as licking and biting of the affected paw. One-way analysis of variance (ANOVA) followed by *post-hoc* Dunnett's multiple comparisons test or Student's *t*-test was used for statistical analysis where appropriate. Grubb's outlier test was used to detect the presence of outliers which were removed prior to analysis. Time course data is presented as mean ± SEM. Summary data for time exhibiting nocifensive behaviour is presented as mean with 95% confidence interval (95% CI). *P* < 0.05 was taken to be statistically significant.

## Results

### The NMDA receptor antagonist, 7-CK, produced localized analgesic effects

7-CK reduced the time exhibiting nocifensive responses at a dose of 2.5  $\mu\text{g}$  (67 s [95% CI, 9, 124];  $n = 5$  with one outlier removed) compared to vehicle (179 s [95% CI, 108, 251];  $n = 5$ ;  $P < 0.05$ ). No difference in algesic responses was detected for 1  $\mu\text{g}$  7-CK (89 s [95% CI, 17, 161];  $n = 5$ ) (Fig. 21). The localization of the effects of 7-CK were investigated by administering 10% hypertonic saline to the front paws. Neither 1  $\mu\text{g}$  (359 s [95% CI, 230, 489];  $n = 8$ ) nor 2.5  $\mu\text{g}$  (335 s [95% CI, 168, 502];  $n = 8$ ) 7-CK produced any statistically significant differences compared to the vehicle group (326 s [95% CI, 211, 441];  $n = 8$ ). The effects of 7-CK were found to affect the hind, but not front paw suggesting a localization of 7-CK to the site of injection (Fig. 22).

### ACBC does not interact with 7-CK to produce increased analgesia

To investigate the interaction between 7-CK and ACBC, 1  $\mu\text{g}$  7-CK was co-injected intrathecally with 57.5  $\mu\text{g}$  and 144  $\mu\text{g}$  ACBC. The combination of 7-CK with either 57.5  $\mu\text{g}$  (101 s [95% CI, 53, 149];  $n = 8$ ) or 144  $\mu\text{g}$  ACBC (74 s [95% CI, 20, 129];  $n = 8$ ) did not produce any statistically significant differences compared to control (57.5  $\mu\text{g}$ : 101 s [95% CI, 53, 149;  $n = 8$ ]; 144  $\mu\text{g}$ : 74 s [95% CI, 20, 129;  $n = 8$ ] vs vehicle: 112 s [95% CI, 31, 194;  $n = 8$ ]) suggesting 7-CK may be antagonizing the effects of ACBC (Fig. 23). 7-CK was then co-administered with a higher dose of ACBC (288  $\mu\text{g}$ ) to investigate possible antagonism. However, a combination of 7-CK with ACBC or ACBC alone produced a statistically significant reduction in time exhibiting nocifensive behaviour (vehicle: 170 s [95% CI, 67, 272;  $n = 5$  with one outlier removed] vs 7-CK with ACBC: 40 s [95% CI, 5, 74;  $n = 5$ ;  $P < 0.01$ ], ACBC: 60 s [95% CI, -2, 122;  $n = 5$ ;  $P < 0.05$ ]) (Fig. 24). This suggests that a higher dose of ACBC is capable of overcoming antagonistic interactions with 7-CK.

### **Confirmation of GABA<sub>B</sub> receptor mediated antinociception in the lumbar intrathecal space**

The time course for the exhibition of nocifensive behaviour following baclofen treatment is shown in Figure 25a. Intrathecal administration of the GABA<sub>B</sub> agonist baclofen produced a statistically significant reduction in the time exhibiting nocifensive behaviour at both 0.025 µg (37 s [95% CI, 13, 61];  $n = 6$ ;  $P < 0.001$ ) and 0.1 µg (2 s [95% CI, -1, 5];  $n = 6$ ;  $P < 0.0001$ ) compared to vehicle (136 s [95% CI, 84, 188];  $n = 6$ ) (Fig. 25b). This observation confirms the presence of functional GABA<sub>B</sub> receptors in the lumbar intrathecal space. The time course for nocifensive behaviour for animals treated with CGP35348, a GABA<sub>B</sub> antagonist is shown in Fig 26a. CGP35348 did not reduce nocifensive behaviour in the hypertonic saline assay at either 0.5 µg (102 s [95% CI, 44, 160];  $n = 6$ ) or 2.5 µg (145 s [95% CI, 145, 100, 189];  $n = 6$ ) compared to vehicle (88 s [95% CI, 36, 139];  $n = 6$ ) (Fig. 26b).

### **Antagonism of baclofen effects by CGP 35348**

CGP 35348 was administered 10 min prior to the injection of baclofen to allow for equilibration of the antagonist. Figure 27a shows the time course of responses in animals pre-treated with CGP 35348 or vehicle. Pre-treatment with CGP 35348 prevented a decrease in time exhibiting nocifensive behaviour compared to vehicle pretreatment (161 s [95% CI, 82, 239;  $n = 5$ ] vs 22 s [95% CI, -7, 50;  $n = 5$ ];  $P < 0.01$ ) indicating CGP 35348 antagonized the effects of baclofen (Fig. 27b).

### **Pre-treatment with CGP 35348 does not affect the effects of ACBC**

Administration of 2.5 µg CGP 35348 prior to 288 µg (250 mM) ACBC did not produce any statistically significant differences between groups (Fig. 28). Animals pre-treated with vehicle exhibited nocifensive behaviour for 92 s (95% CI, 27, 157;  $n = 8$  with one outlier excluded) while animals pre-treatment with 0.5 µg and 2.5 µg CGP 35348 exhibited behaviour for 150 s (95% CI, 95, 205;  $n = 8$ ) and 168 s (95% CI, 82, 255;  $n = 8$ ). This suggests that CGP 35348 does not alter the effects of ACBC.

## Discussion

In this study, the contributions of NMDA and GABA<sub>B</sub> receptors to the effects of ACBC antinociception were investigated. 7-CK and CGP 35348 were selected to provide insight into the activity of ACBC at NMDA and GABA<sub>B</sub> receptors, respectively. Contrary to our hypothesis, we found that when 7-CK and ACBC were combined in the lumbar intrathecal space, the combination did not interact to produce greater antinociceptive effects. Rather, 7-CK appeared to antagonize the effects of a lower dose of ACBC. When administered alone, 7-CK reduced nocifensive responses by acting locally in the lumbar intrathecal space. At a dose that antagonized the antinociceptive effects of baclofen, we found that intrathecal pre-treatment CGP 35348 did not inhibit the antinociception produced by ACBC in the hypertonic saline assay.

ACBC and 7-CK both have been found to act at the glycine site of NMDA receptors to inhibit glycine binding and NMDA-induced currents (Hood et al., 1989; Inanobe et al., 2005; Watson et al., 1989). Despite ACBC acting as a partial agonist and 7-CK as an antagonist at NMDA receptors *in vitro*, both compounds were able to produce analgesia when administered individually. Thus, the assumption was made that ACBC and 7-CK acted through a similar mechanism via NMDA receptors to produce their antinociceptive effects. We hypothesized that by combining two minimally effective doses of compounds acting through the same site, enhanced antinociception would be detected. However, this was not the case and although surprising, this finding is not without precedent. Rao et al. (1990) found that intracerebellar co-administration of ACBC with the NMDA glycine site agonist, D-serine, did not reverse D-serine-induced increases in cGMP levels while the antagonist HA-966 produced a full reversal (Rao et al., 1990). This suggests that the antinociceptive effects of ACBC may be produced via a distinct mechanism compared to NMDA receptor antagonists and does not behave like an antagonist *in vivo*. In support of this, conformational studies of NMDA receptor binding have demonstrated that ACBC

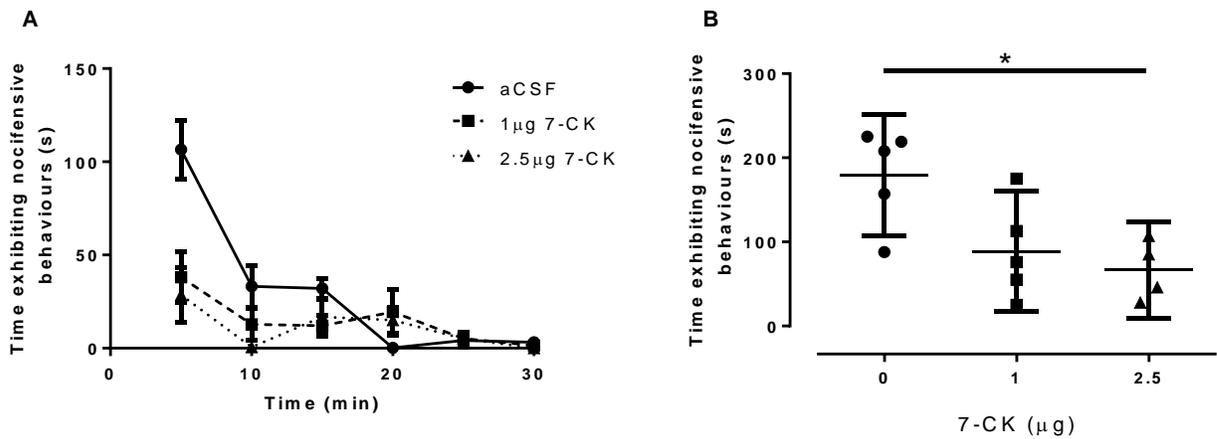
stabilizes a more closed conformation of the glycine binding cleft compared to an antagonist (Dolino et al., 2015; Inanobe et al., 2005). If ACBC and 7-CK required unique NMDA receptor conformations to exert their effects, a combination of the two would effectively antagonize each other by reducing the population of receptors in either conformation. It is possible that competitive antagonism exists at a higher dose of ACBC still produced analgesia suggesting ACBC is capable of overcoming the antagonizing effects of 7-CK and shifting the receptor population towards a more favourable conformation for ACBC. However, further studies are required to explore this interaction.

The role of GABA<sub>B</sub> receptors in mediating the effects of ACBC was investigated due to the structural similarity between ACBC and isovaline which was shown to act on GABA<sub>B</sub> receptors (Cooke et al., 2012; Whitehead et al., 2012). The presence of functional GABA<sub>B</sub> receptors in the lumbar intrathecal space was first confirmed using baclofen. This confirmation was necessary due to a previous observation in our laboratory that intrathecally administered isovaline did not produce antinociception in the murine formalin foot assay. Consistent with reports in the literature, intrathecal baclofen produced antinociceptive effects in rodent pain models when administered into the lumbar intrathecal space indicating the presence of functional GABA<sub>B</sub> receptors (Dirig & Yaksh, 1995; Loubser & Akman, 1996; Wilson & Yaksh, 1978). These effects were abolished by pre-treatment, but not co-administration, of CGP 35348 into the intrathecal space. However, intrathecal pre-treatment with the same dose of CGP 35348 did not inhibit the effects of ACBC. Examination of the data (cf. Results) suggests that there may be a small effect of CGP 35348 on reversing the effects of ACBC and the experiment may have been underpowered for detecting such an effect. It is possible that GABA<sub>B</sub> receptors may play a minor role in facilitating the effects of ACBC.

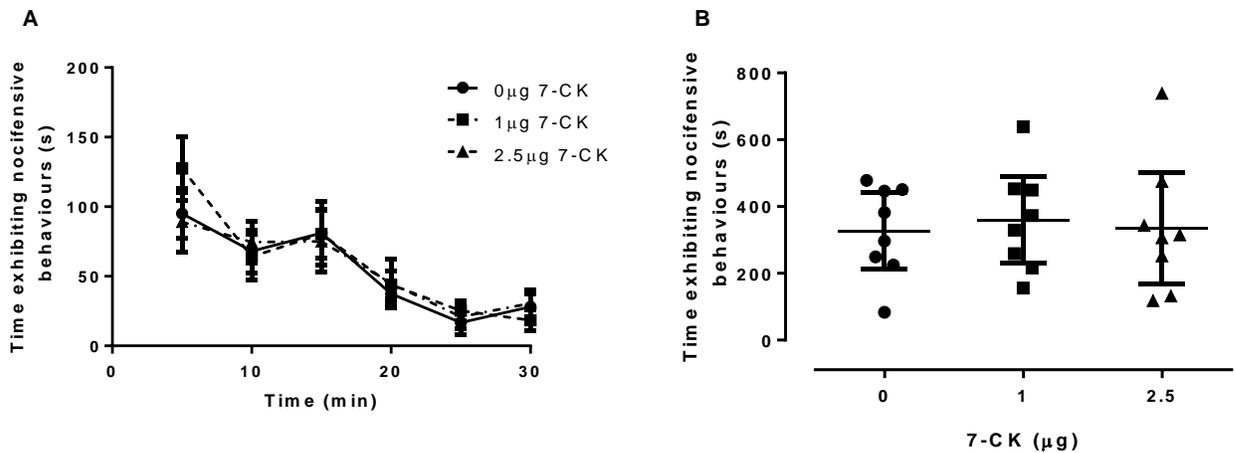
Another potential target receptor for ACBC are metabotropic glutamate receptors (mGluR), specifically, group II mGluRs. Activation of spinal group II mGluRs reduces nociceptive signaling in the cord and alleviates pain in various animal models of nociception (Davidson et al., 2015; Jones et al.,

2005; Lee et al., 2013; Zammataro et al., 2011). The structural prototype of ACBC, isovaline, has been shown to interact with group II mGluRs to produce anti-allodynic effects in mice (Asseri et al., 2015). The similar chemical structure between ACBC and isovaline raises the possibility that ACBC may be able to interact with group II mGluRs as well.

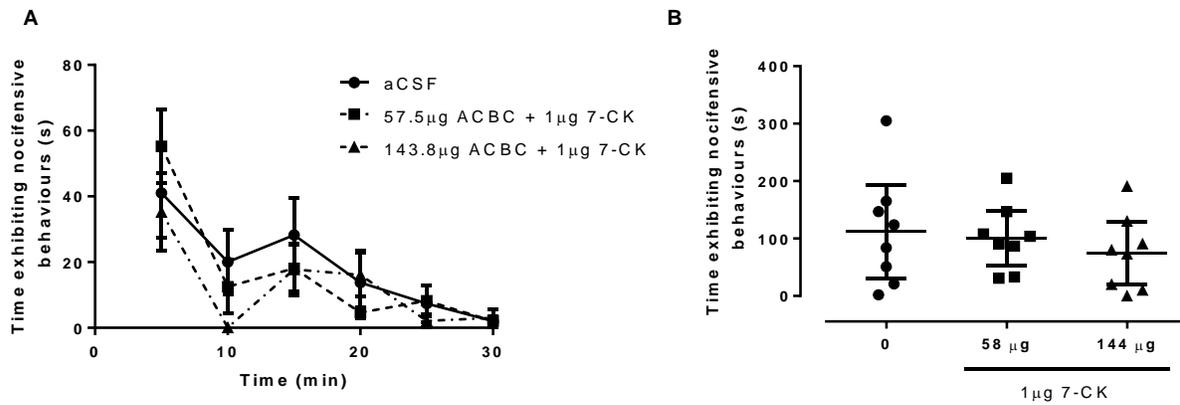
This study provides preliminary evidence for the antinociceptive mechanism of action for ACBC. The findings here suggest a unique action of ACBC at NMDA receptors that is different than the antagonist 7-CK. GABA<sub>B</sub> and  $\mu$ -opioid receptors do not appear to have a primary role in mediating the antinociceptive effects of ACBC. Further investigation is required to elucidate the activity of ACBC at NMDA receptors in whole animals.



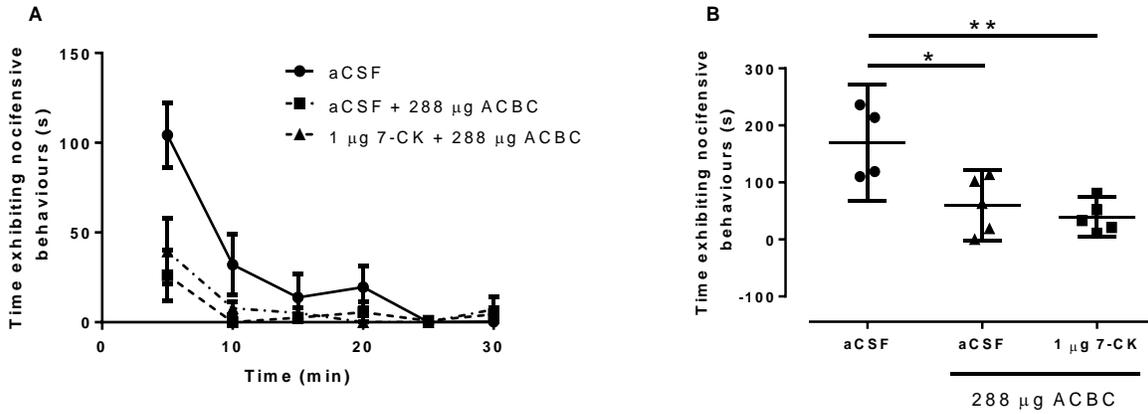
**Figure 21 – Intrathecal 7-CK produces antinociceptive effects in the hypertonic saline assay – A)** Time course of nocifensive behaviour induced by hypertonic saline following intrathecal administration of 7-CK. **B)** 2.5 μg 7-CK produces a statistically significant reduction in the total time exhibiting nocifensive behaviour over 30 minutes. Time course data are presented as mean ± SEM. Total time exhibiting nocifensive behaviour is presented as individual points with summary bars representing mean with 95% CI. \* indicates  $P < 0.05$ .  $n = 5$  per group.



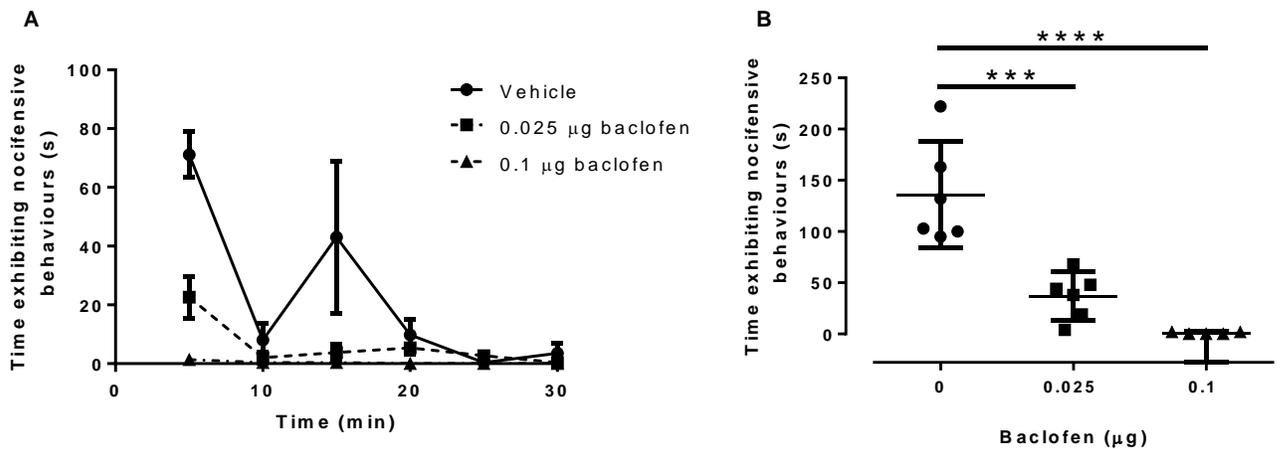
**Figure 22 – Administration of 7-CK into the lumbar intrathecal space at L5-L6 does not affect nocifensive responses of the forepaw –** A) Time course of nocifensive behaviour induced by hypertonic saline. Animals received 0, 1, or 2.5 μg 7-CK. Data are presented as mean ± SEM. B) I.t. 7-CK does not produce any statistically significant changes to the total time exhibiting nocifensive behaviour over the 30 min test period following the injection of 10% hypertonic saline. Each point represents an individual animal with summary bars depicting mean with 95% CI.  $n = 8$  per group.



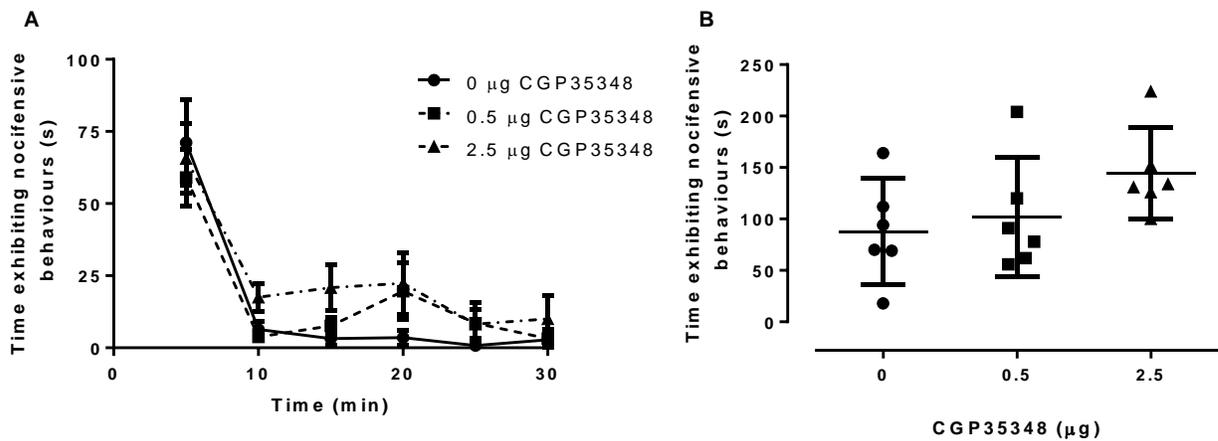
**Figure 23 – Intrathecal co-administration of ACBC and 7-CK do not produce enhanced antinociceptive effects** – A) Time course of nocifensive behaviour induced by hypertonic saline. Data are presented as mean  $\pm$  SEM. B) Co-administration of 1  $\mu$ g 7-CK with either 58 or 144  $\mu$ g ACBC did not produce enhanced antinociception compared to vehicle. Each point represents an individual animal with summary bars representing mean with 95% CI.  $n = 8$  per group.



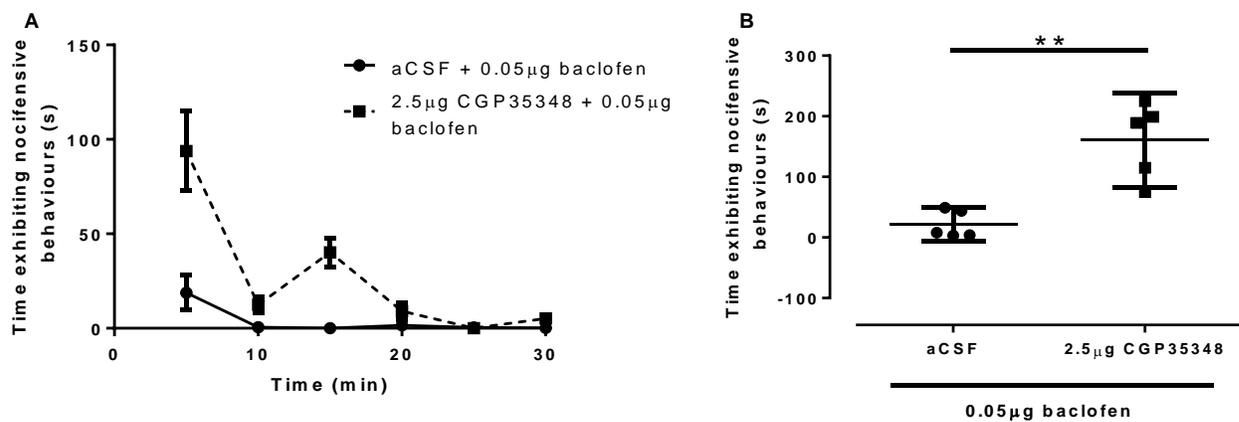
**Figure 24 – A high dose of ACBC intrathecally produces comparable antinociception in the absence or presence of 7-CK** – A) Time course for nocifensive behaviour induced by hypertonic saline. Data are presented mean  $\pm$  SEM. B) Intrathecal ACBC in the absence or presence of 7-CK produced a statistically significant reduction in nocifensive behaviour compared to control. Each point represents an individual animal with summary bars denoting mean with 95% CI. \* indicates  $P < 0.05$ , \*\* indicates  $P < 0.01$ .  $n = 5$  per group with 1 outlier excluded from the control group.



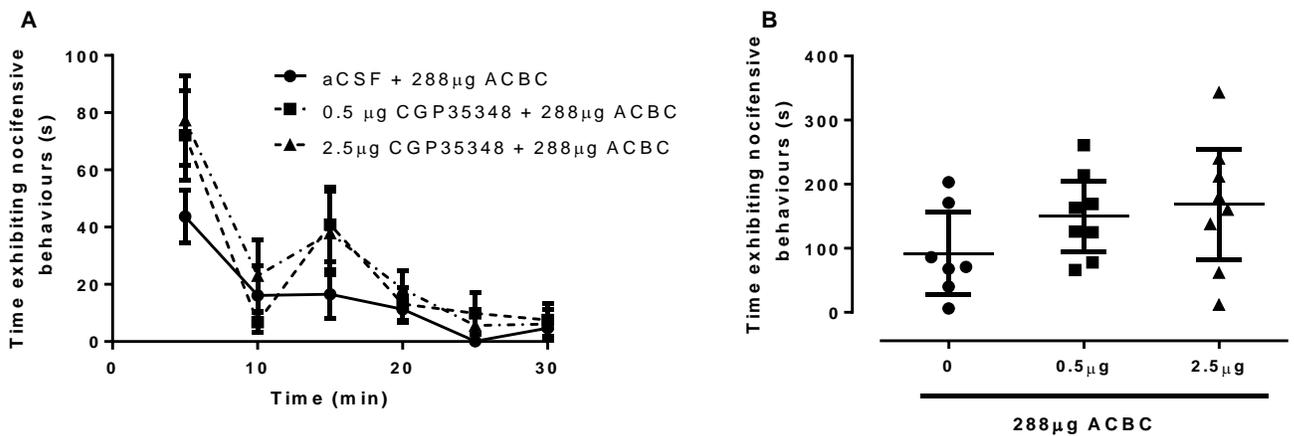
**Figure 25 – Intrathecal baclofen produces antinociception in the hypertonic saline assay – A)** Time course of nocifensive behaviour induced by hypertonic saline following intrathecal administration of baclofen. **B)** Baclofen (0.025 and 0.1 µg) produces a statistically significant reduction in the total time exhibiting nocifensive behaviour. Time course data are presented as mean  $\pm$  SEM. Total time exhibiting nocifensive behaviour is presented as individual points with summary bars representing mean with 95% CI. \*\*\* indicates  $P < 0.001$ , \*\*\*\* indicates  $P < 0.0001$ .  $n = 6$  per group with one animal excluded from vehicle.



**Figure 26 – Intrathecal CGP 35348 alone does not produce antinociceptive effects –** A) Time course of nocifensive behaviour induced by hypertonic saline following intrathecal administration of CGP 35348. B) No statistically significant changes in time exhibiting nocifensive behaviour were detected between animals treated with 0, 0.5, or 2.5 µg CGP 35348. Time course data are presented as mean ± SEM. Total time exhibiting nocifensive behaviour is presented as individual points with summary bars representing mean with 95% CI. *n* = 6 per group.



**Figure 27 – Pre-treatment with CGP35348 antagonizes the effects of intrathecal baclofen – A)** Time course of nocifensive behaviour induced by hypertonic saline following i.t. pre-treatment of CGP 35348 before i.t. baclofen. **B)** Pre-treatment with 2.5 µg CGP 35348 produced a statistically significant increase in the total time exhibiting nocifensive behaviour. Time course data is presented as mean  $\pm$  SEM. Total time exhibiting nocifensive behaviour is presented as individual points with summary bars representing mean with 95% CI. \*\* indicates  $P < 0.01$ .  $n = 5$  per group.



**Figure 28 – Pre-treatment with CGP 35348 does not affect the antinociception produced by ACBC – A)**

Time course of nocifensive behaviour induced by hypertonic saline following i.t. pre-treatment of CGP

35348 before i.t. ACBC. B) Pre-treatment with 0.5 or 2.5  $\mu$ g CGP 35348 produced no statistically

significant changes in the total time exhibiting nocifensive behaviour. Time course data are presented as

mean  $\pm$  SEM. Total time exhibiting nocifensive behaviour is presented as individual points with summary

bars representing mean with 95% CI.  $n = 8$  per group with one outlier removed from control.

## Chapter 5 – General Discussion

The limitations of available analgesics motivate the search for novel compounds with improved properties. This thesis examined the antinociceptive properties of isovaline and isovaline analogs and their potential as spinal analgesics. Isovaline produces antinociception in preclinical models of pain without producing overt behavioural effects thus supporting its possible use as a prototype for developing novel analgesics. In chapter 2, we identified the pharmacophore of isovaline as the cyclobutane conformation that can be adopted by isovaline with the ethyl and methyl groups. This was supported by the presence of antinociception in ACBC where the cyclobutane ring conformation is permanently formed via a bond connecting the ethyl and methyl groups of isovaline. Furthermore, absence or inability to form the cyclobutane ring conformation abolishes antinociceptive activity. In addition, modifications which increase the overall electrostatic size of isovaline eliminates antinociceptive activity suggesting the binding pocket of isovaline is relatively small.

Given the shortcomings in currently available analgesics such as opioids, particularly amongst spinally administered compounds, the compound identified by the structure-activity relationship, ACBC, was investigated as a candidate to address the need for a non-opioid analgesic. In chapter 3, we demonstrated that ACBC produced robust antinociception following intrathecal administration in mice. The other compounds, isovaline and R- $\alpha$ -methylproline, did not produce antinociception following intrathecal administration. In addition, R- $\alpha$ -methylproline caused behavioural abnormalities including licking and biting the site of injection, writhing, and loss of righting reflex. The antinociception was of short duration and found to be reversible. The effects were localized to the lumbar intrathecal region as responses in the hindpaw, but not forepaw, were blocked suggesting a lack of cephalad movement. These effects were not accompanied by adverse effects on respiration or motor activity.

To better understand the antinociceptive effects of ACBC, in chapter 4, we investigated the mechanism of action of ACBC at NMDA and GABA<sub>B</sub> receptors. We demonstrated that ACBC does not behave like an antagonist at NMDA receptors *in vivo*. When co-administered into the lumbar intrathecal space with the NMDA antagonist, 7-CK, additive effects were not observed as hypothesized, but instead reduced the overall antinociception. This was reversed when the concentration of ACBC was increased suggesting a competitive interaction for binding. In addition, the antinociceptive effects of ACBC were not antagonized by pre-administration of the GABA<sub>B</sub> antagonist CGP 35348 suggesting that ACBC does not act through GABA<sub>B</sub> receptors.

## Isovaline as a prototype analgesic

Isovaline has been demonstrated to produce antinociception across a wide range of animal assays of pain (Lee et al., 2013; MacLeod et al., 2010; Whitehead et al., 2015; Whitehead et al., 2012), however, the structural characteristics of isovaline which are responsible for producing its antinociceptive effects were not clear. We have now established a structure-activity relationship study for isovaline and demonstrated the structural elements necessary for producing antinociception and are further discussed below (Fung et al., 2017, Chapter 2). Given the desirable pharmacological properties of isovaline as an analgesic, a solid understanding of the structure-activity relationship for antinociception would lead to other useful compounds for the management of pain. In the work discussed in chapter 2, the compounds examined were investigated at a single dose in one assay of pain. It is possible that the compounds identified as lacking antinociceptive effects may be effective at doses not tested in those experiments and also in assays utilizing other pain modalities. Consistent with this, compounds such as R- $\alpha$ -methylproline exhibited weak antinociceptive effects in the formalin foot assay, but may be more effective in other assays. Interestingly, the antinociceptive effects of isovaline were limited to the formalin foot assay using systemic administration. Isovaline did not appear to produce antinociception after systemic administration in the hypertonic saline assay or after intrathecal administration in the formalin foot assay. These observations suggests that isovaline may be limited to treating pain of a predominantly inflammatory nature.

The compounds investigated in this work represent modifications with relatively large moieties to the chemical structure of isovaline allowing for a diverse library of compounds to be established (Harrold & Zavod, 2013). Given that modifications which increase the size of isovaline abolishes antinociceptive activity, it would be of benefit to examine the effect of smaller modifications such as the addition of a fluorine atom or substitution of other functional groups with smaller alternatives. Although

these experiments have demonstrated the importance of the cyclobutane ring conformation for producing antinociception, it remains unclear how altering the electrostatics of the ring would affect antinociception. Further investigation of isovaline analogs that expand the diversity of the library would serve to deepen the understanding of isovaline's structure-activity relationship.

## ACBC as an analgesic candidate

The experiments described in this thesis have demonstrated the antinociceptive effects of the unnatural amino acid, ACBC. ACBC has been shown *in vitro* to reduce NMDA receptor activation via interaction with the glycine binding site on NMDA receptors (Compton et al., 1990; Hood et al., 1989; Lench et al., 2015; Watson et al., 1989; Watson & Lanthorn, 1990). Ligands that block the activation of NMDA receptors are used clinically to manage pain, chief amongst these drugs is the channel blocker, ketamine (Vorobeychik et al., 2013). The main limitation of full antagonists is the presence of adverse effects at therapeutic doses which affects their tolerability (Vorobeychik et al., 2013). To circumvent these limitations, partial agonists at the glycine site have been proposed as a viable alternative to avoid complete antagonism of the receptor while still exhibiting therapeutic benefit (Koller & Urwyler, 2010). ACBC is a potential candidate that meets these requirements. Previous work has demonstrated that ACBC acts as a partial agonist at the glycine site of NMDA receptors (Dolino et al., 2015; Inanobe et al., 2005). Consistent with this, the work in this thesis has demonstrated that ACBC does not interact in an additive fashion with the NMDA glycine site antagonist, 7-CK, as would have been expected if ACBC was also a full antagonist at the orthosteric site. At antinociceptive doses, ACBC did not produce adverse effects on respiratory rates or motor function suggesting the potential of separating the therapeutic and toxic curves.

This thesis demonstrates the antinociceptive effects of ACBC, but the extent of ACBC's antinociceptive effects remains unclear. The experiments conducted in this body of work were performed in animal models of pain and may not necessarily translate into humans. Furthermore, the animal models used were employing chemical and thermal stimuli to induce pain, thus it is unknown whether ACBC is effective against other modalities of pain. It would be of benefit to study the efficacy of ACBC on other pain assays, in particular, on neuropathic pain models because there is evidence that

NMDA receptor antagonists are effective against neuropathic pain (Aiyer et al., 2017). It would also be of benefit to elucidate the mechanism of action for antinociception and identify potential receptor targets. Despite demonstrating that ACBC does not behave like a full antagonist at the glycine site, it remains unclear how ACBC produces antinociception *in vivo*. Although ACBC did not affect respiration or motor function, there remain other parameters that may be affected by ACBC but were not detected in this work. In particular, further investigations into the impact of ACBC on memory and learning are necessary to better understand the potential adverse effects.

## Future directions

Future directions include investigations into possible synergy between ACBC and morphine and local anesthetics would yield valuable information regarding the use of ACBC as an adjunct to currently employed spinal analgesic strategies. There is evidence that suggests NMDA receptor antagonists and opioids interact to produce greater analgesia (Beaudoin et al., 2014; Dickenson, 1997; Wiesenfeld-Hallin, 1998). To address the pressing need for new analgesics to treat chronic pain, investigating the efficacy of ACBC in this area would be of great benefit. Determining the effects of ACBC in chronic pain models such as neuropathic pain would provide further evidence supporting ACBC as an efficacious drug for treating chronic pain. An indepth investigation into the long-term tolerability of ACBC would be necessary to determine the long-term tolerability of ACBC and produce a comprehensive adverse effect profile to complement the evidence for ACBC's therapeutic effects. In addition, determining the target receptor responsible for mediating the analgesic effects of ACBC *in vivo* would provide a better understanding of its analgesic effects. Further structure-activity relationship into the analgesic effects of ACBC could lead to improved compounds for the treatment of pain. Lastly, after demonstrating the efficacy of ACBC in animals, it would be of utmost importance to translate the findings into humans.

## Conclusions

The work described in this thesis examined the antinociceptive properties of isovaline and isovaline analogs. An initial structure-activity relationship study identified ACBC as an isovaline analog with antinociceptive effects and demonstrated the necessity of the cyclobutane ring conformation for antinociception. We then demonstrated the potential of ACBC as a spinal analgesic and showed that it behaves differently from the NMDA antagonist, 7-CK. Our findings have contributed to the expansion of drugs that can be used for treating pain and the use of ACBC as a prototype analgesic for further development.

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