ANTIALLODYNIA AND SURGICAL IMMOBILITY PRODUCED BY THE NON-PROTEINOGENIC BRAIN IMPERMEANT AMINO ACID, ISOVALINE

by

Ryan Arthur Whitehead

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Abstract

This thesis describes research stemming from investigation of the novel nonproteinogenic amino acid, isovaline. The first chapter is a background for the field of study. The second chapter investigates peripheral $GABA_B$ receptor-mediated mechanisms of action of isovaline, ɤ-aminobutyric acid (GABA), and baclofen. The third chapter details experimental evidence demonstrating that a combination of central hypnosis and peripheral analgesia produces general anesthesia. The fourth chapter describes the development and evaluation of a novel model of human trigeminal allodynia, a feature of intractable and severe pain in trigeminal neuralgia. The mechanism of action of isovaline, as for GABA and baclofen, was found involve peripheral $GABA_B$ receptors, revealed through attenuation of peripheral prostaglandin E_2 (PGE_2)-induced allodynia. This mechanism was tested by reversal of allodynia by the $GABA_B$ antagonist CGP52432 and potentiation of allodynia by the $GABA_B$ positive modulator CGP7930. Immunohistochemical staining showed confluence of $GABA_{B1}$ and $GABA_{B2}$ subunits on free nerve endings and keratinocytes. Peripherally administered isovaline and GABA produced analgesia but no CNS depression, whereas baclofen produced analgesia accompanied by pronounced sedation and hypothermia. In a forced exercise model of osteoarthritic dysfunction isovaline restored joint operability lost presumably due to knee pain.

Next, we hypothesized that co-administration of a peripherally restricted analgesic with a central hypnotic (isovaline co-administered with propofol) would produce general anesthesia in mice. We first demonstrated that isovaline and fentanyl produced surgical analgesia without appreciable sedation. Propofol alone produced hypnosis without analgesia or surgical anesthesia.

When administered with hypnotic doses of propofol, coadministration of isovaline resulted in general anesthesia. Under fixed ratio hypnotic and analgesic dose ratios, propofol-isovaline anesthesia had a markedly higher therapeutic index than propofol-fentanyl. When co-administered with a fixed hypnotic dose of propofol, isovaline in contrast to fentanyl did not have a maximum tolerated dose (MTD).

Next we describe an intracisternal strychnine mouse model of human trigeminal neuropathic pain is described. The model reflects the efficacy of clinical drug treatments as morphine administered intraperitoneally was not effective, but intracisternally injected carbamazepine epoxide provided pain relief. Isovaline applied near the spinal trigeminal nucleus was effective in this model, validating its utility in detecting efficacy of novel analgesics.

Preface

My contributions to chapter two were undertaking the *in vivo* experiments, and the experimental design, analysis and the writing associated with it, and undertook the journal submission processes under supervision of Drs. Bernard MacLeod and Ernest Puil in the Hugill Centre for Anesthesia and Analgesia. The development of the manuscript was also subjected to significant collaborative advice from Drs. Schwarz and Ries. A version of chapter two has been published: Whitehead RA, Puil E, Ries CR, Schwarz SK, Wall RA, Cooke JE, Putrenko I, Sallam NA, MacLeod BA. GABA(B) receptor-mediated selective peripheral analgesia by the non-proteingogenic amino acid, isovaline. Neuroscience 213: 154-60, 2012.

My contribution to chapter three was in the design, conducting, and analysing of experiments and writing associated with it. All experiments were undertaken in the Hugill Centre for Anesthesia and Analgesia. I wrote the manuscript and undertook the journal submission process, under supervision of Drs. Bernard MacLeod, Ernest Puil and Stephan Schwarz co-wrote the manuscript with me. A version of chapter is in preparation for peer-reviewed publication: Whitehead, R.A., Puil, E., Ries, C.R., Schwarz S.K., Fung, T., MacLeod, B.A. Isovaline co-administered with propofol produces general anesthesia in mice. In preparation for submission, 2013.

My contribution to the fourth chapter was in the design, validation of experimental procedures, and analysis of experiments and writing of the manuscript. All experiments were undertaken in the Hugill Centre for Anesthesia and Analgesia. Drs. Bernard MacLeod, Ernest Puil and Stephan

Schwarz co-wrote the manuscript with me. I also contributed, through review of experimental data, blind re-evaluation of data transformed to a new non-parametric allodynia score system, re-analysis of data including new statistical approaches. I wrote the manuscript in collaboration with Drs. MacLeod, Puil and Schwarz and undertook the journal submission process. A version of chapter four has been pubished: Lee IO, Whitehead RA, Ries CR, Schwarz SK, Puil E, MacLeod BA. Evaluation of a novel mouse model of intracisternal strychnine-induced trigeminal allodynia. Can J Anaesth [Epub ahead of print], 2013.

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List of Abbreviations

aCSF	Artificial cerebrospinal fluid
AIB	2-Amino-2-methylpropanoic acid
ANOVA	Analysis of variance
Baclofen	4-amino-3-(4-chlorophenyl)butanoic acid
BHS	Behavioural hypoactivity score
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBZe	Carbamazepine epoxide
CGP 35348	3-aminopropyl(diethoxymethyl)phosphinic acid
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
DAPI	4', 6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
ED_{50}	Effective dose for 50 percent of animals
Fentanyl	N-(1-(2-phenylethyl)-4-piperidinyl)-N-phenylpropanamide
GABA	γ-aminobutyric acid
GDP	Guanosine diphosphate
GIRK	G protein-coupled inwardly-rectifying potassium channel
GTP	Guanosine triphosphate

IPSC	Inhibitory postsynaptic current
Isovaline	2-amino-2-methylbutanoic acid
KCC2	Potassium-chloride co-transporter, isoform 2
KCTD	Potassium channel tetramer domain-containing proteins
LD_{50}	Lethal dose for 50 percent of animals
LOC	Loss of consciousness
LORR	Loss of righting reflex
LOTP	Loss of tail pinch response
MAP-2	Microtubule-associated protein 2
Morphine	(5α,6α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol
NSAID	Non steroidal anti-inflammatory drug
PBS	Phosphate-buffered saline
PNS	Peripheral nervous system
SEM	Standard error of the mean
SS	Stratum spinosum
SG	Subtantia granulosum
Strychnine	Strychnidin-10-one
TIVA	Total intravenous regional anesthesia
TrpV1	Transient receptor potential cation channel subfamily V member 1

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I am also grateful to ongoing support by my family, who have stood by my side throughout my years of education.

Dedication

For family, colleagues and friends

1 General Introduction

Chronic pain, which affects one in five Canadians, is the most common cause of adult disability and is estimated to have a direct health care cost of over $6 billion/year (Statistics Canada, 2008). Commonly used treatments such as opioids produce analgesia but at the cost of limiting central nervous system (CNS) side effects, including sedation, respiratory depression and addiction (Schug and Gandham, 2007; Gold and Gebhart, 2010). Brain-impermeant small amino acids with putative analgesic actions on specific receptors expressed in peripheral nociceptive tissues represent a promising class of compounds for treatment of chronic pain (MacLeod et al., 2010). The novel analgesic isovaline (2-amino-2-methylbutyric acid) exemplifies this group. Examining the analgesic actions and mechanisms of isovaline and related compounds forms the core of this thesis.

This thesis is based on three manuscripts which examine the antiallodynic and analgesic properties of isovaline and related amino acids in laboratory CD-1 mice. The motivation for studying isovaline is based on the following three observations: 1) the demonstration of $glycine_A$ receptors in nociceptive ventrobasal thalamus (Ghavanini et al., 2005); 2) the promising efficacy of lead agent isovaline *in vivo* without detectable CNS effects (Macleod et al., 2010); and 3) the ability of isovaline to increase membrane conductances in neuronal membranes of nociceptive ventrobasal thalamus $GABA_B$ receptors (Cooke et al., 2012). Therefore, we sought to examine the peripheral *in vivo* mechanisms of action of isovaline on PGE_2-induced allodynia. Our primary hypothesis was that isovaline produces antiallodynia via activation of peripheral $GABA_B$ receptors.

1.1 Scope of Thesis

This thesis is organized as follows: the first chapter is a review of pertinent background in pain research, including pain pathways, animal models of pain, analgesic and their receptors, and general anesthesia. The second chapter focuses on the mechanisms of action of the novel peripherally restricted amino acid analgesic isovaline, along with those of the primary inhibitory and brain impermeant neurotransmitter in the mammalian nervous system, ɤ-aminobutyric acid (GABA), and the brain penetrant prototypical $GABA_B$ receptor agonist baclofen. The third chapter assesses the surgical analgesic efficacy of isovaline compared to the opioid fentanyl and examines the relative safety of each preparation. The ability of each of these drugs to produce safe general anesthesia in the presence of propofol induced hypnosis is investigated. The fourth chapter describes the development and validation of an intracisternal strychnine mouse model of human trigeminal neuralgia, using the clinically ineffective agent morphine as a negative control, the clinically effective agent carbamazepine as positive control and the therapeutic drug candidate isovaline, for validation of the model. The main findings and conclusions are discussed in the fifth and final chapter. The remainder of this first chapter provides a more detailed overview of the three aims central to this thesis.

The second chapter is based on the published article “GABA(B) receptor-mediated selective peripheral analgesia by the non-proteinogenic amino acid, isovaline” in *Neuroscience*. Here we examine $GABA_B$ receptor-mediated peripheral analgesia produced by the non-proteinogenic amino acid, isovaline, GABA and baclofen. Recently, we found that the CNS-impermeant amino acid, isovaline, produces analgesia without apparent CNS effects (MacLeod et al., 2010).

Peripherally restricted analgesics are desirable to avoid central nervous system (CNS) side effects of opioids. Nonsteroidal anti-inflammatory drugs produce peripheral analgesia but have significant toxicity. $GABA_B$ receptors represent peripheral targets for analgesia but available selective $GABA_B$ receptor agonists (such as baclofen) cross the blood-brain barrier to produce adverse CNS effects. On observing that isovaline produced $GABA_B$ receptor-mediated conductances in brain slices (Cooke et al., 2012), we hypothesized that isovaline produces peripheral analgesia mediated by $GABA_B$ receptors. We compared the peripheral analgesic and CNS effect profiles of isovaline, baclofen, and GABA (a CNS-impermeant, unselective $GABA_B$ agonist). Anti-allodynic mechanisms actions of isovaline, baclofen, and GABA were assessed using the $GABA_B$ antagonist, CGP52432, and the $GABA_B$ modulator, CGP7930. As indicators of GABAergic action in the CNS, we measured Behavioural Hyperactivity Scores and temperature changes to assess GABAergic actions in the CNS. We tested for potential clinical utility of isovaline in a forced exercise model of induced osteoarthritic dysfunction (Whitehead et al., 2012). Immunohistochemical staining of cutaneous layers of the analgesic test site was used to probe for co-localization of $GABA_{B1}$ and $GABA_{B2}$ receptor subunits on fine nerve endings and keratinocytes.

The third chapter is based on the manuscript entitled “Peripherally restricted isovaline co-administered with propofol produces general anesthesia in mice”, which is at near-submission stage for *Anesthesia and Analgesia*. General anesthesia requires central hypnosis and immobility to noxious stimuli (Zollner, 2008). This study evaluates general anaesthesia produced through a combination of propofol central hypnosis with isovaline-induced inhibition of peripheral nociceptors. We compared the resulting general anesthetic efficacy and side effects to those

produced by propofol co-administered with conventionally used opioid, fentanyl. During this anesthesia, fentanyl crosses the blood-brain barrier to produce respiratory depression, which is one of the leading causes of death resulting from general anesthesia (Bailey et al., 1990). Finally, we compared the relative safety of isovaline-propofol anesthesia to isovaline-fentanyl anesthesia.

The fourth chapter is based on the published paper "Evaluation of a novel mouse model of intracisternal strychnine-induced trigeminal allodynia" in the Canadian Journal of Anesthesia. Here the development and evaluation of an intracisternal strychnine model of trigeminal neuralgia in mice is described. Intractable neuropathic dynamic allodynia remains one of the major symptoms of human trigeminal neuropathy and is considered to be the most excruciatingly painful conditions known. The clinical manifestation of trigeminal neuralgia consists of remitting and relapsing stabbing facial pain in response to touch mediated by aberrant trigeminal nerve function of uncertain etiology, but has been correlated to nerve compression by enlarged blood vessels (Kitt et al., 2000). Intracisternal strychnine injections in rats has been shown to elicit dynamic allodynia in the trigeminal distribution with many of the same features noted in humans, and represents a possible model of trigeminal neuralgia (Miraucourt et al., 2007). The purpose of this study was to validate a mouse model of trigeminal glycinergic inhibitory dysfunction using established positive (carbamazepine epoxide or CBZe) and negative (morphine) controls. The pharmacological actions of the conventional first-line treatment and the clinically ineffective opioid morphine (Miraucourt et al., 2007), were tested against trigeminal dynamic mechanical allodynia produced by intracisternal strychnine. Finally, we tested the effectiveness of isovaline in reducing allodynia produced by glycinergic inhibitory dysfunction

as a means to test for possible efficacy of this model in the detection of novel analgesic drug candidates.

In summary, the second chapter investigates the role of peripheral $GABA_B$ receptors in mediating the anti-allodynic effects of isovaline, GABA and baclofen, and probes for the co-expression of $GABA_B$ receptor subunits in glabrous cutaneous tissue of the mouse footpad. The third chapter investigates the possibility that co-administrations of the peripherally restricted analgesic isovaline to hypnotic doses of propofol could result in general anesthesia in mice. The fourth chapter consists in the development and validation of a mouse model of human trigeminal neuralgia using intracisternally injected strychnine to induce allodynia, wherein we assess the effects of CBZe, morphine and isovaline.

1.2 Background

1.2.1 Definitions of pain, allodynia and hyperalgesia

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Spanswick et al., 2000). While physiological correlates of pain exist, pain is by definition, descriptive and subjective. While pain is often adaptive, inactivity associated with pain avoidance in degenerative conditions such arthritis may lead to faster disease progression than if normal levels of activity were maintained (Conn et al., 2008). Secondary complications of inactivity include obesity and cardiovascular dysfunction. Analgesics play an important role in reducing inflammation and/or pain to increase mobility,

fostering building and maintenance of muscle tone, enhancing joint performance and thereby combating degenerative arthritis. Neuropathic pain is attributable to aberrant pain signalling mechanisms (Woolf and Mitchell, 1994). These syndromes include pathological alterations of peripheral nervous system signalling, complex regional pain syndrome (CRPS), phantom limb pain, trigeminal neuralgia and other conditions associated with nerve injury or trauma.

In order to adequately treat chronic pain, it is imperative to gain a thorough understanding of physiological mechanisms responsible for maladaptive pain (Keefe et al., 1989). Historically the vast majority of drug discoveries have been generated by the intersection of chemistry with basic and clinical pharmacological sciences; drug research has contributed more to medicine than any other scientific factor (Drews, 2000). However, from the standpoint of analgesic drug development, present mechanistic pharmacological demonstrations of antinociceptive actions of drugs are not adequate to preclude the possibility of detrimental CNS effects produced by generalized activation of physiological processes unrelated to pain (Misra et al., 2005).

Currently, both allodynia (pain responses to normally non-painful stimuli) and hyperalgesia (heightened pain responses to normally painful stimuli) represent aspects of chronic pain that are not fully understood; however, it is known that tissue injury is one of the main underlying causes of hyperalgesia and is accompanied by inflammation (Keefe et al., 1989). In the early 20th century, Lewis divided hyperalgesia into two classes – primary and secondary hyperalgesia. While the former occurs at a specific site of injury, the latter occurs in the surrounding areas which are undamaged. Many peripheral pain signalling mechanisms have been proposed to

explain distinct types of hyperalgesia which have differing relationships with subsequent CNS processing and central sensitization (Woolf, 2011).

1.3 Ascending pain pathways

Pain is a function of afferent nociceptive input from the periphery; pain signals are transmitted into the dorsal horn of the spinal cord and subsequently to higher order pain processing centers. This peripheral input is processed in the spinal cord and the brain. Peripheral input can initiate central sensitization to peripheral stimuli (Woolf, 2011) and continual peripheral nociceptive input can contribute to cognitive decline and brain atrophy (May, 2008). These considerations argue for the development of peripherally restricted analgesics for treatment of chronic pain conditions such as osteoarthritis (Konttinen et al., 2005). This is an unconventional and when considered in comparison to molecular modification strategies devised in development of GABA-like drugs such as baclofen and gabapentin, which instead have intentional and specific chemical modifications to allow entry to the CNS to produce analgesia. However, development of novel analgesics based on identification of CNS targets such as that observed in nociceptive ventrobasal thalamus can provide the basis for development of novel analgesics which may also act in the periphery (Ghavanini et al., 2005). While some classes of brain penetrant drugs produce pain relief without substantial side CNS effects, notably NSAIDs, acetaminophen and triptans (for migraine headaches) their principal analgesic effects are a result of peripheral actions and effects on vasculature/meninges, not brain tissue. While ongoing treatment regimens are widely prescribed for treatment of osteoarthritis, NSAIDs only provide approximately 15%

reduction in pain while severe gastrointestinal and renal effects are observed (Bjordal et al., 2004).

Spreading secondary hyperalgesia induced by injections of high temperature mimetic transient vanilloid receptor agonists (TRPV1) can be blocked by local anesthetic pre-treatment in skin surrounding the site of injury, indicating that in addition to primary afferent nociception, peripheral mechanisms can be involved in the development of secondary hyperalgesia and allodynia (Woolf, 2011). Injections of TRPV1 agonists such as capsaicin (the "hot" compound in chili peppers) have been shown to induce allodynia which occurs in humans as well as in rats (Roberts et al., 2006). Under such conditions, C fibres become sensitized as a result of inflammation (Devor, 1991). Many endogenous substances have been demonstrated to be involved in decreasing pain thresholds under a variety of circumstances and are therefore candidates of interest in studying hyperalgesia and allodynia (Dickenson et al., 1997). These substances include: bradykinin, serotonin (5-HT), GABA, glycine, glutamate, prostaglandins (PGs), leukotrienes, substance P, tumor necrosis factor alpha, cyclic adenosine monophosphate (cAMP), and nerve growth factor (NGF).

Various noxious stimuli depolarize peripheral fine nerve endings and cutaneous tissues (Zhao et al., 2008). The depolarization of primary afferent fibers results in transmission of action potentials to the spinal cord via the cell bodies of the dorsal root ganglia (DRG). These signals undergo processing by spinal targets including modulation by interneurons (Todd, 2010). Afferent signals from the periphery may also bypass processing in the dorsal root ganglia and

undergo the first stage of processing in spinal interneurons. After modulation by spinal interneurons the signal is further transmitted to pain processing regions in the ventrobasal thalamus and higher order cortical regions. Primary afferent nociceptive fibres are comprised of two distinct classes, Aδ and C fibres, based on differences in gross morphology, diameter and conduction velocity. The axons of Aδ fibres (3-8 μm in diameter) have thin myelination facilitating fast reflex responses to pain such as hot element or extreme cold prior to psychological perception of such pain (Basbaum et al., 2009).

C fibres are much thinner (0.2 – 1.5 μm diameter), unmyelinated, and conduct neuronal signals at a slower rate (0.5 – 2.5 m/s). By comparison, Aδ fibres transmit signals at a much higher speed (2 to 4 m/s, Basbaum et al., 2009). The rapid conduction velocity of Aδ fibres compared to that of C fibres - a consequence of the former's relatively large diameter and myelination, is consistent with evolutionary arguments for rapid pain responses (Sneddon et al., 2003). Aδ fibres respond to dangerous stimuli requiring immediate action to ensure survival and have evolved to achieve this. In contrast, C fibre fine nerve endings are nonspecific and respond to a variety of noxious stimuli, transmitting dull non-discriminating pain arising from substances such as K^+, acetylcholine, proteolytic enzymes, serotonin, prostaglandins, substance P and histamine.

1.3.1 Neospinothalamic tract

The neospinothalamic pain transmission tract mediates sharp pain, commonly referred to as "pin-prick pain", transmitted along Aδ fibres which synapse in the dorsal horn and the lamina I region in which dendrites of spinothalamic neurons are present. The axons of second order neurons

decussate in the anterior white commissure zone and ascend via the contralateral side along the lateral spinothalamic tract (Garcia, 2009). These axons terminate in the ventroposterolateral (VPL) and ventroposteroinferior nuclei (VPFL) of the thalamus among other brain regions. The VPL thalamus integrates pain signals and provides discrimination between frequency and pain information (Sinclair et al., 1991) before transmitting the signal to the primary somatosensory cortex (S1) (Liu et al., 1989).

1.3.2 Paleospinothalamic tract

The paleospinothalamic tract is more primitive evolutionarily, and ascends bilaterally up the spinal cord (Garcia, 2009). Nociceptive fibers, mostly of the unmyelinated C-type, synapse in the substantia gelatinosa (laminae II & III) of the spinal cord. Second-order interneurons are receptive to multiple types of afferent information, receiving direct input from both mechanoreceptors and thermoreceptors (Garcia, 2009). These fibers synapse with neurons in laminae IV to VIII which ascend bilaterally along 3 tracts: the spinomesencephalic/spinoreticular tract (which terminates at the mesencephalic reticular formation), the spinotectal tract (which terminates in the periaqueductal gray and hypothalamus) and the spinohypothalamic tract (terminating at the intralaminar (IL) thalamus). The tracts are collectively referred to as the anterospinothalamic tract. Paleospinothalamic neurons innervate emotional affect centers (including the limbic system, cingulate cortex, hypothalamus, basal ganglia, insular cortex and S2) (Garcia, 2009).

1.3.3 Archispinothalamic tract

The archispinothalamic tract is the oldest nociceptive system (Garcia, 2009). Dorsal root afferents synapse with interneurons in the substantia gelatinosa which synapse in laminae regions IV to VII with spinal tract neurons. Nociceptive impulses then ascend bilaterally along the multi-synaptic tract to the mesencephalic reticular formation, periaqueductal gray, thalamus, hypothalamus and limbic system.

1.4 Translation pain testing preparations *in vivo*

Many animal models of pain have demonstrated predictive translational efficacy and are widely used in the development of new pain drugs, and to advance understanding of the pathophysiological and etiology of pain (Le Bars et al., 2001). While pain is subjective by definition, quantitative surrogates of pain can be measured to provide meaningful information about the analgesic efficacy of various compounds in different pain models. For example, some assays measure changes in frequency or intensity of paw withdrawal responses to noxious stimuli (Le Bars, 2001). Other assays evaluate the degree of nocifensive behaviour (defined as any defensive response to pain) produced by acute noxious stimuli. Standard assessments of acute hypersensitivity to pain include von Frey mechanical allodynia tests, the hot water tail-flick test and hot plate tests in rodents (Le Bars, 2001).

1.4.1 Tail-flick assays

In tail-flick tests, the tip of a rat or a mouse's tail is placed under a heat source such as radiant heat, or is submerged in a hot water bath. In this test, latency to withdrawal (also known as tail flick latency) is measured. Tail withdrawal is a nociceptive spinal reflex; severing or pharmacologically blocking nerve conduction of the upper spinal cord does not influence tail flick response time effects (Grossman et al., 1982). The variance is very low in this model, which is one reason why it forms the basis for recent pilot studies probing local mechanisms of action of novel analgesics including isovaline. Nociceptive afferent fibres from the tail enter between the sacral S4 and coccygeal Co3 segments of the spinal cord, which transmit signals to dorsal horn interneurons and to subsequent relay centres. These interneurons are synaptically connected to motor neurons of the segmental lumbar L4 to coccygeal Co3 areas that innervate muscle control over the tail (Garcia, 2009).

Underlying mechanisms for thermal nociception include activation of specific temperature-sensitive receptors, which occurs in many fibres. Temperature-dependent increases in neuronal responses are reflected by increased motor output (Le Bars et al., 2001). In rodents, neurons first begin to respond at 36°C, but an actual tail flick response occurs at 40°C. From this baseline, the intensity follows a non-standard stimulus-response relationship, with increased responses until a plateau is reached between 48 and 54°C. This is taken to be a saturating response temperature. When morphine is administered at 1 to 2 mg/kg, direct suppression of sacral spinal neuronal activation is achieved with ensuing tail flick response blockade (Le Bars et al., 2001). However,

many non-opioid analgesics such as NSAIDs (aspirin, naproxen, ibuprofen, and indomethacin) are ineffective in blocking tail flick responses (Spencer, 1976).

The tail immersion test is considered to be among the most robust and consistent of all tail-flick assays (Le Bars et al., 2001). This test is advantageous as it allows for a perfectly uniform heating of the tail and raises cutaneous temperature in a linear fashion. It also facilitates use of tourniquet constriction devices which can be harnessed to localize drug effects (Pau, 2000). The observed responses elicited by this test are insensitive to tail pigmentation, and the exact positioning of the heat source facilitates a high degree of reproducibility over time. In opioid analgesia, the water bath is generally set near the upper limit of 55°C to ensure detection. Temperature can be varied for specific purposes such as testing of partial agonists (Spencer, 1976).

1.4.2 Acute chemical pain

Rodents injected with formalin exhibit a biphasic pattern of paw licking and flinching, which can be used as a model of both acute and chronic pain (Le Bars, 2001). Formalin is normally administered in 0.5-15 % formalin solution, injected subcutaneously into the paw of a mouse or rat (Le Bars, 2001; Kassuya, 2007). The pain behaviour in the formalin model is characterized by the scoring of posture, frequency or duration of nocifensive response. The early phase I nociceptive response lasts approximately five minutes and is followed by a period of quiescence. A second phase of painful behaviour initiates at approximately 20 min and continues for 60 to 90 minutes (Le Bars, 2001). These two phases can be selectively inhibited by different classes of

drugs. Phase I licking responses are based on direct activation of C-fibers by peripheral formalin, but prostaglandin induced inflammation is associated with phase II responses (Hunskaar and Hole, 1987). Opioid based analgesics such as morphine and codeine have been shown to inhibit both phase I and phase II responses, while indomethacin and naproxen affect phase II, but not phase I responses. The non-proteinogenic amino acid isovaline has been shown to reduce both phases without detectable adverse CNS effects (MacLeod et al., 2010). Furthermore, NBQX, an alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor antagonist is capable of selectively ablating phase I formalin responses without motor dysfunction. The NMDA receptor antagonist 2-amono-5-phosphono valerate (AP5) can selective inhibit the second phase, attributable to inhibition of rat dorsal horn neurons (Hunter, 1994). Direct administration of PGE_2 into the mouse hindpaw produces allodynia to pressure induced by von Frey filaments, lasting approximately 30 minutes (Kassuya et al., 2007). Isovaline has been previously shown to attenuate phase II licking responses in this model (MacLeod et al., 2010).

Mechanical allodynia induced by PGE_2 can be assessed by measuring responsiveness to application of calibrated von Frey pressure filaments. PGE_2 mimics many aspects of inflammatory pain and has the added advantage of minimizing animal discomfort, over alternatives such as formalin (Kassuya et al., 2007). Allodynia resulting from PGE_2 administration can be quantified using a series of calibrated filaments applied to the plantar surface of the mouse or rat hindpaw (Reis and Duarte, 2006; Kassuya et al., 2007). This model was selected for a portion of the *in vivo* studies as it provides a reproducible means to assess local inflammatory pain, which is relevant to the study of peripheral drug action. It also facilitates reduced animal suffering over many alternative models.

1.4.4 Chronic pain

Chronic pain refers to painful conditions, but can include repeated acute pain events or pervasive uninterrupted pain signals (Richards et al., 2013). Chronic pain describes pain that is sustained beyond the natural time course required for healing. From a public health and human welfare standpoint, chronic pain associated with progressive terminal illness or joint malfunction is a huge burden to society, and costs an estimated $635 billion in the United States alone (Gaskin et al., 2012), with proportionate costs in Canada..

Chronic pain is not restricted to those with advanced diseases; over one third of all respondents indicate that they experience pain on a daily basis (Manchikanti et al., 2003). The negative impact of chronic pain on quality of life poses a substantially larger economic impact than the pain-independent economic impacts of cancer and cardiovascular diseases taken together (Breivik et al., 2006). Chronic pain can also arise from central pain states (including those resulting from central sensitization from repetitive peripheral afferent input). The CNS can become sensitized and activated without external stimuli (Woolf, 2011). An example of this phenomenon is “phantom limb pain”, in which individuals experience pain that originates from a non-existent limb (Ramachandran, 2000). Damage to afferent nociceptive circuitry causes abnormal signal processing. Currently used analgesics and sedatives are not effective for treatment of these unusual conditions; however, novel psychological techniques designed to create the illusion of intact limbs can provide relief (Ramachandran et al., 2000). Truly chronic pain models use nerve cutting or intra-articular ligament transaction ligation to induce long-term damage resulting in continuous pain (Le Bars, 2001).

1.4.5 Osteoarthritis

Osteoarthritis (OA) is a degenerative joint disease involving degradation of articular cartilage and subchondral bone; it affects over 80 percent of the population over the age of 75 (Busija et al., 2013), and commonly occurs in the knee, hand and hips. Analysis of x-rays from affected joints show loss of articular cartilage, subchondral remodeling and joint misalignment. Through the course of disease progression, joint flexibility is impaired until complete replacement is undertaken (Fernihough et al., 2004). However, alternatives such as exercise and strengthening can reduce many of the debilitating effects of osteoarthritis, combined with usage of non-steroidal anti-inflammatory drugs (NSAIDs). These measures are only partially effective. The most incapacitating aspect of osteoarthritis is not so much joint dysfunction as the accompanying pain which leads to inactivity, further increasing joint degeneration and muscle atrophy which exacerbates chronic pain (Fernihough et al., 2004). It has been estimated that more than 10 percent of the world population over 60 years of age suffers from osteoarthritic pain (WHO Scientific Group on the Burden of Musculoskeletal Conditions at the Start of the New Millennium, 2003). Sensitization of afferent peripheral nociceptive pathways is thought to be the principal cause of osteoarthritic pain, but central mechanisms are also involved (McDougall, 2006).

Both peripheral and central neuronal pathways undergo plasticity during inflammation which gives rise to hyperalgesia and allodynia. During induction of osteoarthritis, normally inactivated afferent nociceptors are recruited as a result of inflammation and tissue injury (Le Bars, 2001), resulting in peripheral sensitization. Another change which has been observed is reduced activation thresholds for synovial membrane nociceptors and mechanoreceptors (Le Bars, 2001).

In arthritic rodents, afferent firing rates of neurons increase in response to normal movement and to flexion hyperextension (McDougall, 2006). The CNS can become sensitized as a result of increased peripheral input, reducing the pain threshold for afferent signals from the periphery (Woolf, 2011).

Current models of osteoarthritis range from spontaneous models in aged mice to genetically modified rodents and experimentally-induced models involving ligament ligation or excision. Injections of noxious substances intra-articularly (including the metabolic inhibitor monoiodoacetate) reproduce many aspects of arthritic pain (Bove et al., 2003). Past research has focused predominantly on pathological joint changes and chemical mediators for osteoarthritis to better understand the initiation and progression of osteoarthritic dysfunction (Ameye and Young, 2006). The pain aspect of osteoarthritis has not been a major focus of past models; however it has been shown that intra-articular administration of monosodium iodoacetate into rat knee results in pain behaviours and biochemical joint changes that are similar to those seen in human osteoarthritis (Le Bars et al., 2001). Monoiodoacetate inhibits glyceraldehyde-3-phosphate dehydrogenase, disrupting glycolysis and resulting in chondrocyte death. The cartilage degeneration mimics many aspects of osteoarthritis seen in humans (Fernihough et al., 2004).

Two phases of pain commonly occur in the model. First, an acute inflammatory response occurs during the week following MIA injection. In the chronic stage of the model, heightened sensitization to pain is not a result of inflammation, but rather peripheral sensitization. Chronic osteoarthritic pain occurs after 14 days and lasts in excess of 2 months (Combe et al., 2004).

Other behavioural aspects of osteoarthritis include mechanical hyperalgesia, static allodynia and weight distribution differences between ipsilateral and contralateral paws. These behavioural manifestations of arthritis are reversible by administration of conventionally used analgesics such as morphine, but are not substantially altered by peripherally acting NSAIDs (Fernihough et al., 2004).

While treatments are presently focused on reducing inflammation and producing analgesia, currently used therapies are only partially effective (McDougall, 2006); many individuals continue to experience a high level of pain (Michael et al., 2010). It is therefore critical to develop alternative safer therapies with reduced side effects and increased blockade of pain. Many analgesics with novel mechanisms are in the pipeline; however very few have focused on peripheral selectivity as an overarching prerequisite for analgesic drug development.

1.4.6 Models of allodynia

The concept of allodynia refers to the state of sensitization to otherwise innocuous stimuli and was first introduced based on the observation that large undamaged areas of skin in the surrounding area of a local cutaneous injury can become hyperalgesic to mechanical stimuli (Sandkühler, 2009). Hyperalgesia refers to a state of increased pain from a stimulus that normally provokes pain whereas allodynia refers to pain due to a stimulus that does not normally provoke pain (International Association for the Study of Pain). Hyperalgesia occurs at the site of local injury (which is also subject to primary hyperalgesia) as well as the surrounding normal and undamaged tissues (Holtman et al., 2012). Gentle touch elicits a heightened to pain response (for ex-

ample, responsiveness to a painful pinprick). Both hyperalgesia and allodynia have been observed directly within the area of primary/secondary hyperalgesia. It has been proposed that spreading hyperalgesia to surrounding undamaged tissue areas is mediated by peripheral nerves.

PGE_2 is one of the main constituents of the "inflammatory soup" referring to a host of compounds released in response to injury or under conditions of inflammation. PGE_2 is part of the arachidonic acid pathway and its metabolic formation is downstream of the cyclo-oxygenase pathways that NSAIDs inhibit. Injection of intraplantar PGE_2 in low quantities is commonly used to produce sensitization to otherwise non-painful stimuli, such as low force von Frey filaments which are undetectable under normal conditions. In this model, PGE_2 is injected in nanomolar quantities; allodynia emerges during a 15 minute period and lasts for up to one hour (Kassuya et al., 2007). Co-injection of locally acting analgesic substances such as isovaline, GABA or baclofen allows for assessment of local pharmacological mechanisms of action. Allodynia elicited by local injected PGE_2 is referred to as static allodynia, because the type of hypersensitivity observed is hypersensitivity to static applied touch such as pressure.

1.4.7 Tourniquet intravenous regional analgesia

A tourniquet is a constriction device which compresses blood vessels around an extremity of interest, preventing blood flow to and from the area distal to tourniquet applications. Post-surgical pain has been reported by individuals after surgical procedures in which pneumatic tourniquets have been used. Here a severe dull aching pain directly at the site of tourniquet application was reported. This type of pain is generally referred to as tourniquet pain (Gielen et

al., 1991). While the underlying mechanisms of tourniquet pain are subject to further investigation, tourniquets can be utilized as powerful experimental tools to better understand peripheral pain mechanisms and the physiology of pain and allodynia. Tourniquet usage in rodents is less invasive than injection of the many noxious substances used to produce allodynia, including capsaicin, formalin, nociceptin and mustard oil (Calo et al., 1998). Others models, such as spinal nerve ligation of the L5 and L6 segments in rodents, have also been asserted to be inhumane by comparison.

The application of a tourniquet around the tail or limb of an animal such as a rat has been found to be very effective in localizing drug action and in producing post-ischemic reperfusion allodynia (Pau, 2000). Post-ischemia reperfusion allodynia is observed after tourniquet application for 60 min or less to the base of the tail (Gelgor et al., 1986). Tourniquets can allow for selective assessment of peripheral drug actions when drugs are injected intravenously into the tail, after the tourniquet has been applied and before the onset of ischemia (Pau, 2000). This technique has been used in other animals as well, including rats (Gelgor et al., 1986), to induce hyperalgesia and allodynia.

Localized drug actions can be assessed using a tail tourniquet in the period prior to allodynia-inducing ischemia; previous experiments have indicated a 10 minute window period before ischemic effects are seen. Here blood is trapped in the tail and is completely isolated from systemic circulation (Pau, 2000). This could allow for the assessment of cutaneous specific actions of inhibitory agents such as GABA, isovaline, or baclofen. Additionally, the possibility of

systemic injections and ensuing effects of ischemia reperfusion-induced allodynia facilitate assessment of the role of specific nociceptive fibres and interactions between peripheral and central nervous systems in the perception of pain. This tool constitutes a means for further evaluation of the local effects of isovaline, and forms the basis of ongoing studies.

1.5 Analgesics, inhibitory amino acids and their receptors

Isovaline was originally identified as a potential analgesic compound based on the discovery of $glycine_A$ receptors in ventrobasal nociceptive thalamus (Ghavanini et al., 2005). While isovaline had the highest analgesic efficacy of several glycine analogs tested with no observable adverse effects (MacLeod et al., 2010), its neuronal effects were unexpectedly found to be produced via G protein coupled effects to $GABA_B$ receptors, instead of glycine receptors previously predicted.

Neurotransmitter receptors can be classified into two major categories: ionotropic and metabotropic receptors - both are activated by specific ligands. Ionotropic receptors form an ion channel pore, which opens upon activation by a ligand, allowing ions such as Na^+, K^+, or Cl^- to flow (Smits et al., 2012). In contrast, activated metabotropic receptors modulate multi-step intracellular signaling cascades, typically initiated by a G protein coupled to the receptor, providing indirect linkage to ion channels (Jessell et al., 2000).

The four classes of inhibitory receptors that are most relevant to this thesis are: $GABA_A$ (Smits et al., 2012), $glycine_A$ (Lynch, 2004), $GABA_B$ (Bettler et al., 2004) and metabotropic glutamate

(mGluR) receptors (Bonsi et al., 2005), which have been characterized in the spinal cord, ventrobasal thalamus and select peripheral tissues.

1.5.1 Non-steroidal anti-inflammatory drugs and acetaminophen

NSAIDs including over-the-counter drugs such as aspirin (acetylsalicylic acid), ibuprofen and naproxen, are the most widely used class of analgesics for treatment of pain. The chief advantages of NSAIDs include the absence of many CNS effects, and for the case of aspirin, anticoagulant health benefits.

While largely successful in reducing inflammation and its associated pain, NSAIDs are not adequately efficacious for chronic or neuropathic pain and produce adverse gastrointestinal side effects (Hooper et al., 2004). Acetaminophen (Tylenol), while NSAID-like in structure, is not anti-inflammatory and acts by unknown mechanisms to provide more effective relief for some types of headache pain. However, it produces acute liver toxicity at approximately 10 times the recommended therapeutic dose, which significantly limits its amenability to chronic usage.

Salicylates originated from the ancient discovery that willow park, boiled and administered in tea, produce analgesic and anti-inflammatory effects. This observation by Hippocrates dates back to 400BC, in which documentation on the usage of willow bark and characterization of its effects in a variety of types of pain were recorded. Birch tree branches (which contain similar compounds), have also been used throughout history in Nordic saunas, as a means of producing

topical pain relief (Nordskog et al., 2010). An active ingredient in Willow Bark, salicylic acid, is the main metabolite of Aspirin. While the mechanistic framework of salicylic acid was originally unknown, the observed anti-inflammatory effects of willow bark gave rise to the refinement and development of arguably the world's primary analgesic drug class.

NSAIDs reduce inflammation by inhibiting COX-1 and COX-2 enzymes, which convert arachidonic acid into prostaglandins to produce inflammation among other functions. Blockade of COX-1 and COX-2 reduces inflammation and pain associated with excessive inflammation. Adverse effects of NSAIDs include gastrointestinal bleeding and ulcers, and renal dysfunction due to generalized COX-1 (Norojan et al., 2012). COX-2 selective NSAIDs have been developed (including celecoxib and rofecoxib) to bypass these effects and reduce the formation of gastric ulcers (Drazen, 2005). However, they produce effects similar to COX-1 inhibitors in the kidney and cause a significantly increased risk of thrombotic myocardial infarctions (Rodriguez et al., 2000).

1.5.2 Opioids

Opioids are the main class of analgesics for treatment of severe pain, and are widely used in the management of postoperative pain, and during anesthesia to ensure immobility and analgesia to incision stimuli and visceral operating procedures (Ezici et al., 2013). While endogenous opioids have been identified, the prototypical exogenous opiate morphine was originally isolated from opium, with origins of recreational abuse in ancient India and Chinese opium lounges. As in the case of NSAIDs, although for divergent purposes, the underpinnings of morphine's discovery

and further refinement were based exclusively on historical usage. Modern opioids have been developed to produce desirable fast onset pharmacokinetics: these include fentanyl (i.v. half-life, 2.5 min) and remifentanyl (i.v. half-life, 12 min), which are commonly used as anesthetic adjuvants (Alves et al., 2007). Usage of opioids also has the disadvantage of yielding rapidly induced drug tolerance, hyperalgesia (Juni et al., 2006), hyperactivity (Schnur et al., 1987) and addiction (Kirsh et al., 2012). Efforts to develop peripherally restricted opioid agonists for treatment of pain are currently being undertaken (Janson et al., 2003).

1.5.3 Gabapentinoids

Gabapentinoids are a classic of analgesics based on the structures of gabapentin and pregabalin (Yu et al., 2013), which are analogs of GABA designed to enter the CNS. Gabapentin, the first agent developed by Pfizer, was originally intended to treat epilepsy. Gabapentin is essentially GABA with a cyclic pentyl group, which facilitates crossing through the blood-brain barrier (BBB) into the CNS to produce GABAergic inhibition. However, gabapentin has not been shown to act on the same receptors which GABA acts on. Instead its mechanism involves inhibition of Ca^{2+} channels in the CNS (Davies et al., 2007). While gabapentin is approved for alleviation of fibromyalgia and neuropathic pain, gabapentin's common side effects consist of dizziness, fatigue, weight gain, drowsiness, and peripheral edema (swelling of extremities) and increased risk of suicide (US FDA Announcement); these effects are observed in all ages; additionally, in children 3-12 years old, mild-to-moderate mood swings, hostility, concentration problems, and hyperactivity are noted. Hepatotoxicity and renal impairment have also been observed, although

these effects are less frequent (Lasso-de-la-Vega et al., 2001). Gabapentin is not recommended for patients with renal impairment due to accumulation and toxicity.

Pregabalin is the newer anticonvulsant and analgesic gabapentinoid sold by Pfizer, originally invented by Richard Bruce Silverman from Northwestern University. Recent studies have indicated that pregabalin is effective for treatment of chronic pain arising from fibromyalgia and spinal cord injury; it is currently one of only two drugs approved specifically for treatment of fibromyalgia (the other being duloxetine Capitals are used for trade names) (Crofford et al., 2005). The United States' Food and Drug Administration has approved pregabalin as an adjunct treatment for patients with partial onset seizures, post herpetic neuralgia, along with neuropathic pain resulting from diabetic peripheral neuralgia. It is also used in the treatment of generalized anxiety disorder (Bandelow et al., 2007). The chemical structure of pregabalin is distinct from gabapentin – its onset anxiolytic effects confer several advantages over competing agents such as benzodiazepines. The most common adverse effects of pregabalin, which occur in over 10 percent of patients, are dizziness and drowsiness, while other common side effects include blurred vision, euphoria, confusion, vivid dreams, libido changes, irritability, dry mouth, constipation, and vomiting. Similarly to gabapentin, pregabalin can also cause withdrawal after long-term usage if discontinuation is abrupt. When used for treatment of epilepsy, withdrawal can exacerbate seizures. Less frequently, depression, lethargy or agitation is observed. While the compound differs considerably in structure from gabapentin, the mechanism of action is presumed to act much in the same way, through binding to the α2δ subunit of the voltage-dependent calcium channel in the CNS. Additionally pregabalin is thought to reduce the release

of neurotransmitters including glutamate, norepinephrine, substance P and calcitonin gene-related peptide (CGRP) (Micheva et al., 2006).

1.5.4 Glycine

Glycine is the smallest amino acid and commonly facilitates tight turns in protein α-helices. Glycine is one of the principal inhibitory neurotransmitters and has been shown to be involved in modulation of pain (Ghavanini et al., 2005). While glycine receptors had originally been assumed to be found only in caudal regions of the neuraxis (Ghavanini et al., 2005), they were recently identified in ventrobasal nuclei of the thalamus suggesting a more involved role in modulation of pain. The most ubiquitous subtype of glycine receptor is the $glycine_A$ receptor, which contains the primary unmodulated, i.e., orthosteric, binding sites for agonists and antagonists glycine and strychnine, respectively.

$Glycine_A$ receptors are heteropentamers, comprised of both α and β subunits (Lynch, 2004). The α subunits are responsible for ligand-binding whereas the β subunits are responsible for trafficking receptors via interaction with gephyrin anchoring protein (Lynch, 2004). Some functional glycine receptors are made exclusively of homomeric α subunits (Kirsh et al., 1995); however, this subtype is not strongly expressed in adult neuronal cells (Kirsch et al., 1995). β subunits do not form functional homomers (Bormann et al., 1993), as shown by their inability to bind ligands such as glycine and strychnine (Lynch, 2004). Glycine receptors are assembled with stoichiometry of either 3α and 2β subunits or 2β and 3α subunits (Lynch, 2004). Additionally, 4 distinct α subunits have been identified (α1-4) in addition to a single additional distinct beta

subunit; in the brain, the α1 subunit is the most prominent isoform (Lynch, 2004). Most functional glycine receptors expressed in synapses are composed of heteromers. In young animals, α2 subunits are prevalent – this expression drops over time (Becker et al., 1988). On the other hand, the α1 subunits increase with age (Lynch, 2004). The expression of α3 subunits seems to be largely restricted to nociceptive areas of nervous system circuitry, specifically the spinal dorsal horn; this was determined via experiments indicating that knockout mice for this subunit were immune to intrathecal inflammatory hyperalgesia induced by prostaglandin E_2 (Harvey et al., 2004). The expression of the α4 subunit is restricted to prenatal development (Harvey et al., 2000). There are two commonly used antagonists for glycine receptors: strychnine and the Cl^- channel blocker picrotoxin (an antagonist of $GABA_A$ and $GABA_C$ receptors, with higher potency than strychnine). Strychnine is the most commonly used selective antagonist for glycine receptors, and has potent proconvulsant effects *in vivo*. The binding site for strychnine is the glycine α1 subunit (Lynch, 2004). Glycine receptors, like $GABA_A$, are ionotropic, permeable to Cl^- ions when activated. The activation of glycine receptors in juvenile animals leads to depolarization of the neuronal membrane whereas activation of glycine receptors in adult animals hyperpolarizes membranes (Wang et al., 2005). The discovery of glycine receptors in nociceptive thalamus was critical in the development of isovaline as a promising amino acid analgesic (Ghavanini et al., 2005). Glycine receptors are also known to be present in the brainstem trigeminal nucleus, innervating facial regions, and implicating possible involvement in trigeminal neuralgia. Trigeminal neuralgia is a severely painful remitting and relapsing disorder, considered to be the most excruciatingly painful condition known (Malosio et al., 1991). The targeting of trigeminal glycine receptors may be relevant for development of novel models to screen potential therapeutics for treatment of trigeminal dynamic allodynia in humans.

1.5.5 ɤ-aminobutyric acid (GABA)

ɤ-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in mammalian and invertebrate nervous systems. GABA is an endogenous agonist for both $GABA_A$ and $GABA_B$ receptors, and is synthesized on demand in the brain (Watanabe et al., 2002). GABA does not cross the blood-brain barrier and active mechanisms exist to import and export GABA to maintain the necessary concentrations in the brain to avoid widespread disruption in the function of various neural networks (Kakee et al., 2008). GABA itself could be a promising peripherally restricted analgesic, acting on peripheral $GABA_B$ and $GABA_A$ receptors (Jasmin et al., 2004). However, speculation has arisen regarding its potential efficacy, specifically, that hyperalgesic actions may arise from $GABA_A$ receptor activation under neuropathic pain conditions due to gradient reversal in the KCC2 K^+ Cl^- transporter (Hasbargen et al., 2010).

1.5.6 $GABA_A$ receptors

$GABA_A$ receptor-mediated antinociception involves actions on spinal and peripheral $GABA_A$ receptors (Knabl et al., 2008). $GABA_A$ receptors are ionotropic Cl^- channel coupled receptors and their activation under normal physiological conditions produces inhibition of neuronal firing due to transient equilibration of Cl^- down its concentration gradient. $GABA_A$ receptors are pentamers composed of heterogeneous subunits arranged to form a chloride permeable pore. $GABA_A$ receptor activation generally results in inhibitory post-synaptic currents (IPSCs). The consequences of Cl^- chloride channel activation depend on the gradient of chloride on either side of the membrane. Under neuropathic pain conditions the KCC2 K^+-Cl^- pump is upregulated, switching the Cl^- gradient to the opposite direction, postulated to result in hyperalgesia in

response to $GABA_A$ receptor activation. Furthermore, the Cl^- gradient is shifted in an age-dependent fashion; lower concentrations of Cl^- exist outside the cell in neonates than in adults. Spinal $GABA_A$ receptors have also been linked to hyperalgesia based on studies involving intrathecal administration of the $GABA_A$ receptor antagonist bicuculline. Such differential expression can lead to reduced likelihood of firing in the postsynaptic neuron by raising thresholds for action potential generation. However, synaptic and extrasynaptic $GABA_A$ receptors contain different proportions of each isoform, one of many considerations which make it difficult to predict whether the effects of chloride gradient direction reversal would lead to inhibition or excitation (Enna and McCarson, 2006).

1.5.7 $GABA_B$ receptors

$GABA_B$ receptors play an important role in the mechanism of action of isovaline and in the rationale for the development of peripheral restricted $GABA_B$-modulated analgesia. Unlike $GABA_A$ receptors, $GABA_B$ receptors are metabotropic G protein-coupled receptors, meaning their activation results in modulation of intracellular G protein-coupled signalling cascades. To form an intact receptor they are express as heterodimers which consist of $GABA_{B1}$ and $GABA_{B2}$ receptor subunits (Kaupmann et al., 1998). Each constituent subunit is a 7-transmembrane spanning entity with an extracellular area known as the “Venus flytrap domain” wherein a bound ligand results in the closing of two extracellular domain lobes (see Fig 1.1; Galvez et al., 1999). The $GABA_{B1}$ subunit contains the main orthosteric agonist binding site. The receptor is activated by ligand binding at this $GABA_{B1}$ subunit whereas the $GABA_{B2}$ subunit is an activator of G-proteins that inhibit adenylyl cyclase by means of a coupling mechanism to intracellular G-

proteins linked to downstream activation of K^+ channels. An important function of the $GABA_{B2}$ receptor subunit is facilitating the trafficking of the $GABA_{B1}$ receptor subunit to the cell membrane. This was demonstrated by the observation that cells without $GABA_{B2}$ subunits have the tendency to accumulate large volumes of $GABA_{B1}$ receptors (Kaupmann et al., 1998). If the $GABA_{B2}$ receptor subunit is inserted into the cell membrane by itself, it is usually unable to function as a receptor largely due to its inability to bind ligands (Couve et al., 1998). Another consideration is the possibility raised by the suggestion that the $GABA_{B1}$ receptor subunit may be expressed as two distinct subtype isoforms: $GABA_{B1a}$ and $GABA_{B1b}$ subunits. The chief difference between these two subunit subtypes is the existence of N-terminal interaction motifs (Bettler and Tiao, 2006). The $GABA_{B1a}$ subtype is localized in axon terminals while the $GABA_{B1b}$ subtype is not present in the presynaptic terminal based on a lack of required coding domains (Biermann et al., 2010). Efforts have been undertaken to identify pharmacologically distinct $GABA_B$ receptor isoforms (Cunningham and Enna, 1996). However, these experiments have not been reproduced, and are not well supported by current computer models of receptor action (Bettler and Tiao, 2006). There is therefore no current scientific consensus supporting the existence of pharmacologically distinct $GABA_B$ receptors.

$GABA_B$ receptor agonists include the endogenous neurotransmitter GABA and the prototypical, selective, brain penetrant $GABA_B$ receptor agonist baclofen ((RS)-4-amino-3-(4-chlorophenyl) butanoic acid), which can be used to identify the presence of $GABA_B$ receptors (Bowery et al., 1980). GABA and baclofen are differentiable based on their mechanisms of $GABA_B$ receptor activation: GABA requires the presence of Ca^{2+}, while baclofen does not (Galvez et al., 2000). There are two known positive allosteric modulators of the $GABA_B$ receptor: GS39738 and

CGP7930, both of which bind the hepta-helical area located on the $GABA_{B2}$ receptor subunit domain (Adams et al., 2007). These drugs do not activate the $GABA_B$ receptor when applied in isolation but amplify responses of orthosteric agonists acting on the $GABA_{B1}$ subunit. A host of $GABA_B$ receptor antagonists exist which prevent activation of $GABA_B$ receptors including: CGP35348 and 2-OH saclofen along with newer agents such as the highly potent CGP52434 used in this study (Lanza et al., 1993).

Activation of $GABA_B$ receptors leads to a downstream cascade of G protein-coupled events, and the exchange of a molecule of guanosine triphosphate (GTP) for one of guanosine diphosphate (GDP); the change in conformation allows both G-protein subunits (Gα and Gβɤ) to modulate their respective downstream proteins (Fig 1.1) (Brown and Sihra, 2008). The Gβɤ subunit directly activates inwardly rectifying K^+ (GIRK) channels (Bettler et al., 2004). The Gβɤ subunit directly influences the activity of Ca^{2+} channels, leading to their closure (Callaghan et al., 2008). Distinct populations of K^+ channels can also be activated through second messenger cascades through Gα subunit dissociation including activation of leak K^+ channels and outwardly rectifying K^+ channels (Saint et al., 1990). The Gα subunit is an inhibitor of adenylyl cyclase producing reduced cyclic adenosine monophosphate (AMP) concentrations with resultant lowering of protein kinase A (PKA) concentrations (Hill, 1985). This modification leads to downstream changes in a variety of transcription factors and protein kinases (Diversé-Pierluissi et al., 1997). Activation of adenylyl cyclase is terminated by endogenous GTPase activity associated with the Gα subunit. Upon exchange of one GTP molecule for a GDP, Gα re-associates with the Gβɤ subunit, the G-protein cascade. GTPase properties of the Gα subunit are

influenced by G-protein signalling regulatory proteins (RGS), enhancing rates of G-protein activation, and inactivation (Fowler et al., 2007).

Due to multiple downstream mechanisms, $GABA_B$ receptor functions can be influenced by changing various intracellular enzyme concentrations. For example, Protein Kinase A (PKA) has been demonstrated to be involved in regulating desensitization of $GABA_B$ receptors. An externally imposed increase in PKA activity is reduced after $GABA_B$ receptor activation, promoting $GABA_B$ receptor desensitization (Couve et al., 2002). PKA-induced desensitization results from internalization of $GABA_B$ receptors and phosphorylation of a $GABA_{B2}$ amino acid residue. However, inhibition of N-methylmaleimide-sensitive factor (NSF) proteins known to be associated with $GABA_B$ receptors prevents $GABA_B$ receptor desensitization (Pontier et al., 2006). Activation of the $GABA_B$ receptors leads to the activation of Src kinase (Diversé-Pierluissi et al., 1997). Src kinase can either increase or decrease (Fadool et al., 1997) the amount of K^+ current activated by $GABA_B$ receptors, and includes modification of the outwardly rectifying transient A-type K^+ channels Ia current along with inwardly rectifying Kir channels (Fadool et al., 1997). There are a total of 15 different $GABA_B$ receptor subtypes found on both pre- and post-synaptic membranes. Pre-synaptic $GABA_B$ receptors can be autoreceptors or heteroreceptors depending on whether they are expressed on inhibitory or excitatory terminals (Bettler and Taio, 2006). Presynaptic $GABA_B$ receptors inhibit neurotransmitter release primarily through inhibition of Ca^{2+} influx into presynaptic terminals (Mintz and Bean, 1993). Receptors coupled to K^+ channels appear to be less common at presynaptic sites; and the tendency is for postsynaptic $GABA_B$ receptors to be coupled to K^+ channels (Luscher et al., 1997).

In the CNS, $GABA_B$ receptors are expressed opposite to postsynaptic terminals around the base of dendritic spines in synapses which predominantly transmit glutamate (Kulik et al., 1998). Thus, postsynaptic $GABA_B$ receptor conductance activation diminishes the magnitude of excitatory postsynaptic potentials (Bettler and Tiao, 2006). The activation of postsynaptic $GABA_B$ receptors arises from spillover of GABA released at GABAergic synapses (Bettler and Tiao, 2006). Upregulation of ambient GABA release is a major factor responsible for the fading of pain perception over time presumably through activation of postsynaptic $GABA_B$ receptors and the ensuing inhibition of afferent nociceptive drive (Enna and McCarson, 2006).

1.5.8 Isovaline

Isovaline is a non-proteinogenic α-amino acid that is a structural analog of both glycine and GABA (Fig 1.2), originally as a racemate with enantiomeric excess for the L-conformation in a meteorite in Murchison, Australia (Kvenvolden et al., 1971). The observed excess has also been noted in a small cohort of related amino acids which are believed to have been involved in the origin of enantiomeric excess in early evolution of biology on earth, preserved in all extant organisms (Pizzarello et al., 2000). Isovaline is not found in humans or mammals but is synthesized in abiotic peptides constructed by filamentous fungi (Elsila et al., 2011). This class of fungi includes the genera Emericellopsis and Trichoderma, which are able to synthesize antibiotics that contain peptides with α-aminoisobutyrate (AIB) (Brückner et al., 2009). The synthesized antibiotics range from 4 to 19 amino acids in length and are capable of yielding many different functions including but not limited to increased bacterial cell wall permeability

(Raap et al., 2005). Both AIB and isovaline are actively transported by cells of the gut (cf. Christensen, 1962). Isovaline produces antinociception in a variety of rodent models of pain without any detectable CNS effects (MacLeod et al., 2010). Upon intravenous administration, R- and S-isovaline were found to reduce chronic phase II formalin induced nocifensive behaviour with equivalent potency. Intrathecal administration of isovaline has additionally been shown to effectively attenuate phase II model of formalin pain (MacLeod et al., 2010). Strychnine, a $glycine_A$ receptor antagonist, does not attenuate isovaline-induced conductance increases in nociceptive ventrobasal thalamus *in vitro* (Cooke et al., 2009). This was corroborated by the observation that isovaline-induced analgesia was not reduced after treatment with strychnine (MacLeod et al., 2010). These antinociceptive properties are promising from the standpoint of the development of novel analgesics and suggest that isovaline may be the first in class analgesic based on the prototype of small α-amino acid inhibitory neurotransmitter structural derivatives for treatment of a variety of pain-related disorders.

1.6 General anesthesia

Anesthesia refers to the state of being without sensation, including the absence of sensation or movement in response to painful stimuli such as surgical incision (Sonner, 2004). Injection or inhalation of relevant neuro-inhibitory agents is generally necessary to induce anesthesia (although alternative approaches have been attempted historically, including ingestion of large amount of alcohol) (Ballantine, 1970). Many advances have been made since the advent of modern anesthesia to optimise safety and reduce postoperative complications (Theodorsson et al., 2005). This has been achieved through finely tuned co-administration of specific drugs, with

infusions tailored to maintain homeostasis, reduce autonomic responses to surgical stimulation, and attain unconsciousness (Evers et al., 2006). Usually unconsciousness is sufficient to achieve amnesia. The main goals of general anesthesia are creation of a reversible condition of comfort, quiescence and physiological stability in a patient before, during and after performance of a procedure that would otherwise be painful, frightening or hazardous (Sonner, 2004). Current advances in anesthesiology have facilitated increasingly advanced surgeries which can now be safely performed in healthy patients. There is, however, an ongoing need to make further improvements in anesthesia safety. While estimates for mortality rate of perioperative anesthesia vary widely, but in the aggregate is estimated to be approximately 1 death per 100,000, the risk is much higher for elderly or unwell patients, who continually present high risk scenarios (Lagasse, 2002).

1.6.1 Neuropharmacological mechanisms of general anesthesia

While many studies have been undertaken, there is no unified theory describing the physiological basis of general anesthesia. The earliest theory of anesthesia, termed the Meyer-Overton lipid theory lipid theory of anesthesia, was developed in the 20th century. It postulates that all anesthetics exert their effects through perturbation of the physical properties of neuronal cell membranes and that the degree of lipid solubility is the primary determinant of anesthetic potency (Missner and Pohl, 2009).

The lipid theory has been gradually replaced by theories asserting that anesthetic action is a result of binding directly to specific receptors which confer precise changes in conductance

changes, leading to depression of CNS networks (Yamakura et al., 2001). More specifically, anesthetics have been shown to bind ligand-gated ion channels, namely, $GABA_A$ and $glycine_A$ receptor-coupled chloride ion channels. Binding events at the $GABA_A$ receptor increase conductance to Cl^- ions and enhance inhibitory neurotransmission leading to CNS depression (Yamakura et al., 2001). Nearly all anesthetics potentiate $GABA_A$ currents but many also produce $GABA_A$ receptor-independent events (eg. ketamine or nitrous oxide) implying that multiple receptor systems are involved in producing general anesthesia, including: N-methyl-D-aspartate (NMDA) and nicotinic acetylcholine (nACh) receptors (Yamakura et al., 2001). The effects of general anesthetics on ionotropic K^+ channels also play an important role in anesthesia (Ries and Puil, 1999).

A comprehensive understanding of consciousness is integral to an adequate understanding of anesthesia (Alkire et al., 2005). However, there is no rigorously defined explanation of consciousness; both consciousness and lack of consciousness are plagued similarly by lack of understanding (Searle, 1998). However, recent progress has been made in understanding whole-brain activity from a computational modeling standpoint (Eliasmith et al., 2012). Despite these advances, it has not been possible to adequately reconcile the computational and neuropharmacological frameworks due to scaling issues, and difficulty in modeling groups of firing neurons. From a physiological standpoint, excitation of thalamocortical neurons during wakefulness plays a crucial role in consciousness (Delacour, 1997); recent work has identified thalamocortical neurons as potential mediators of unconsciousness produced by inhalational agents (Ries and Puil, 1999). Indeed, the hippocampus has also been argued to be a potential contributor to cortical activation (Delacour, 1997) and is inhibited by a variety of anesthetics

(Miu & Puil, 1989). Separation of hypnotic and analgesic components of general anesthesia is a tangible and critical first step in the development of superior safer anesthetics, and in furthering understanding of the mechanisms of anesthesia.

1.6.2 Pharmacokinetic decay properties of common general anesthetics

The rate of attenuation of anesthesia can be influenced by a variety of factors including but not limited to the rate of elimination of the anesthetic from the brain and body tissues (Levy and Gibaldi, 1971). There are different advantages in administration of inhalational versus intravenous anesthetic preparations. The chief advantage of inhalational agents is that elimination through the lungs results in major decreases in blood and tissue concentration. In general the blood: gas partition coefficients (the index of anesthetic solubility in blood) of commonly used inhalational anesthetics have been determined as follows: halothane (2.3), isoflurane (1.4), sevoflurane (0.6), nitrous oxide (0.47), and desflurane (0.42) (Stoelting, 2006). Anesthetics with lower blood and lipid solubility confer more rapid recovery; drugs that are more highly soluble accumulate in body fat (Stoelting, 2006). As the duration of anesthesia increases, the extent of accumulation of anesthetic also increases (Stoelting, 2006). The relationship between the duration of anesthesia and recovery also depends on the age and physical characteristics of the patient.

The chief pharmacokinetic parameters which influence the clearance of intravenous general anesthetics consist of the degree of solubility in lipid membranes, the degree of protein binding, and the rate of drug metabolism (Levy and Gibaldi, 1971). Common injectable anesthetics

include propofol, the barbiturates methohexital and thiopental, and non-barbiturate propofol. The intravenous hypnotic agent propofol is slowly released into the bloodstream resulting in prolonged effects on the brain (Stoelting, 2006). Propofol undergoes hepatic metabolism and is subject to rapid clearance (Stoelting, 2006). The clearance time of propofol is not influenced appreciably by the duration of anesthesia (Shafer et al., 1988). Even for surgeries in excess of 8 hours wherein propofol is continually infused, its elimination half-time is under 40 minutes. For this reason, propofol is considered to be desirable from a pharmacokinetic standpoint, allowing for rapid awakening and return of cognitive function. Propofol results in considerably faster recovery than thiopental for ambulatory surgeries (Greenberg et al., 2003).

1.7 Brief overview

Manuscript 1 (Chapter 2) focuses on peripheral $GABA_B$ receptor-mediated antinociception against PGE_2-induced allodynia injected into cutaneous hindpaw tissues of in mice. The peripherally restricted $GABA_B$ agonist isovaline, and unselective endogenous agonist GABA along with the centrally and peripherally acting selective $GABA_B$ receptor agonist, baclofen, were tested for anti-nociceptive properties. Anti-allodynic dose dependency was assessed for all three agents, and mechanism of action was assessed using specific the specific $GABA_B$ receptor antagonist CGP52432 and positive allosteric modulator CGP7930. To probe for the site of local anti-allodynic action, we used immunohistochemistry to characterize the expression profile of $GABA_B$ receptor subunits co-localized in glabrous cutaneous tissue of mouse hindpaw. Next, we tested for the sedative properties associated with analgesic and supra-analgesic doses of isovaline, GABA and baclofen. We subsequently examined the duration of anti-allodynic action

of isovaline and the ability of peripherally injected isovaline to produce functional restoration in a novel forced exercise model of intra-articular monoiodoacetate (MIA)-induced arthritic dysfunction.

Manuscript 2 (Chapter 3) focuses on producing safe general anesthesia through co-administration of isovaline with hypnotic propofol. Propofol is a commonly used intravenous hypnotic induction agent used to induce unconsciousness; however propofol does not produce sufficient analgesia to yield immobility to surgical stimuli, requiring co-administration of analgesic opioid adjuvants (commonly fentanyl or remifentanyl) to achieve immobility (Alvez et al., 2007). Our primary hypothesis was that isovaline would produce surgical analgesia (loss of response to Haffner tail clip) in mice with a superior safety profile than fentanyl. We assessed the relative therapeutic indices for general anesthesia produced by isovaline-propofol versus fentanyl-propofol. We also examined the presence of additive or synergistic analgesic of effects of isovaline and fentanyl when combined with propofol and maximum tolerated doses.

In Manuscript 3 (Chapter 4) we developed and validated a model of trigeminal dynamic allodynia of glycinergic inhibitory dysfunction of the brainstem, testing clinically effective and ineffective agents, a carbamazepine metabolite and morphine, respectively. The efficacy of peripheral isovaline does not preclude CNS efficacy for treatment of disorders such as trigeminal neuralgia and addiction (systemic injection of a structurally modified isovaline might achieve this). Our primary hypothesis was that intracisternal strychnine would produce allodynia (Lee et al., 2013) with a similar treatment-response profile to that seen in human trigeminal neuralgia.

We assessed the efficacy of isovaline injected intracisternally with strychnine. Both qualitative side-by-side allodynia comparisons and quantitative allodynia scores were employed for each drug comparison group.

1.8 Figures

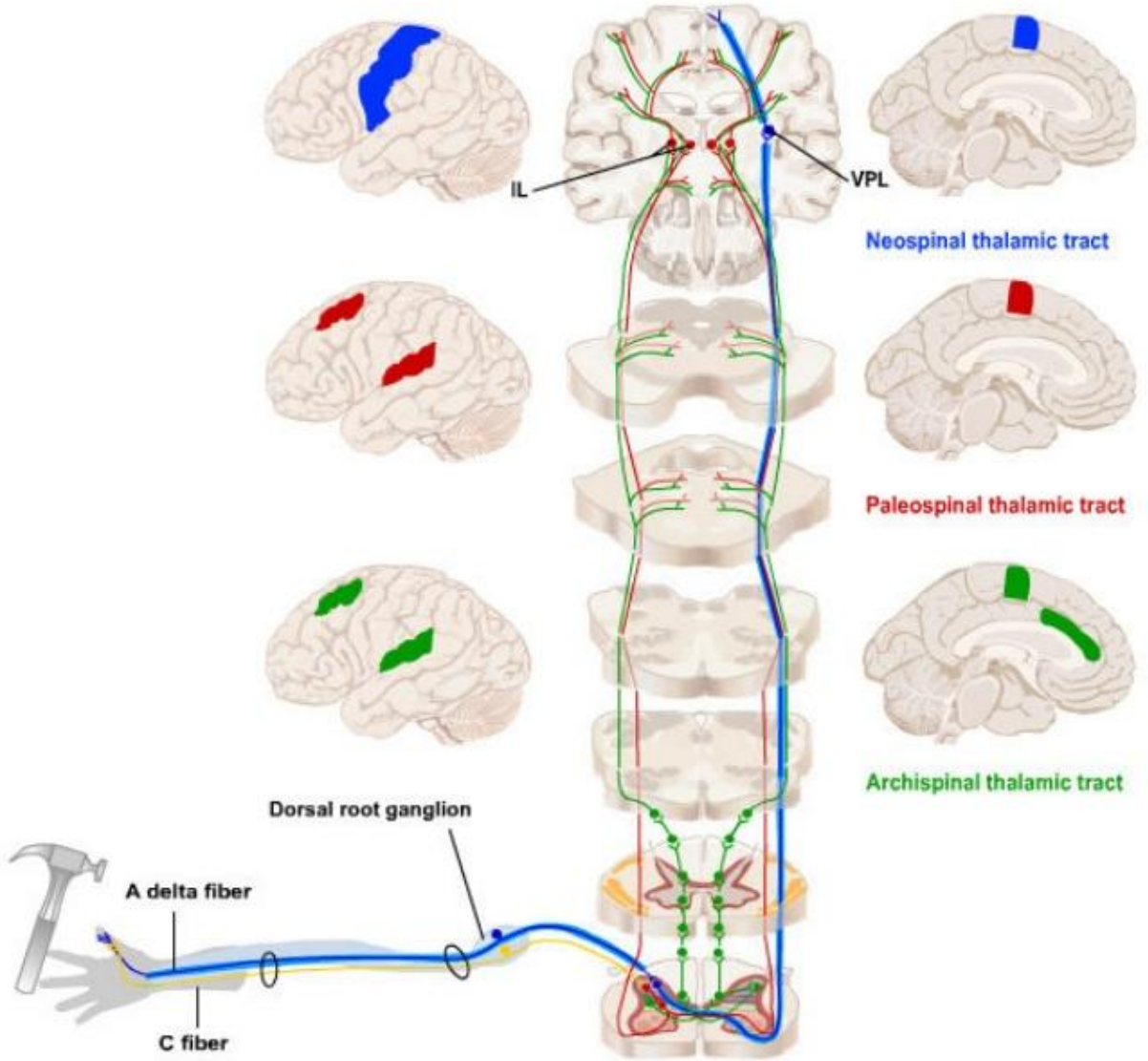

Figure 1.1 Schematic depicting the three nociceptive pathways. The blue afferent indicates afferent signals from the neospinal thalamic tract which are relayed on both A delta fibers and C fibers and are relayed up the spinal cord through interneurons. The archispinal and paleospinal thalamic tracts are indicated in green and red, respectively (modified from Garcia, 2009).

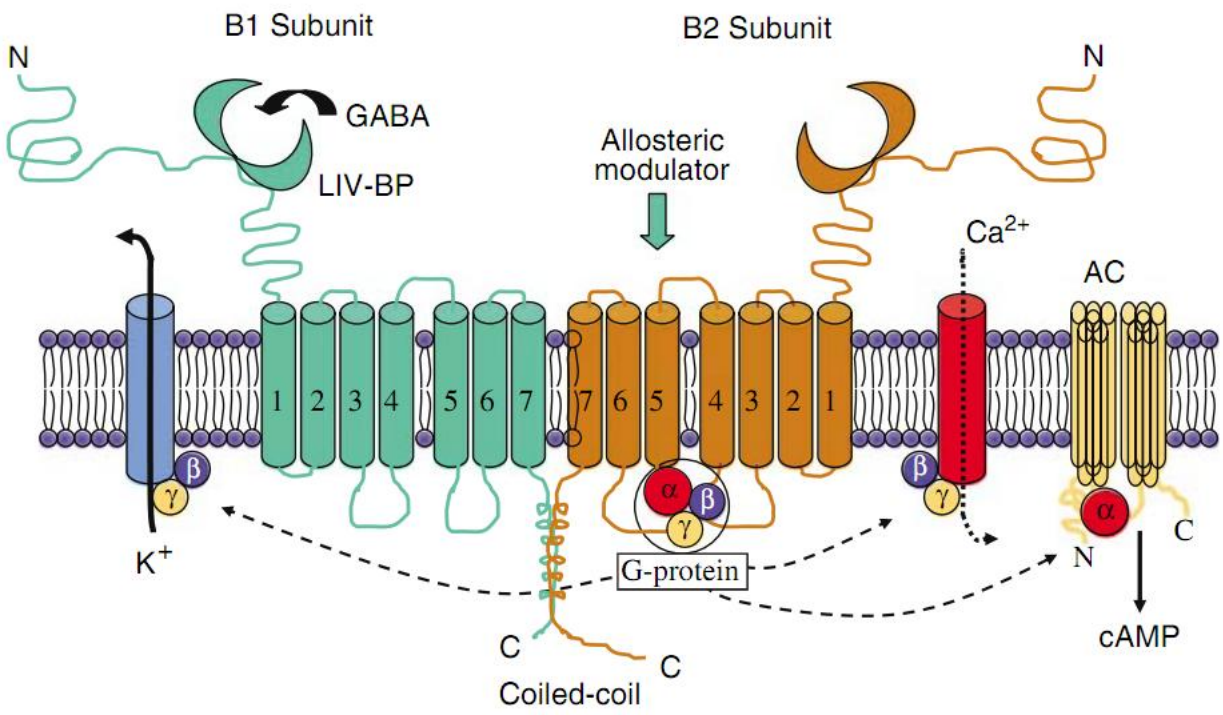

Figure 1.2 Pictorial representation of the $GABA_B$ receptor. The binding of a ligand such as GABA or baclofen to the orthosteric binding domain of the $GABA_{B1}$ subunit induces a conformational shift which alters the binding properties of Gα and Gβγ subunits causing an exchange of a GDP for a GTP at the Gα subunit. The Gβγ is released and subsequently goes on to activate K^+ currents indicated as positive, and inhibits Ca^{2+} channels indicated as negative and resulting in lower cAMP levels and hence lower PKA levels. The ability of the Gα subunit to act as a GTPase is increased by regulators of G protein signalling proteins (commonly referred to as RGS). Upon re-association of Gα and Gβγ subunits, receptor actions are henceforth terminated. GDP = guanosine diphosphate; GTP = guanosine triphosphate; GABA = γ-aminobutyric acid; cAMP = cyclic adenosine monophosphate; PKA = protein kinase A. (Source, Wikimedia Commons)

Figure 1.3 Chemical structures of glycine, isovaline and ɤ-aminobutyric acid (GABA). $Glycine_A$ receptors were originally identified in nociceptive ventrobasal thalamus (Ghavanini et al., 2006), prompting a search for glycine analogs as drug candidates. Isovaline, which is structurally similar to glycine and GABA, is an agonist of the $GABA_B$ receptor and was identified as a promising drug candidate (MacLeod et al., 2010).

2 [1] GABA(B) receptor-mediated selective peripheral analgesia by the non-proteinogenic amino acid, isovaline.

2.1 Introduction

There is an urgent need for drugs that relieve pain without also producing confusion, sedation, respiratory depression, or addiction (Schug and Gandham, 2007; Gold and Gebhart, 2010). Small molecules that produce peripherally restricted analgesia could meet this need since these side effects are caused by drug actions inside the central nervous system (CNS). Structural analogs of inhibitory neurotransmitters such as ɤ-aminobutyric acid (GABA) which do not cross the intact blood-brain barrier are likely candidates. Previously, we investigated several analogs including 2-amino-2-methylbutanoic acid (isovaline). Both the R- and S-enantiomer of isovaline had anti-allodynic effects in mouse pain models without producing signs of acute toxicity (MacLeod et al., 2010). While the mechanism of antinociception remains unknown, results suggested that isovaline produced antinociception through central actions when administered centrally and through peripheral actions when administered peripherally. Our recent *in vitro* studies in the CNS demonstrated that metabotropic $GABA_B$, and not ionotropic GABA or glycine receptors, mediate the inhibitory actions of R-isovaline on thalamic neurons (Cooke et al., 2009; 2012). Isovaline actions were similar to the actions of the prototypical $GABA_B$ agonist, baclofen. Both were blocked by $GABA_B$ antagonists and potentiated by a positive allosteric modulator of

[1] A version of this chapter has been published. Whitehead RA, Puil E, Ries CR, Schwarz SK, Wall RA, Cooke JE, Putrenko I, Sallam NA, MacLeod BA. (2012). GABA(B) receptor-mediated selective peripheral analgesia by the non-proteinogenic amino acid, isovaline. *Neuroscience* 213: 154-160.

$GABA_{B1}$ subunits. We hypothesized for the current *in vivo* study that $GABA_B$ receptors are involved in producing the analgesic effects of intraplantar isovaline.

In the periphery, $GABA_B$ receptors exist on sensory and autonomic neurons, as well as on Schwann cells and other non-neuronal cells (reviewed by Magnaghi, 2007). $GABA_B$ receptors on small primary afferent neurons with Aδ and C fibres regulate nociceptive transmission in peripheral tissues and the spinal cord (Desarmenien et al., 1984; Takeda et al., 2004). While $GABA_B$ receptors on the cell bodies of sensory ganglia and synapses in laminae I–IV of the dorsal horn may participate in nociception (cf. Carlton and Coggeshall, 1998), anatomical evidence for $GABA_B$ receptors on the fine nerve endings is lacking (cf. Magnaghi et al., 2008). Using monoclonal antibodies, $GABA_{B1}$ and $GABA_{B2}$ receptor subunits have been demonstrated in rat knee synovial membrane (Tamura et al., 2009). While it is not known if $GABA_B$ receptors are present in cutaneous tissue, we proceeded to study the peripheral receptor-mechanism of isovaline antinociception. In doing so, we set out to determine the effects of isovaline, GABA and baclofen and their interactions with $GABA_B$ antagonists and a $GABA_B$ modulator on allodynia induced by intraplantar prostaglandin E_2 (PGE_2). Previous studies demonstrated both central and peripheral anti-hyperalgesic effects of baclofen on PGE2-induced allodynia (Reis and Duarte, 2006). In contrast to baclofen, CNS concentrations of isovaline would likely remain negligible since (within the limits of detection) carbon^{14}-labelled isovaline injected intraperitoneally in mice does not cross the blood–brain barrier (Shiba et al., 1988; 1989). Consistent with peripheral disposition, subcutaneous injections of isovaline at 10-fold the analgesic dose do not produce changes in behaviour or motor function (MacLeod et al., 2010). Here, we confirm a lack of CNS effects using a battery of CNS function tests and the

measurement of body temperature (cf. MacLeod et al., 2010). We performed immunohistochemistry to determine the presence of $GABA_{B1}$ and $GABA_{B2}$ subunits in cutaneous tissue. Lastly, we studied the potential clinical applicability of our findings by determining the effects of isovaline in a mouse model of osteoarthritis.

2.2 Methods

2.2.1 Animals

With approval from the Animal Care Committee of The University of British Columbia, we studied female CD-1 mice that weighed 25–30 g. In forced exercise experiments, male C57Bl/6 mice were used. Mice were housed 12 in each cage for 1 week before the experiment, in an environmentally controlled room with ambient temperature of 21 ^{0}C and relative humidity at 55%. Animals were maintained on a reversed dark-light cycle with free access to food and water.

2.2.2 Drugs

Inorganic chemicals, RS-baclofen, GABA, CGP52432, CGP7930, sodium monoiodoacetate (MIA), diclofenac, and PGE_2 were purchased from Sigma Chemical Company (St. Louis, MO, USA). RS-isovaline (base and HCl salt) was synthesized by Biofine International Inc. (Vancouver, BC, Canada). Drugs were prepared in 0.9% saline for injection. The pH of drug solution was 7.3–7.4.

2.2.3 Experimental design

Experiments were conducted using a blinded, randomized, and controlled design. Where appropriate, drug and control solutions were injected in an order determined by computer randomization. Each animal was used only once. Experiments were conducted between 09:00 and 16:00 h in a quiet room at stable room temperature.

2.2.4 Peripheral anti-allodynia and $GABA_B$ pharmacology

To study $GABA_B$-receptor involvement in intraplantar PGE_2-induced allodynia, we utilized selective $GABA_B$ agonist, antagonist, and allosteric modulator agents. Intraplantar PGE_2 was used to elicit nociceptive response (0.1 nM, 20 µl; cf. Kassuya et al., 2007), and was injected subcutaneously (s.c.) into both hindpaws. To test the nociceptive responses, mice were habituated for 10 min on an elevated wire mesh platform prior to each experiment. The wire mesh platform allowed access to the plantar surface of the paw. Von Frey filaments of increasing stiffness (0.008–6.0 g) were applied to the plantar hindpaw before, and several times after intraplantar PGE_2 injection. The paw withdrawal threshold (g) was defined as the von Frey filament mass at which at least 5 of 6 consecutive intraplantar stimulations elicited robust and readily detectable paw withdrawals. When a given filament application induced a paw withdrawal, a period of 5s was allowed before subsequent stimulation. We determined dose–responses for the brain-penetrant, selective $GABA_B$ agonist, baclofen; the brain impermeant, non-selective agonist, GABA; and the brain-impermeant, putatively selective $GABA_B$ agonist, isovaline. All $GABA_B$ receptor agonists tested were injected into only the ipsilateral paw, whereas PGE_2 was always injected into both paws. Next, we established the effects of the

specific $GABA_B$ antagonist, CGP52432, on the analgesia produced by baclofen, GABA and isovaline. We then determined the effects of the selective $GABA_B$ allosteric modulator, CGP7930, on the responses to an approximate ED_{10} of each agonist. Saline and compound controls were determined in the absence of PGE_2. CGP52432 or CGP7930 were injected 5 min prior to co-injection of PGE_2, and either saline, isovaline, GABA, or baclofen (Adams and Lawrence, 2007).

2.2.5 CNS effects assessment

To study possible CNS depression of peripherally administered isovaline (as well as GABA and baclofen) we performed a battery of five tests to yield the Behavioural Hypoactivity Score (BHS; Heal et al., 1981):

1. Posture: Lowering of tail and abdomen.

2. Passivity: Struggle in response to picking up the mouse by the skin of the back.

3. Tactile responsiveness: Response to a finger pinch of the torso.

4. Body sag: Maintenance of grip on inverted wire mesh.

5. Gait: Slowing and rolling gait.

Each behavioural parameter was rated 0, absent; 1, slight; 2, moderate; and 3; severe. The total BHS was computed as the sum of the scores.

We also assessed the effects of the above agents on body temperature as an indication of GABAergic action in the CNS. For this purpose, we applied a ThermoFocus infrared thermometer (Tecnimed; Varese, Italy) beam to the mouse underbelly (Saegusa and Tabata, 2003). Three temperature measurements were averaged before and after injections of isovaline and the $GABA_B$ agonists.

2.2.6 Forced exercise assay

Experimental osteoarthritis was induced in the right knees of male C57Bl/6 mice under isoflurane anesthesia by infra-patellar injection of MIA (0.1 mg in 10µl of 0.9% sterile saline) (Harvey and Dickenson, 2009). The control group received saline. Osteoarthritis developed in 1–2 weeks. The mice were placed in a motorized wheel system (Lafayette Instrument Co., IN, USA) and videotaped while undergoing forced exercise at a low speed (3 m/min). A blinded observer counted the number of slips/falls *post hoc* from the video footage. Only mice demonstrating impaired function (P15 slips over 15 min) were used in the experiments. After 24 h, animals were randomly assigned to receive isovaline (80 mg/kg in 0.2 ml of 0.9% sterile saline) or sterile saline by intraperitoneal injection. The NSAID, diclofenac, was used as a positive control. Fifteen minutes later, a 15-min session of forced exercise was recorded.

2.2.7 $GABA_B$ receptor immunohistochemistry

Since isovaline has recently been shown to interact with $GABA_B$ receptors in thalamic brain slices (Cooke et al., 2012), we set out to establish the presence of $GABA_B$ receptors at the site of peripheral nociception, the free nerve endings. For this purpose, formalin (5%) was injected subcutaneously under the plantar surface of the hindpaw of a mouse under isoflurane anesthesia. A section of dermal tissue of the hindpaw was subsequently excised using a 2-mm-diameter punch biopsy. Dermal tissue was then embedded with Tissue-Tek embedding medium (Sakura Finetek, Torrence, CA, USA) and frozen in liquid nitrogen. Sagittal sections were made at 30 µm thickness and stored at 21 ^{0}C.

Immunohistochemistry was performed on sections post-fixed in 4% formaldehyde for 10 min. The sections were permeabilized with 0.1% Triton X-100, blocked with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and incubated in primary antibody overnight at 4°C in PBS solution containing 0.1% BSA. The primary antibodies were mouse anti-$GABA_{B1}$ subunit (1:100; ab55051; Abcam, Cambridge, MA, USA), rabbit anti-$GABA_{B2}$ subunit (1:100; ab75838; Abcam, Cambridge, MA, USA), and the specific marker of neuronal processes; chicken anti-microtubule-associated protein 2 (MAP-2) (1:100; ab75713; Abcam). 40-6-Diamidino-2-phenylindole (DAPI) was used as a DNA marker to identify the requisite materials for assembly of $GABA_B$ receptor protein. Sections were then incubated in goat anti-mouse Alexa 546, goat anti-rabbit Alexa 488, and goat anti-chicken 633 secondary antibodies for 1 h at room temperature (Invitrogen, Burlington, ON, Canada) followed by DAPI nuclear counterstain. Sections were cover-slipped with Prolong Gold (Invitrogen) and allowed to cure overnight

before imaging. Images were captured using a FluoView 1000 confocal microscope (Olympus Corporation, Tokyo, Japan; Plan-Apochromat objectives). Image brightness was modified slightly to enhance visualization using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA, USA).

2.2.8 Intravenous regional analgesia

In order to test for distal local analgesia, we set out to determine isovaline's effects in a mouse model of intravenous regional analgesia. A tourniquet, as described in section 1.3.7 is a device that compresses blood vessels when applied around a limb or tail to isolate circulation. The tourniquet used here was constructed from a piece of 3 mm long plastic C-Flex tubing (Cole-Parmer Instrument Company, Vernon Hill, Illinois). The outer and inner diameters of the tubing were 1/8th inch and 1/16th inch respectively, with a wall thickness of 1/32nd inch. A custom-made plexi-glass device was used. The device had 4 pieces of 3mm thick acryclic glass mounted on a stand. Gaps between these 4 pieces of acrylic were made just wide enough to insert a surgical blade. Holes (diameter 1/8th inch) were drilled through the four pieces of acrylic glass to allow insertion of the C-Flex plastic tubing. To cut the C-Flex tubing at a consistent length of 3mm, tubing was inserted through on the holes until flush with the opposite end. The surgical blade was then inserted through the gaps between the acrylic glass slides. Once cut, the 3mm C-Flex tubing was applied 1 cm from the base of the mouse tail, constraining blood flow. Immediately after application either 50µl of saline or isovaline was injected into the lateral tail vein. After a 5 minute period to allow drug action and before onset of ischemia, tail-flick latency was assessed three consecutive times for each animal in a 55ºC hot water bath.

2.2.9 Statistical analysis

Data were analyzed and plotted using Prism 5 software (GraphPad, San Diego, CA, USA). For comparisons between groups, we used the Kruskal–Wallis test followed by Dunn's post-test and ANOVA with the Bonferroni or Dunnett's post-test as appropriate. For statistical outlier identification, we utilized Grubbs' test. Unless mentioned otherwise, data are presented as mean ± SEM; $P < 0.05$.

2.3 Results

2.3.1 Antiallodynic effects of isovaline and $GABA_B$ receptor agonists

Isovaline, GABA, and baclofen dose-dependently attenuated PGE_2-induced allodynia (Fig. 2.1A). The average baseline von Frey threshold was 0.41 ± 0.02 g. The estimated ED50s were 10 µmol, 10 nmol, and 100 nmol for isovaline, GABA, and baclofen, respectively. Whereas isovaline produced a locally restricted anti-allodynic effect at 5 µmol, we did not observe a locally restricted decrease in mechanical hypersensitivity at the doses tested for GABA or baclofen.

The selective $GABA_B$ receptor antagonist, CGP52432 (Lanza et al., 1993), blocked the analgesic effects induced by near maximally effective doses of isovaline, GABA, or baclofen (Fig. 2.1B). Injected prior to PGE_2 alone, CGP52432 did not increase PGE_2-induced sensitivity. The selective positive allosteric $GABA_B$ receptor modulator, CGP7930, significantly increased the potency of

the $GABA_B$ agonism by isovaline, GABA, and baclofen (Fig. 2.1C). CGP7930 injected alone produced no changes in the threshold for PGE_2-induced sensitivity.

2.3.2 CNS effects

Neither isovaline nor GABA produced CNS depression, even at doses greater than 10-fold that which blocked allodynia. Baclofen produced significant CNS depression at twice the dose required to reverse allodynia, and at six times that dose rendered mice completely flaccid and unresponsive (Fig. 2.2A). No change in body temperature was produced by isovaline or GABA, at greater than 20-fold the antiallodynic doses. However, baclofen at six-fold its approximate ED_{90} for analgesia produced a 1.5ºC temperature decrease (Fig. 2.2B).

2.3.3 MIA-induced osteoarthritis

Mice without osteoarthritis had an average of <1 slips/falls per 15 min in the forced exercise wheel (Fig. 2.3). Mice with induced osteoarthritis treated with saline (i.p., n = 6) had 51 ± 4 slips per 15 min compared to mice after isovaline administration (80 mg/kg i.p., n = 6), which had 12 ± 3 slips and diclofenac (50 mg/kg i.p., n = 3) which had 7.3 ± 4.7 slips (Fig. 2.3).

2.3.4 Cutaneous $GABA_B$ receptor subunits

Fluorescence immunohistochemistry with antibodies against $GABA_{B1}$ and $GABA_{B2}$ subunits to identify $GABA_B$ receptors in the skin demonstrated staining for both subunits within the stratum

spinosum of epidermal tissue, at the local test site (shafted arrows in Fig. 2.4A, B). In order to test whether $GABA_B$ receptors were located on free nerve endings, we used the neuron-specific tubule marker, MAP-2 (cf. De Camilli et al., 1984). We observed co-expression of both $GABA_B$ receptor subunits in MAP-2 positive neuronal processes, particularly in the stratum basale of the epidermis (see arrowheads in Fig. 2.4C, E). These processes exhibit the characteristic longitudinal staining pattern used to identify fine afferent nerve endings (Messlinger, 1996). Keratinocytes also exhibited strong co-expression of $GABA_B$ receptor subunits in the stratum spinosum, and did not express MAP-2 (Fig. 2.4C, shafted arrow). In summary, $GABA_{B1}$ and $GABA_{B2}$ subunits were associated with keratinocytes and fine nerve endings.

2.4 Discussion

This study in mice demonstrates for the first time that the analgesia produced by isovaline results from activity on peripheral $GABA_B$ receptors. Isovaline and the $GABA_B$ agonists, GABA and baclofen produced dose dependent analgesia, blocking PGE_2–induced allodynia. In an absence of CNS mediation, this suggests redistribution from the local site of injection to the contralateral site. A low dose of isovaline (5 µmol) decreased allodynic responses to PGE_2 only at the intraplantar site. The antiallodynic effects of all three $GABA_B$ agents tested were attenuated by the $GABA_B$ antagonist, CGP52432, and potentiated by the $GABA_B$ modulator, CGP7930. In another model, isovaline restored limb function in mice with induced osteoarthritis. Isovaline produced no CNS depression such as temperature changes indicative of central GABAergic action. In contrast, the prototypical $GABA_B$ agonist, baclofen, had both peripheral and CNS actions. We established the existence of co-expressed $GABA_{B1}$ and $GABA_{B2}$ receptor subunits in

keratinocytes and fine nerve endings in tissue from the site of analgesia. These results suggest peripherally restricted agonists, mediated or modulated by peripheral $GABA_B$ receptors, have a role in the treatment of pain.

The $GABA_B$ receptor-mediated mechanism was apparent from the reduction of isovaline-induced analgesia by the $GABA_B$ antagonist, CGP52432, and a greater potency of isovaline on co-administration with the $GABA_B$ modulator, CGP7930. Since CGP52432 (17 nmol) did not completely block the analgesic effect of isovaline, we cannot rule out a contribution from additional receptor mechanisms. On intraplantar injection, isovaline produced selective peripheral analgesia, in contrast to the prototypical $GABA_B$ agonist, baclofen, which produced a combination of peripheral analgesia and CNS effects. Immunostaining for $GABA_{B1}$ and $GABA_{B2}$ receptor subunits at the intraplantar site implicated $GABA_B$ receptors on keratinocytes and fine nerve endings in the peripheral analgesia.

2.4.1 $GABA_B$ receptor-mediation of analgesic effects

In this study, isovaline attenuated PGE_2-induced allodynia by actions that were similar to those of GABA and baclofen. The rank order of potency was GABA > baclofen > isovaline. The $GABA_B$ antagonist, CGP52432, prevented the antiallodynic effects of isovaline. CGP52432 also antagonized the antiallodynic effects of GABA and baclofen, consistent with a previous study using saclofen antagonism in rat (Reis and Duarte, 2006). An absence of tonic $GABA_B$ activity was indicated by the absence of any effect on PGE2-induced allodynia by the antagonist (CGP52432) or the positive allosteric $GABA_B$ modulator (CGP7930) when administered alone.

On co-injection, CGP7930 potentiated the effects of subanalgesic doses of isovaline, GABA, and baclofen. CGP7930 is thought to act at a $GABA_{B2}$ subunit site on the heterodimeric $GABA_B$ receptor (Adams and Lawrence, 2007). The above data suggest that isovaline's analgesia was mediated by cutaneous $GABA_B$ receptors.

2.4.2 Peripheral site of analgesic effects

The attenuation of PGE2-induced allodynia by isovaline or GABA was not accompanied by CNS side effects, as measured with the BHS (Heal et al., 1981) and body temperature changes. These observations are consistent with an inability of isovaline and GABA to cross the blood–brain barrier (Shiba et al., 1988, 1989; van Gelder and Elliot, 1958). Baclofen appreciably crosses into the CNS, and was found to produce CNS depression and hypothermia at analgesic doses. Previous observations showing the lack of effect of isovaline on the ability to remain on the roto-rod, a test of motor co-ordination and balance (MacLeod et al., 2010), are consistent with these findings. GABA also produces peripheral antiallodynic effects at $GABA_B$ receptors, but concomitant actions at $GABA_A$ receptors under conditions of neuropathic pain may limit its clinical usefulness. The latter ionotropic receptor actions result in significant side effects such as hypotension and bradycardia, and may exacerbate the response to painful stimuli (Vonkeman and van de Laar, 2010; Carlton et al., 1999).

2.4.3 Cutaneous immunostaning of $GABA_B$ receptor subunits

The present study provides the first demonstration of $GABA_{B1}$ and $GABA_{B2}$ receptor subunits in cutaneous tissue. The subunits were co-expressed with MAP-2 positive neuronal processes, particularly in the stratum basale of epidermis (cf. De Camilli et al., 1984). The processes are morphologically similar to free nerve endings, exhibiting the characteristic longitudinal staining pattern used to identify fine afferent nerve endings (Messlinger, 1996). Keratinocytes, which did not express MAP-2, exhibited strong co-expression of $GABA_{B1}$ and $GABA_{B2}$ subunits in the stratum spinosum. While fine nerve endings are the chief functional constituent of peripheral nociceptors, keratinocytes also participate in pain signalling (Radtke et al., 2010). The existence of a GABA synthesis enzyme (glutamic acid decarboxylase 67) in neighbouring fibroblasts may imply an ability of cellular elements to modify nociception (Dubin and Patapoutian, 2010; Ito et al., 2007). Our demonstrations of functional $GABA_B$ receptors suggest that isovaline and GABA involve one or more $GABA_B$ receptor-expressing peripheral structures in cutaneous tissues.

2.4.4 Implications for clinical utility of isovaline

Isovaline restored mobility in an osteoarthritic model that mimics the main features of human osteoarthritis, including pain, gross and histo-pathology, and relief with diclofenac therapy (Clements et al., 2009). We did not establish the time course for restoration of function in this arthritic model which was associated with a brief period of inflammation. In chronic disease, acute inflammation may alter blood–brain barrier permeability properties which might allow entry of a peripherally restricted drug into the CNS (Erickson et al., 2012). However, no inflammation was present in the MIA model at the time of testing (Bove et al., 2003) and the

afflicted mice did not show any signs of CNS depression. This is consistent with the observation from the point of view of human therapeutics that osteoarthritis generally manifests itself as a degenerative as opposed to inflammatory condition. Therefore, the relief of osteoarthritic dysfunction may have resulted from activation of $GABA_B$ receptors expressed in the knee synovium (cf. Tamura et al., 2009). The finding that isovaline acts primarily through a peripheral mechanism is of potentially great clinical significance and further testing must be done. Actions at this site avoid adverse CNS effects such as respiratory depression, sedation, and addiction produced by opioids. Due to isovaline's high chemical stability (Elsila et al., 2011) and inability to be incorporated into proteins, potential involvement in the production of toxic proteins is negligible. Combinations of such peripherally restricted agents with conventional analgesics should improve the therapeutic index.

2.4.5 Summary and conclusions

We have shown that $GABA_B$ receptors mediate selective peripheral isovaline analgesia. Isovaline-induced analgesia originated in the peripheral nervous system and was unaccompanied by CNS effects. Isovaline's ability to restore mobility of osteoarthritic mice demonstrates its potential utility in the treatment of arthritis. Peripherally restricted isovaline is a promising first-in-class analgesic without CNS effects.

2.5 Figures

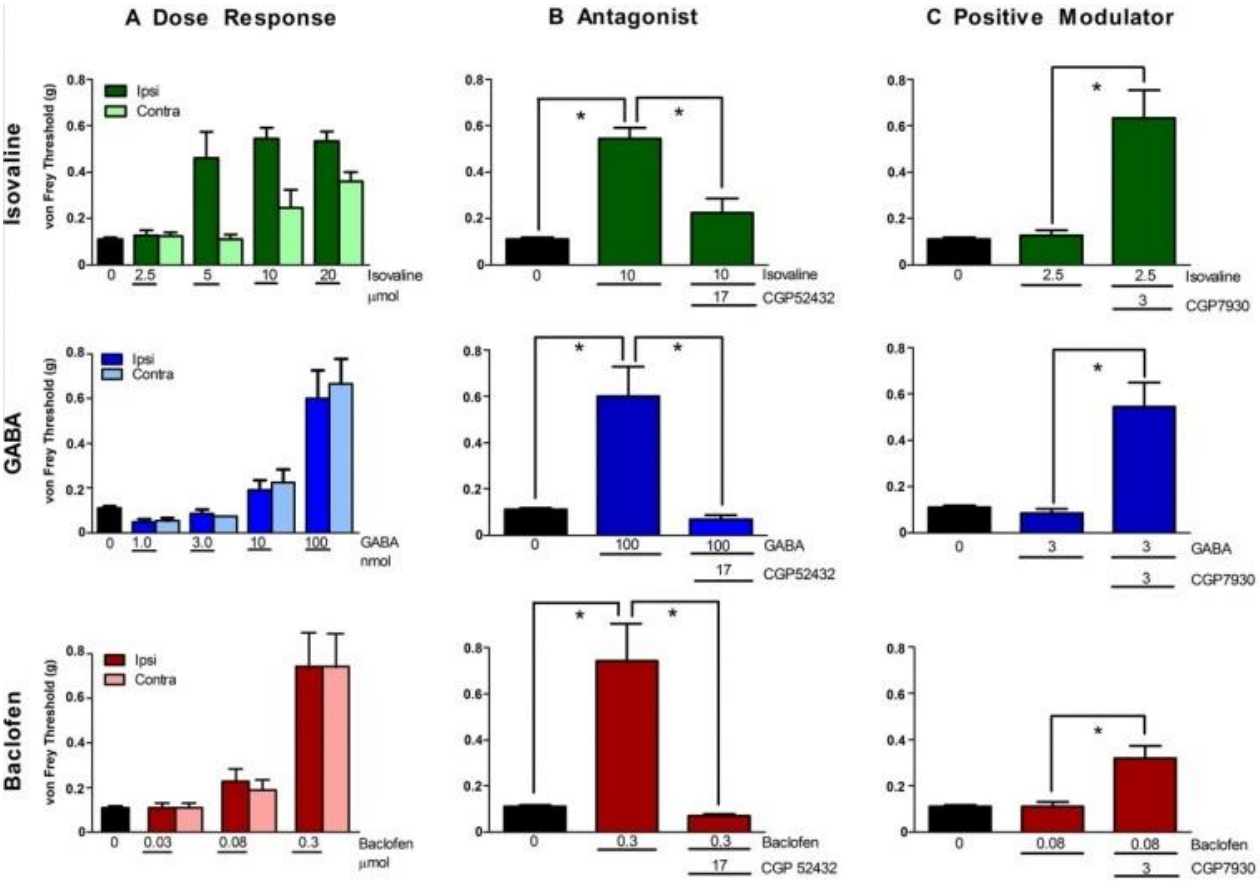

Figure 2.1 Isovaline, GABA, or baclofen analgesia and the $GABA_B$ receptor. (A) Antiallodynic dose-dependency using von Frey hairs to assess mechanical nociception threshold. (B) Effect of the receptor antagonist, CGP52432 (on near maximally effective dose). (C) Effect of the positive allosteric modulator, CGP7930 (on response to approximate agonist ED10). Results are means ± SEM (n = 6 for each group; P< 0.05 [Kruskal–Wallis test followed by Dunn's post-test]). Grubbs' test for outliers was utilized. For forced exercise, ANOVA with Dunnett's multiple comparison was used.

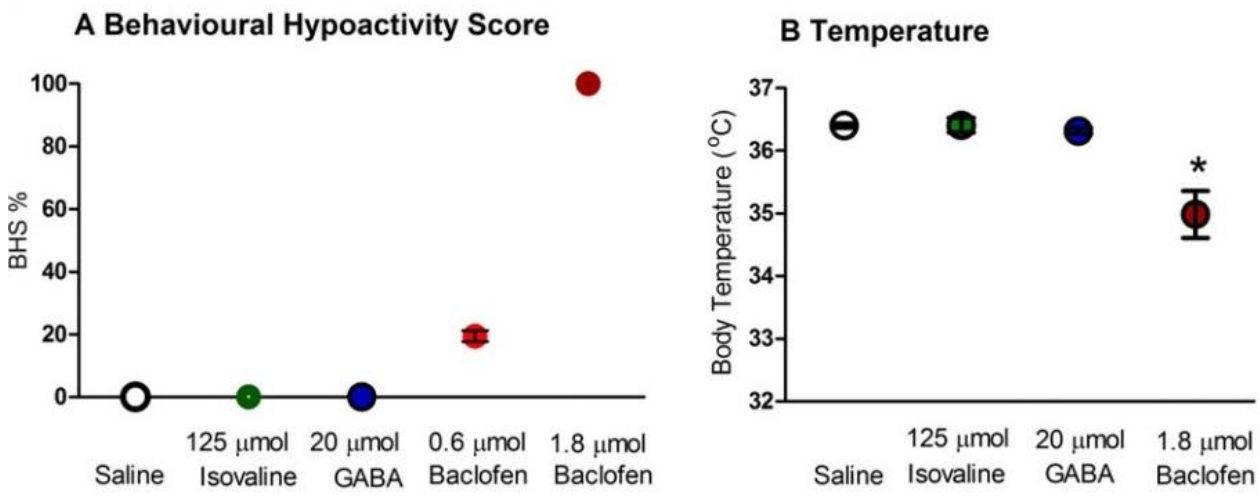

Figure 2.2 Hypothermia and Behavioural Hypoactivity Score (BHS) produced by $GABA_B$ agonists. Doses expressed in µmol (n = 6 per drug; mean ± SEM; P< 0.05 for difference from saline [Kruskal–Wallis test followed by Dunn's post-test]).

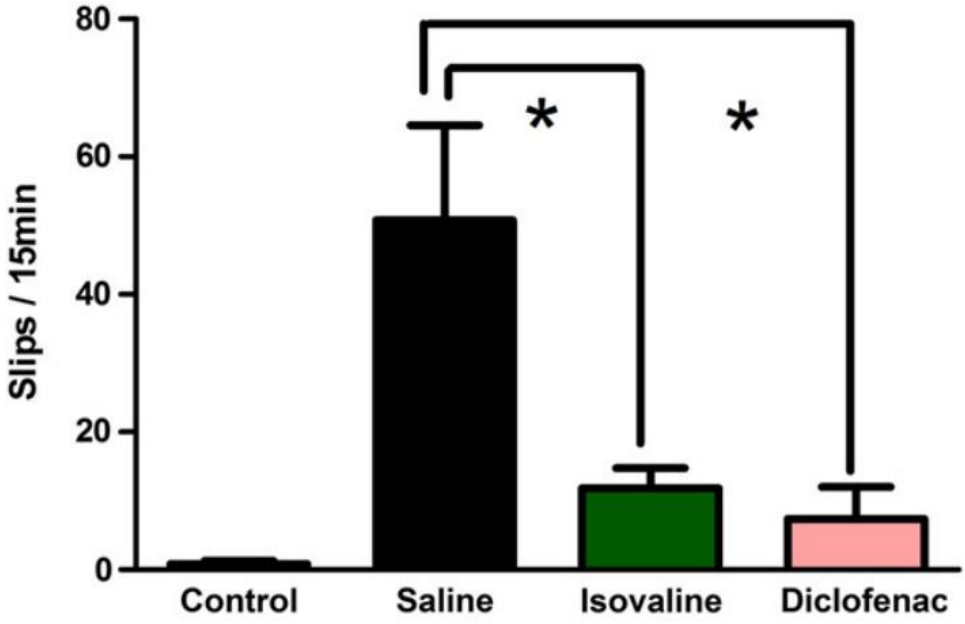

Figure 2.3 Isovaline-induced functional restoration of function in arthritic mice. MIA-induced arthritis was present in all groups except control. Isovaline treatment significantly reduced the number of slips compared to the saline group, ($P < 0.05$; $n = 6$), diclofenac also reduced the slips ($P < 0.05$; $n = 3$); ANOVA with Dunnett's multiple comparison.

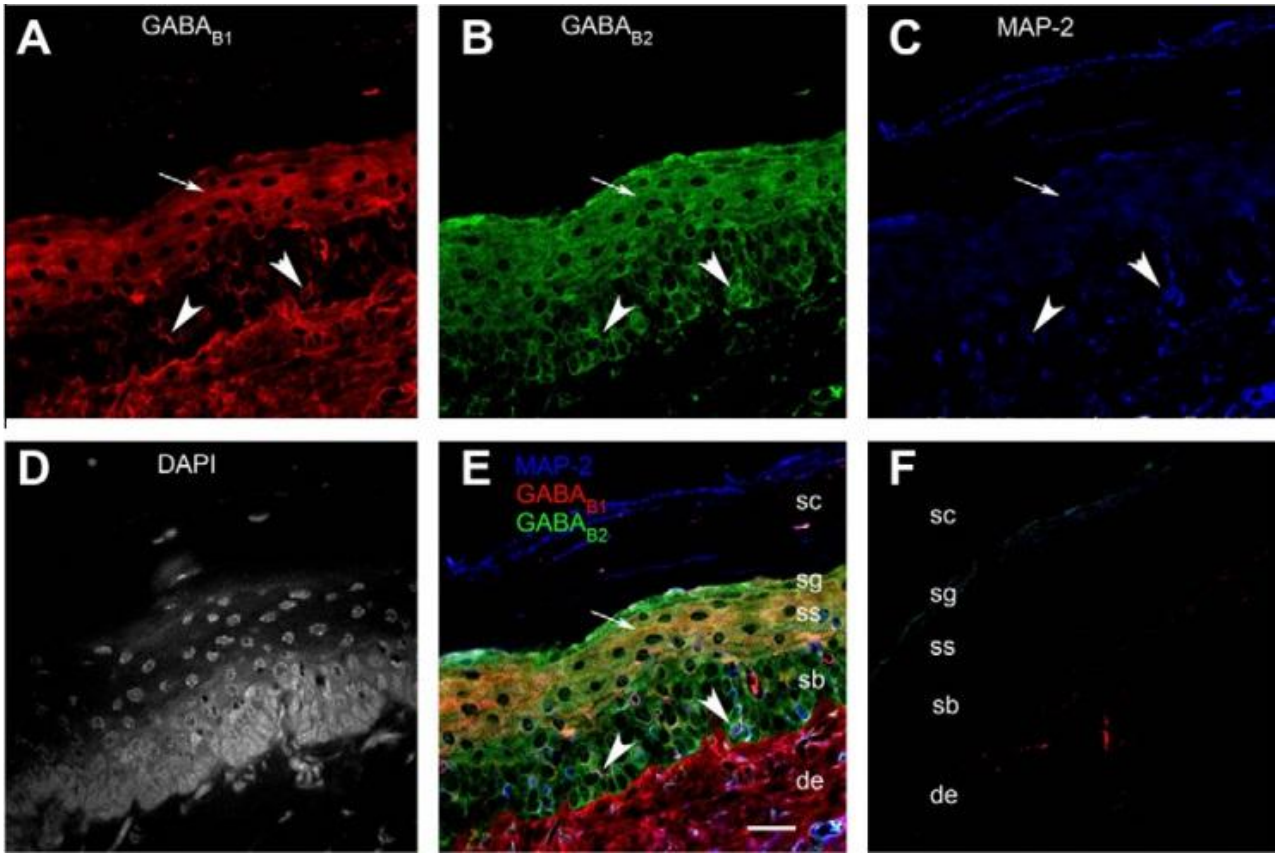

Figure 2.4 Immunohistochemical demonstrations of cutaneous $GABA_B$ receptors. (A) $GABA_{B1}$ receptor subunits (red). (B) $GABA_{B2}$ receptor subunits (green). (C) Staining for MAP-2 positive neural processes (blue). (D) DAPI fluorescence nuclear staining for DNA (greyscale). (E) Confluence of $GABA_{B1}$ and $GABA_{B2}$ (yellow), and MAP-2 (blue) (F) negative control. Imaging settings were identical for panels except (D) Scale bar in (E) is 20 µm and applies to all panels. sc, stratum corneum; sg, stratum granulosum; ss, stratum spinosum; sb, stratum basale; de, dermis.

3 A Novel Alternative to Opioids in General Anesthesia: Evaluation of the Efficacy and Safety of Isovaline Compared to Fentanyl as an Adjuvant to Propofol in Mice *In Vivo*

3.1 Introduction

General anesthesia is very safe for healthy patients, with less than 1 death occurring per 250,000 patients (Catchpole et al., 2008). During 50 years from the first well documented controlled study of anesthetic safety death rates have been reduced by a factor of 160 (Beecher & Todd, 1954). The continual advancements in general anesthetic safety have been achieved through use of improved analgesic and hypnotic agents and through optimization of drug combinations to produce loss of consciousness, blockade of responsiveness to noxious stimuli and muscle relaxation while minimizing side effects. However, severely debilitated patients remain at high risk during anesthesia.

The two principal constituents of general anesthesia are loss of consciousness and immobility (Sanders et al., 2012). The sedative/hypnotic agent, propofol (2, 6-diisopropylphenol), induces a loss of consciousness by modulating central inhibition produced by γ-aminobutyric acid (GABA) at $GABA_A$ receptors (Nishikawa et al., 2011). While the induced unconsciousness is accompanied by some degree of analgesia, propofol is unable to produce surgical analgesia except at doses that produce intolerable side effects in humans and rodents (humans, Hudetz, 2012; rodents, Alves et al., 2007). A disadvantage of currently used opioids such as fentanyl and remifentanyl is that they readily cross the blood-brain barrier, producing adverse effects such as respiratory depression (Dahan et al., 2010). A peripheral analgesic that does not have

appreciable distribution to the central nervous system (CNS) should increase safety by minimizing the use or side effects of opioids.

We have discovered that isovaline (2-amino-isobutyric acid), a blood brain-barrier (BBB) impermeant amino acid, produces analgesia in mice (Whitehead et al., 2012), which may have relevance form improved anesthetic practice. Isovaline analgesia results from activation of $GABA_B$ receptors (Whitehead et al., 2012; Cooke et al., 2012), suggesting additive actions with propofol (Tallarida et al., 2001). Here we have assessed the anesthetic effects resulting from co-applications of isovaline, fentanyl and propofol *in vivo*, measuring hypnosis, immobility to a noxious stimulus, relative therapeutic indices, respiratory side effects and motor performance in a rotorod assay of conscious sedation. Isovaline has not been tested nor approved for human use. Fortunately rodent models of anesthetic actions are reproducible and have been validated for predictive efficacy in humans (Sonner et al., 1999). A rodent model was chosen to test our primary hypothesis that isovaline combined with a hypnotic dose of propofol produces surgical anesthesia in mice without intolerable side effects. Our first hypothesis was that isovaline would produce analgesia in rodent models of surgical stimuli. Our secondary hypothesis was that co-administrations of isovaline and propofol would produce surgical anesthesia with less respiratory depression than fentanyl co-administered with propofol. Finally, our third hypothesis was that isovaline co-administered with low doses of propofol would produce conscious sedation.

3.2 Materials and Methods

3.2.1 Animals

114 adult female CD-1 mice weighing 20-25 g were housed in Animal Resource Unit of The University of British Columbia (UBC). The mice were kept at controlled room temperature (21ºC) and humidity (55%), housed in groups of 4 per cage with a 12 hour light/dark cycle, and had free access to food and water. Experiments were conducted according to guidelines of the Canadian Council on Animal Care. Details of the protocol pertaining to this project were approved by UBC's Animal Care Committee.

3.2.2 Drugs

Propofol was purchased from Tocris Biosciences (Minneapolis, MN), fentanyl citrate from McNeil Laboratories Ltd. (Don Mills, Ontario, Canada) and isovaline hydrochloride from Biofine International Inc. (Vancouver, BC). Physiological saline (0.9% NaCl) was used for dilution of fentanyl and isovaline.

3.2.3 Experimental procedures and design

Mice were gently restrained and injected intraperitoneally according to standard procedures (Hurst & West, 2010). After each injection, mice were placed individually in separate cages for observation, continued video recording and subsequent testing. The restraint and injections were always performed by the same experimenter. All observations were re-assessed through video

analysis by an independent, blinded observer. Because of the binary nature of the up-and-down method, our protocol eliminated mice when there was disagreement regarding the response. For example, when observations made by the two observers were inconsistent, such data were not incorporated in the study.

3.2.4 Assessment of anesthesia

Each animal received a single injection of propofol, isovaline, or fentanyl, or propofol concurrently with either isovaline or fentanyl. Dixon's up-and-down method was used for determination of fifty percent effective doses (ED_{50}) for either hypnosis, loss of tail clip response or sedation. Each animal was used only once. Data are presented as means with 95 % CI. Hypnosis was assessed by measuring the inability of a supine mouse to right itself, which is hereafter referred to as Loss of Righting Reflex (LORR). Immobility, a surrogate measurement of analgesia, was defined by a loss of purposeful response towards an applied Haffner tail clip applied 1 cm from the base of the tail (Langford et al., 2010), which is hereafter referred to as Loss of Response to Tail Clip (LORTC). General anesthesia was defined as the presence of both LORR and LORTC. Maximum Tolerated Dose (MTD) was the dose at which mice became apneic, if respiratory rate was reduced to less than 1/s. For assessment of conscious sedation, mice were placed on a rotating horizontal rod maintained at consistent speed. Time differences taken for mice to fall from the rod were used as a measure of motor dysfunction associated with sedation produced by a given drug. Conscious sedation was defined as a significant decrease in time on the rotorod without abolishing LORR (Hayes and Tyers, 1983). The maximum tolerated dose (MTD) was defined as the dose producing a respiratory rate of less than one breath per

second or multiple apneic periods or death. To provide a general assay on therapeutic index, dose ratios were fixed propofol-isovaline and propofol-fentanyl with starting doses being the LORR ED_{50} and LORTC ED_{50} for propofol and either isovaline or fentanyl, respectively.

3.2.5 Experimental protocol

We studied the effects of isovaline, propofol, isovaline with propofol, and fentanyl with propofol (see Table 1). Doses were administered according to Dixon's up-and-down method (Dixon, 1965) to determine ED50s for hypnosis, surgical analgesia, conscious sedation and MTD. Co-administrations of propofol and isovaline were prepared as follows (see Tables 1-3): the hypnotic ED50 of propofol was co-administered with varying doses of isovaline using Dixon's up-and-down method to determine isovaline's LORTC ED_{50} in the presence of an LORR ED_{50} dose of propofol. Next, the analgesic ED50 of isovaline was co-administered with varying doses of propofol using Dixon's method to determine the LORR ED_{50} of propofol in the presence of an analgesic dose of isovaline or fentanyl. Combinations of propofol and fentanyl were administered using the same methodology. The absence of movement in response to tail clip combined with LORR defined general anesthesia.

3.2.6 Data analysis

Table 1-3 illustrate the conditions assessed in this study. Dixon's up-and-down method was used to determine ED50s, and significance was assessed by comparing the means with 95% confidence intervals for LORR and LOTP. Significance was determined to be equivalent to $p <$

0.05 if 95% confidence intervals did not overlap. Conscious sedation data were recorded, graphed and assessed for significance using the Mann-Whitney test in GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

3.3 Results

3.3.1 Propofol alone

The hypnotic ED50 dose of propofol was found to be 124 mg/kg; 95% CI 84 to 164 mg/kg (Table 3.1; Fig. 3.1A). Consistent with previous reports, propofol was unable to achieve surgical anesthesia when administered alone (Table 2; Figure 1B). Animals became moribund prior to attainment of analgesia to an applied tail clip.

3.3.2 Propofol-isovaline anesthesia

Isovaline produced analgesia (ED_{50} 350 mg/kg; 95% CI 245 to 455 mg/kg) (Table 1, Fig 3.2) while producing surgical analgesia with a reduced dose requirement (ED_{50} 96.2 mg/kg; 95% CI 88 to 104.4 mg/kg) when co-administered with the LORR ED_{50} of propofol (124 mg/kg) (Fig. 3.2) ($P<0.05$). At maximum soluble doses, isovaline did not produce hypnosis (Table 1). The LORR ED_{50} for propofol was the same in both the presence (mean 124 mg/kg 95% c.i. 84-144) and absence of LORTC ED_{50} doses of isovaline (120 mg/kg; 95% CI 80-175) ($P<0.05$). When added to conscious sedating doses of propofol, isovaline did not exacerbate propofol induced rotorod deficits (Fig. 3.5).

3.3.3 Propofol-fentanyl anesthesia

Fentanyl produced surgical analgesia (ED_{50} 0.35 mg/kg; 95% CI 0.23 to 0.47 mg/kg) while producing surgical analgesia with a reduced dose requirement (0.124 mg/kg 95 % CI 0.11 to 0.14 mg/kg) when co-administered with the LORR ED_{50} of propofol (124 mg/kg) ($P<0.05$) (Table 2; Fig. 3.1). Fentanyl did not produce LORR when administered alone prior to the emergence of intolerable side effects to < 1/s (data not shown). The LORR ED_{50} for propofol was in the same both in the presence and absence of LORTC ED_{50} doses of fentanyl ($P>0.05$).

3.3.4 Maximum tolerated dose

Fentanyl was determined to have a maximum tolerated dose (MTD) (ED_{50} 1.36 mg/kg; 95% CI 1.05-1.67) (Table 1) limited by respiratory depression. As the MTD neared, respiration slowed and was dominated by shallow thoracic breathing. There were no side effects for isovaline at 5 g/kg (maximum soluble dose) and no MTD was obtainable (Fig. 3.3). The therapeutic ratio ED_{50} for fixed dose ratios of propofol-fentanyl anesthesia was 2.2; 95% CI 1.96 to 2.6, markedly lower than for fixed dose ratios of propofol-isovaline anesthesia (ED_{50} 4.29; 95% CI 3.82-4.76) ($P<0.05$) (Fig. 3.3).

3.4 Discussion

3.4.1 Peripheral and central components of anesthesia

Anesthesia has been postulated to be composed of distinct hypnotic and analgesic components.

Analgesia in anesthesia can occur in the brain, spinal cord or the periphery (Sonner et al., 2004). Currently used clinical opioids act principally in the CNS to produce analgesia, which results in unwanted respiratory depression. While NSAIDs produce peripheral analgesia, they are not sufficiently efficacious to produce immobility on application of surgical stimuli. We have discovered a novel class of peripheral analgesics, defined by selective $GABA_B$ receptor activation and an inability to cross the blood-brain barrier. This allows us to demonstrate that a selective hypnotic agent, propofol, when combined with a peripherally selective analgesic, isovaline, produces general anesthesia. While not precluding peripheral side effects such as gastrointestinal impairment, peripheral selectivity entirely avoids the limiting central side effects of opioids, which include respiratory depression and sedation. Isovaline, because of its peripheral activity, did not show any side effects at maximum attainable doses (>50 times its effective dose) (MacLeod et al., 2010). Here we show that co-administrations of isovaline and propofol produces robust general anesthesia with enhanced safety compared with preparations based on fentanyl. Whereas volatile anesthetics do not require adjuvants to produce immobility, their potency for hypnosis is much higher than for analgesia indicating that they would also benefit from the addition of peripherally restricted adjuvant agent (Sonner et al., 1999). As corroborated by previous studies (Alves et al., 2007), the effects of propofol alone by intraperitoneal route are unpredictable and are not conducive to producing analgesia sufficient for surgical stimuli. In the first part of the study, we observed that propofol produced hypnosis whereas it was unable to produce any hypnosis prior to emergence of unacceptable side effects. Whereas isovaline and fentanyl were both capable of producing analgesia in mice, neither analgesic produces a hypnotic effect.

3.4.2 Postoperative hyperalgesia and addiction

Postoperative hyperalgesia occurs after opioid usage during surgery, and also as a result of postoperative pain management, leading to progressively increasing dose requirements and associated side effects (for which tolerance does not develop) (Juni et al., 2006). The time course of isovaline analgesia during electrophysiological recording of membrane conductance in nociceptive ventrobasal thalamic neurons is at least 2 h in duration and much longer than opioids or baclofen (Cooke et al., 2012), suggesting that any tolerance to isovaline might emerge over a much longer time scale than for morphine. While isovaline does not enter the CNS, the kinetics of receptor activation may be the same as no functionally distinct $GABA_B$ receptors have been observed and may be assumed to be the same from a kinetics standpoint in the CNS and PNS.

3.4.3 Synergistic properties of isovaline and fentanyl in anesthesia

Interestingly, analgesic potencies of isovaline and fentanyl were found to be enhanced when administered with propofol at hypnotic ED_{50} doses. While the implications on the mechanisms of analgesic synergy remain unknown, isovaline and fentanyl act via distinct mechanisms to produce analgesia, implying that the mechanisms of presumed additivity with propofol are distinct. This suggests the possibility that multiple combinations of analgesics in addition to isovaline in the development of novel analgesics may result in enhanced analgesia. Indeed it has been previously demonstrated that α_2-agonists such as medetomidine and dexmedetomidine are known to reduce dose requirements of propofol to achieve loss of consciousness in a variety of species (Alves et al., 2007). Whereas volatile anesthetics do not require adjuvants to produce immobility, their potency for hypnosis is much higher than for analgesia indicating that enhanced

anesthetic properties would be produced by the addition of peripherally restricted adjuvant. As corroborated by previous studies, the effects of propofol alone by intraperitoneal route are unpredictable and unable to produce analgesia (Alves et al., 2007). However, while isovaline and fentanyl were both capable of producing analgesia in mice, neither analgesic was capable of producing hypnotic effects. While the mechanisms of analgesic synergy remain unclear, these findings suggest that co-administrations of multiple peripheral analgesics could improve analgesic potency and further increase anesthetic safety.

3.4.4 Safety through use of peripheral analgesics in anesthesia

In terms of safety the therapeutic index for propofol-isovaline anesthesia was substantially higher than for propofol-fentanyl anesthesia, suggesting that isovaline represents a superior candidate as an adjuvant in anesthesia compared to opioids. In contrast to isovaline, fentanyl and related opioids such as remifentanyl are potent analgesics but their rapid metabolism makes continued active maintenance during anesthesia necessary and also limits their use in management of postoperative incision pain.

3.4.5 Summary and conclusions

We have shown that peripheral isovaline mediates safer propofol anesthesia in mice, compared to the potent opioid adjuvant, fentanyl. Both isovaline and fentanyl produced synergistic analgesia with propofol, suggesting that distinct drug combinations may be a fruitful subject of further investigation. Isovaline did not exacerbate rotorod deficits seen under propofol conscious

sedation, further demonstrating potential for increased safety in anesthesia. The peripherally restricted analgesic isovaline is a promising first-in-class novel anesthetic adjuvant without detectable CNS effects.

3.5 Tables and figures

LORR (Hypnosis)					
Propofol $ED_{50\ LORR}$	***Isovaline $ED_{50\ LORR}$***	***Fentanyl $ED_{50\ LORR}$***	***Propofol $ED_{50\ LORR}$ added to $ED_{50\ LORTC}$ Isovaline***	***Propofol $ED_{50\ LORR}$ added to $ED_{50\ LORTC}$ fentanyl***	***Comments***
120 mg/kg 95% c.i.80-175	*NO LORR at maximal Soluble dose*	*NO LORR at maximal tolerated dose*	*124 mg/kg 95% c.i.84-144*	*124 mg/kg 95% c.i.84-144*	*No decrease in ED50 LORR in presence of isovaline or fentanyl*

LORTC (analgesia)					
Propofol	***Isovaline***	***Fentanyl* ED_{50}**	***Isovaline ED50 added to ($ED_{50\ LORR}$ Propofol)***	***fentanyl* ED_{50} *added to ($ED_{50\ LORR}$ Propofol)***	***Comments***
NO LORTC at maximal tolerated dosedose	*350 mg/kg 95% c.i.245-488*	0.35 mg/kg *95% c.i. 023-0.47*	*120mg/kg 95% c.i.40-195*	*0.124 mg/kg 95% c.i. 0.11-0.14*	*Isovaline and propofol Analgesia potentiated by propofol*

Maximum Tolerated Doses (MTD) and Therapeutic Index				
Isovaline	***Fentanyl***	***Isovaline-propofol Therapetic Index***	***Fentanyl –propofol Therapeutic Index***	***Comments***
None at 5 g/kg (maximum soluble dose)	*1.36 mg/kg 95% c.i.1.05-1.67*	*4.29 95% c.i.3.82-4.76*	*2.2 95% c.i.1.96-2.0*	*Fentanyl confers reduced therapeutic ratio compared to isovaline*

Table 3.1 Anesthesia produced by propofol, fentanyl and isovaline. The degree of hypnosis produced by propofol was not influenced by ED_{50} analgesic doses of isovaline or fentanyl. Propofol hypnosis increases the analgesic potency of isovaline and fentanyl. Isovaline-propofol anesthesia is safer than fentanyl-propofol anesthesia.

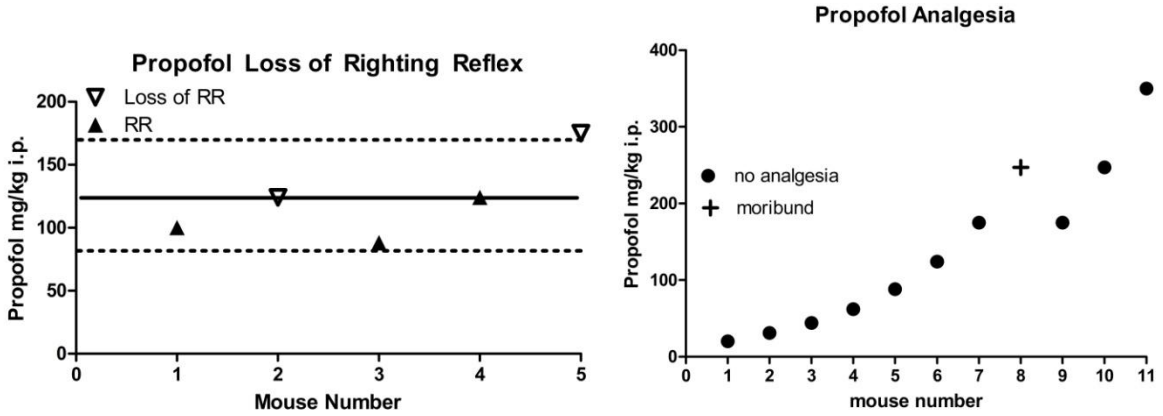

Figure 3.1 Propofol loss of righting reflex and analgesia. Propofol produces LORR but no loss of tail pinch (LOTP). Doses expressed in mg/kg (n = 5 for LOC and n = 12 for LOTP; mean ± 95% CI). Propofol LOC ED_{50} was determined to be 124 mg/kg. Propofol did not produce LOTP prior to MTD.

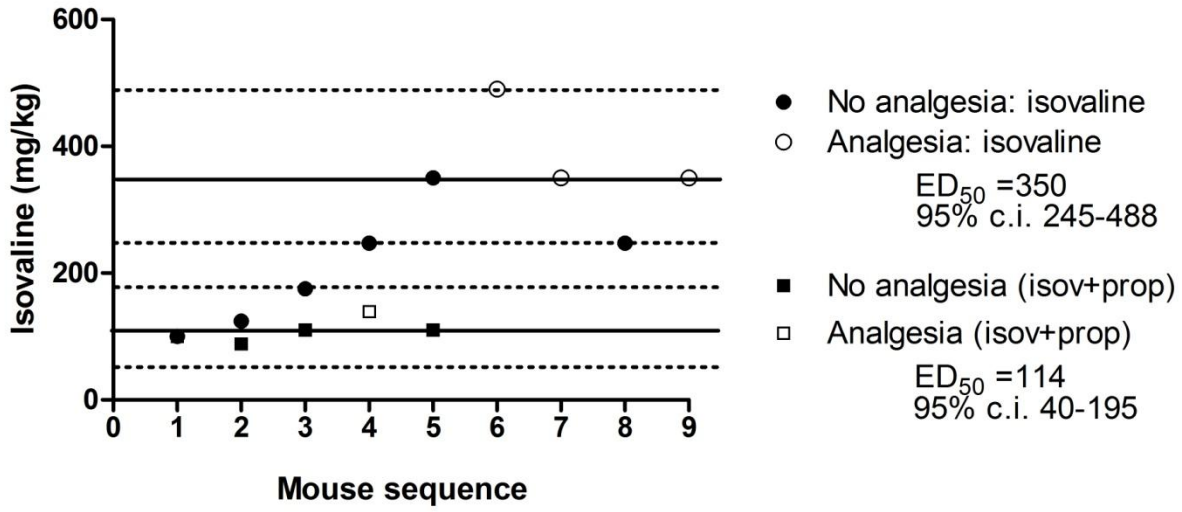

Fig. 3.2 Analgesia produced by isovaline and propofol-isovaline. Doses expressed in mg/kg for co-incidence of LOC and LOTP for isovaline and propofol-isovaline (n =10 for isovaline; and n = 6 for isovaline-propofol; means ± 95% CI).

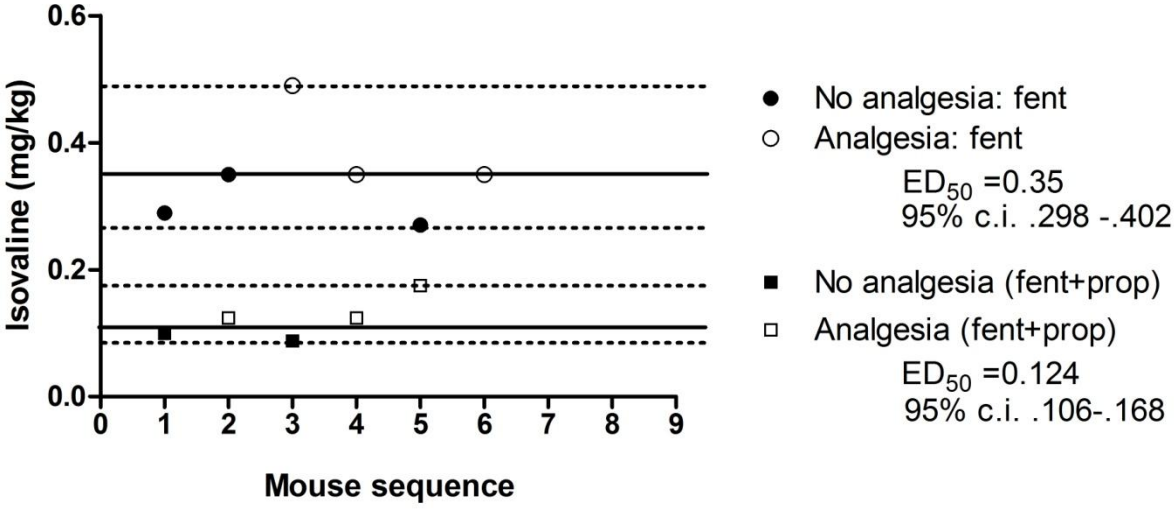

Fig. 3.3 Analgesia produced by fentanyl and propofol-fentanyl. Doses expressed in mg/kg for co-incidence of LOC and LOTP for isovaline and propofol-isovaline (n = 6 for fentanyl; n = 6 for fentanyl-propofol; means ± 95% CI).

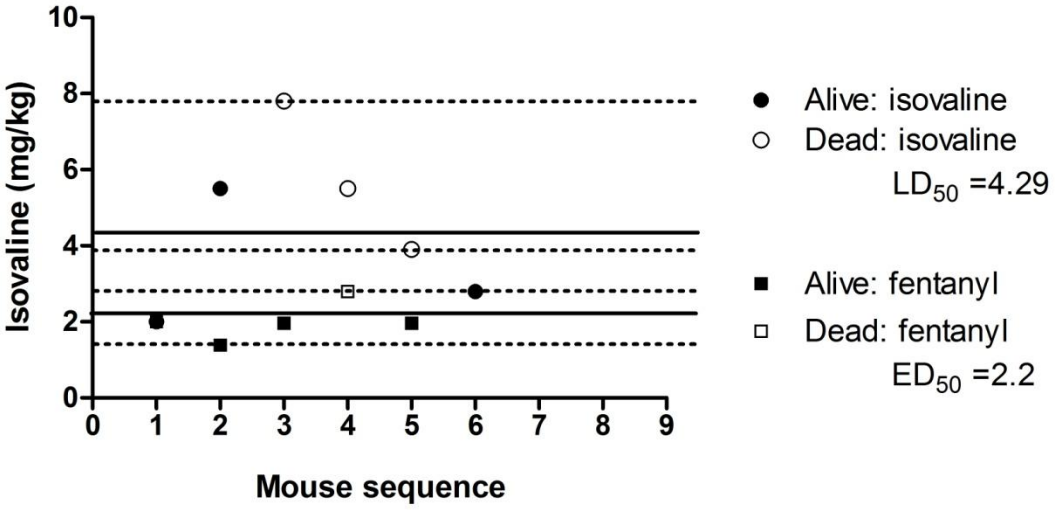

Fig. 3.4 Therapeutic indices for propofol-isovaline and propofol-fentanyl. Doses expressed in mg/kg for co-incidence of LOC and LOTP for isovaline and propofol-isovaline (n = 5 for isovaline; n = 5 for fentanyl; means ± 95% CI).

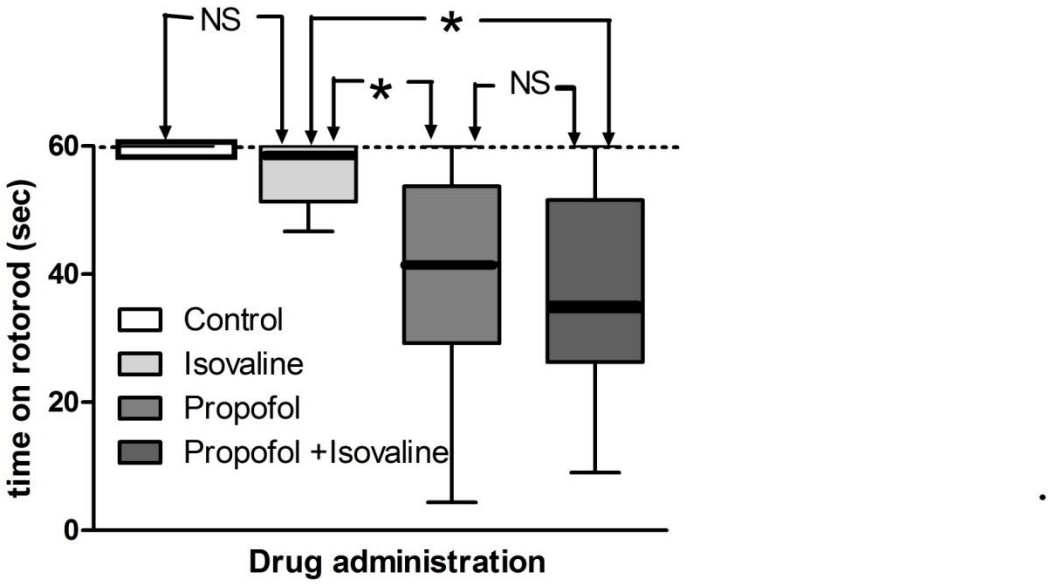

Fig. 3.5 Rotorod performance under conscious sedation. Doses expressed in mg/kg (n = 6 for each group; means with SD). Propofol disrupted rotorod performance and rotorod performance deficits were not accentuated by isovaline (350 mg/kg).

4 [2]Evaluation of a Novel Mouse Model of Intracisternal Strychnine-Induced Trigeminal Allodynia

4.1 Introduction

There is a pressing need for effective therapies for intractable dynamic mechanical allodynia in conditions such as trigeminal neuralgia (Costigan et al., 2009). Indeed, trigeminal neuralgia is widely considered to be the most excruciatingly painful condition in existence for humans, which consists of remitting and relapsing stabbing pain in facial regions in response to light touch, mediated by aberrant local trigeminal nerve function (Kitt et al., 2000). Studies of trigeminal neuralgia drug treatments in humans are difficult to implement due to the episodic and variable nature of the disorder (Miraucourt et al., 2007). There is therefore a need for accurate, predictive and cost effective animal models for development of novel drug candidates. Recently, trigeminal glycinergic inhibitory dysfunction has been shown in rats to produce localized dynamic allodynia, a central feature of human trigeminal neuralgia (Okeson, 2005; Miraucourt et al., 2009). While electrophysiological, behavioural, and pharmacological studies *in vivo* have shown that this produces many of the features of clinical trigeminal neuropathies, such a model has not tested for possible validity as a potential screen or model of drug treatment responsiveness in human trigeminal dyniamic allodynia (Dickenson et al., 2006). Nonetheless, clinically ineffective morphine has also been shown to be ineffective in reversing rat dynamic mechanical allodynia resulting from strychnine-induced glycinergic inhibitory dysfunction. This suggests a

[2] A version of this chapter has been published. Lee, I.O., Whitehead, R.A., Ries, C.R., Schwarz, S.K., Puil, E., MacLeod, B.A. Evaluation of a novel mouse model of intracisternal strychnine-induced trigeminal allodynia. Canadian Journal of Anesthesia. [Epub ahead of print]. 2013

possible relationship to the human disorder (Okeson, 2005; Miraucourt et al., 2009). Here we evaluate an alternative mouse preparation of intracisternal strychnine-based of trigeminal neuropathic pain and examine the effects of aCSF, morphine and carbamazepine expoxide (CBZe) in order to assess predictive efficacy for screening drugs which may treat human trigeminal dynamic allodynia. CBZe is more water soluble than carbamazepine and has been shown to be of equivalent potency to carbamazepine in humans, making it a more desirable compound for experimental usage (Tomson et al., 1984). The evaluation and development of a mouse preparation analogous to the rat model of trigeminal glycinergic inhibitory dysfunction takes advantage of the availability of genetic mouse variants, resulting in lower costs for animals and animal housing, and reduced drug dose requirements. The comparative efficacy of clinical effective and ineffective agents has not been studied for trigeminal strychnine-induced glycinergic inhibitory dysfunction (van Kleef et al., 2009). Here we test a putatively novel mouse model of human trigeminal dynamic allodynia by comparatively assessing the effects of the first-line clinically efficacious epoxide of carbamazepine along with the clinically ineffective drug, morphine.

4.2 Materials and methods

4.2.1 Animals

Adult female CD-1 mice weighing 20-25 g were housed in an approved facility with a 12-hr light/dark cycle and free access to food and tap water. The experiments were conducted in accordance with the Guidelines of the Canadian Council on Animal Care and were approved by The University of British Columbia Animal Care Committee.

4.2.2 Prior experiments

Intracisternal (i.c.) injections were conducted such that needle placement was directed into the cleft between the occiput and atlas vertebra through intact skin. The needle tip was inserted into the cleft such that near-vertical position was maintained, and was then rotated forwards such that the bent portion was kept in close contact with the internal surface of the occiput for the entire length. After injection of 5 μl drug solution, the syringe was held in the same position at an angle of 45-55^{o} for 5 seconds to minimize fluid outflow. In a set of prior experiments, the accuracy of the injection method was tested using 1% methylene blue solution, allowing assessment of dye distribution in intracisternal space. Dye was distributed in the cisterna magna up to the occipital surface of the brain in 98 percent of 20 mice tested. In pilot experiments, we investigated the effects of i.c. glycine on strychnine-induced allodynia. A (5 μl) solution of 200 μM (0.3344 μg) strychnine in artificial cerebrospinal fluid (aCSF, composition below) was coinjected with 0, 20, 200 or 400 μM glycine into the cisterna magna. An additional pilot study was done using glycine, which could be from a logical standpoint a useful treatment to alleviate glycine$_A$ receptor synaptic blockade induced by strychnine. However, it had previously been observed that i.c. glycine injections in cats (65 μM) resulted in pronounced respiratory depression (Holtman et al., 1982). Here were observed that glycine at 20 μM [n = 1] 200 μM [n = 1] and 400 μM [n = 3] co-injected with i.c. strychnine did not attenuate strychnine induced allodynia. Consistent with Holtman's observations, the highest dose of glycine (400 μM) produced pronounced respiratory depression and death in 3 or 3 animals, presumably resulting from activation of glutamate receptor activation of the glycine$_B$ site, and resulting in discontinuation of these experiments.

4.2.3 Response measurement to intracisternal injections

Mice were anesthetized with an isoflurane/air mixture in a 500 ml induction chamber. A rubber mask was used to maintain halothane anesthesia and to immobilize the head. Isoflurane was administered through a Mapleson D circuit with a Drager vaporizer. To maximize the opening to the cisterna magna, the neck was flexed over a modeling clay form and the space between the occiput and C1 segment was identified by palpation with the index finger (Lee et al., 2011). A 10 μl microvolume precision syringe was used (Hamilton, Reno, NV, USA) with a 26 gauge needle that had a non-coring beveled tip partially covered by polyethylene (PE-10) tubing to limit penetration to 4 mm. Dynamic allodynia was restricted to the trigeminal innervated facial regions. No morbidity was observed (such as piloerection, immobility, decreased responsiveness to touch, or decreased respiratory rate lower than 20% below baseline levels).

4.2.4 Drug administration

Three separate experiments were performed for a total of 36 mice. Each animal was used only once. Experiments were conducted between 09:00 and 16:00 in a quiet room. All experiments were recorded by video camera (Canon, Tokyo, Japan) and saved to a computer file. Drug dosage is reported here as the molarity in total volume of injected solution (5 μl). Even after sonication of carbamazepine for >20 min, carbamazepine did not result in a homogeneous emulsion. We therefore used the water-soluble active carbamazepine metabolite, carbamazepine epoxide (CBZe; Sigma Chemical, St. Louis, MO, USA), which has comparable pharmacological properties in humans (Tomson et al., 1984). The maximal soluble dose of CBZe (3.2 μM) had no obvious systemic side effects as indicated by an absence of piloerection. Strychnine and CBZe were dissolved in aCSF containing (in mM): 124 NaCl, 26 NaHCO3, 1.25 NaH_2PO_4, 2.5 KCl, 2

$MgCl_2$, and 2 $CaCl_2$, and 10 dextrose at pH = 7.3-7.4. Strychnine and CBZe were injected intracisternally. Morphine (Reckitt & Colman, Toronto, ON, Canada) was dissolved in saline (3 mg/kg) and was injected subcutaneously at 5 ml/kg body weight. Since intracisternal morphine has been shown to be ineffective against glycinergic inhibitory dysfunction, this administration of an effective anti-allodynic dose systemically served as a control for nonspecific mechanical allodynia. All drug combinations were injected 10 min prior to recordings.

4.2.5 Experimental design

Drug and control (aCSF) solutions were injected in random order in order as determined by a computer. Injections were prepared and coded by an independent investigator from the experimenter, allowing for blinding to group allocation. One mouse of each of 6 pairs in each experiment randomly received one injection for each comparison. Mice were placed on opposite sides of a transparent plastic cage (30 cm long, 15 cm high, and 15 cm wide), divided by an opaque internal wall. The maximum time variation between control and drug injections was 30s. Blinded and randomized drug allocation was determined by random sequence using a computer randomizer, and solutions were prepared by an independent experimenter. Recovery from anesthesia occurred less than 4 min after discontinuation of halothane. Assessments were made at 4, 6, 8, 10, 12 and 14 minutes.

4.2.6 Allodynia testing

We tested for dynamic mechanical allodynia by observing the response to lightly brushing an 8 cm long PE-10 tube against the grain of the animal's fur in trigeminally innervated regions as

detailed below. Flinching, scratching, and/or agitation (quantified by discrete criteria) were indicative of dynamic mechanical allodynia. The brushing was applied first to the face and then to the neck, followed by the back, forelimbs and hind limbs. There were no allodynic responses in the back, fore and hind limbs, areas not in the anatomical distribution of the trigeminal nerve. Each experiment was reviewed through videotaped record by an independent observer. The absence of response to stimulus within the distribution of the trigeminal nerve was scored as 0; eye squinting or backward folding of ears as 1; head withdrawal as 2; and face scratching as 3. 12 In treatment groups, we determined each individual animal's allodynia score as the sum of the scores at each of the six two minute intervals. The minimum cumulative allodynia score for each mouse was zero and the maximum was 18; results are presented as percentage of this maximum attainable allodynia score with mean and standard deviation or standard error for each of the treatment groups. In a separate set of experiments we recorded the global impression as to which mouse in a pair of mice had the more severe allodynia. To determine if generalized analgesia was produced, we observed tail-flick responses to a curved 50 mm vascular tail clamp (Bulldog type Serrefine; International Fine Science Tools Inc., North Vancouver, B.C., Canada).

4.2.7 Statistical analysis

For each of the three experiments the allodynia scores were compared between 2 drug groups using Student's two-sample *t*-test was used in each of the two drug groups in the three individual experiments, chosen based on previous observations of normally distributed data sets. While no specific sample size calculation was undertaken, our previously observed low variance with experiments involving intracisternal injections provided adequate justification for discrimination

between experimental outcomes. Data were analyzed using SigmaStat, v. 3.01 (Systat Software Inc.; Chicago, IL, USA). 3 groups of 6 mouse pairs (36 mice total in these comparisons) were tested to objectively determine relative pain scores in a dichotomous disequilibrium side-by-side comparison. These data were analyzed using the binomial comparison test, a well-established method to compare exact test of the statistical significance of deviations from a theoretically expected distribution of observations into two discrete categories.

4.3 Results

4.3.1 Effects of strychnine compared to artificial cerebrospinal fluid (aCSF) control

In the preliminary study 200 μM intracisternal strychnine, the largest dose that produced allodynia without convulsions or agitation was chosen to induce reproducible dynamic allodynia. There were no changes in responses to tail clamp. Allodynia lasted from the time of emergence from anesthesia to greater than 20 min after strychnine injection (Fig. 4.1A). Neither strychnine (200 μM) nor aCSF produced motor abnormalities or convulsions. Between stimuli, animals appeared behaviorally normal and did not exhibit excessive grooming, piloerection, or abnormal movement. None of the 6 animals injected with aCSF showed allodynia over the entire test period but 3 showed transient initial allodynia lasting less than 4 min (Fig 4.1A). Strychnine produced a significant increase in individual animal's allodynia scores (17.10 ± 2.81) compared to aCSF ($p < 0.05$) (Fig. 4.1B) and greater comparative allodynia in 6 of 6 pairs of mice over all time periods compared to those injected with aCSF alone (Table 2).

4.3.2 Effects of CBZe on strychnine-induced allodynia

In the absence of strychnine, CBZe (4 ng intracisternally) produced no apparent sensory or motor deficits based on qualitative observation (data not shown). The dose was chosen based on relative potency of CBZe to carbamazepine. A normal tail-flick response to a vascular clamp was present at each measurement time. All 6 mice coinjected with CBZe and strychnine showed markedly reduced allodynia scores over all time periods (Fig. 4.2A). CBZe co-injected with strychnine reduced the mean cumulative allodynia score compared to that associated with strychnine alone (Fig. 4.2B). In 6 of 6 pairwise comparisons, allodynia in animals receiving CBZe was lesser compared to those injected with strychnine alone (Table 4.2).

4.3.3 Effects of subcutaneous morphine on strychnine-induced allodynia

Morphine (3 mg/kg s.c.) co-injected with intracisternal strychnine did not change mean allodynia scores compared to strychnine alone (Fig 4.3). Since the onset time of morphine analgesia is within the time frame recorded, the lack of effects cannot be attributed to allowing for inadequate onset time. In pairwise assays, allodynia compared to strychnine alone was lesser in 3 pairs and greater in 3 pairs, indicating no effect ($p = 1.0$) (Table 4.1).

4.4 Discussion

In this study, CBZe selectively decreased intracisternal trigeminal mechanical allodynia induced by glycinergic inhibitory dysfunction while morphine did not, validating this preparation as a mouse model of human trigeminal mechanical allodynia. The similarities of intracisternal

strychnine-induced glycine disinhibition to clinical trigeminal neuropathy include the presence of dynamic allodynia and its restriction to the distribution of the trigeminal nerve distribution (Okeson, 2005). Strychnine did not produce changes in sensorimotor activity in areas other than the trigeminal distribution, allowing for assessment of nervous system dysfunction specific to the trigeminal nerve.

Models of trigeminal allodynia such as infraorbital nerve constriction (Mansikka et al., 2004) produce secondary changes in the CNS resulting from peripheral nerve damage, while intracisternal strychnine does not produce peripheral nerve destruction. These features allow for reproducible assessment of glycinergic inhibitory dysfunction in trigeminally innervated regions from the standpoing of achieving parallel drug efficacy. In the treatment of trigeminal neuropathy in humans, the water-soluble active carbamazepine metabolite carbamazepine-10, 11-epoxide, has similar or greater potency than its parent drug carbamazepine, and contributes to its antineuralgic effects (Tomson et al., 1984). Systemic administration of carbamazepine is widely documented and would be expected to achieve similar brainstem therapeutic target levels when taken orally. However, systemic administration could complicate behavioural observations as there are several active metabolites of carbamazepine with differing potencies and side effects. The predominance of CBZe due to inhibition of its efflux transport mechanisms by the other metabolites results in decreased elimination of the epoxide (Tomson et al., 1984). While the efficacy of CBZe is higher in our model than for carbamazepine in human trigeminal neuralgia the demonstration of, and the ability to localize the effect, was felt to be of greater importance than establishing analogous dosing potencies. Morphine is presumed to accentuate allodynia via excitation several mechanisms; however, the lack of effectiveness of intracisternal morphine on dy-

namic allodynia as commonly occurs in clinical trigeminal neuralgia was not attributable to glycine receptor activation (Vos et al., 1994). Subcutaneous morphine (at 3 mg/kg or less) has been shown to block peripheral mechanical allodynia without sedation, allowing morphine to serve as a negative control condition for the presence of non-specific peripheral mechanical allodynia (Miraucourt et al., 2009; Vos et al., 1994). To examine the involvement of intracisternal glycine receptors in modulation of allodynia we injected i.c. glycine and observed excitation attributable to co-activation of excitatory glutamate receptors (Vos et al., 1994). Acute injection of strychnine into the cisterna magna of mice has been previously demonstrated to produce dynamic trigeminal mechanical allodynia and has previously been shown to be of supraspinal origin based on observation of effects specifically due to localized injections in the cistern magna (Miraucourt et al., 2007). These observations correlate well with the occurrence, type, and receptive field of dynamic allodynia observed in human trigeminal neuralgia. Our pairwise binary disequilibria design is a useful technique for identification of drug effects. Given the presence of glycine receptors in the trigeminal nuclei and the presence of glycine α2 receptor neuronal gene (Zarbin et al., 1981; Puri et al., 2011), the simplest explanation for the observed allodynia is that strychnine blocks brainstem $glycine_A$ receptors (Betz et al., 2006; Holtman et al., 1982). The brainstem trigeminal nucleus is the most probable site of action of strychnine in our study as strychnine applied to the trigeminal nucleus caudalis enhances responses in rostral trigeminal nuclei evoked by innocuous facial tap (Davidoff et al., 1969). Direct action on the glycinergic receptor to produce allodynia is unlikely for a number of reasons. Co-administration of glycine with strychnine did not produce any antiallodynic effect at 20 μM or 200 μM but 400 μM produced respiratory depression that lead to death in 3 cases. This respiratory depression has been previously reported for glycine (Holtman et al., 1982). We have verified that the novel amino acid isovaline blocked

strychnine-induced allodynia (MacLeod et al., 2010) despite not acting on iontropic receptors but rather through metabotropic receptors (Whitehead et al., 2012). Similarities between baclofen and carbamazepine have been noted, in addition to superficially evident molecular similarities in ring structures and amide groups (Fromm et al., 1987). The prototypical $GABA_B$ receptor agonist baclofen has also been shown to be effective against animal models and in human trigeminal neuralgia (Terrence et al., 1983), and must be tested in the future in this model to determine the efficacy of $GABA_B$ receptor agonism in treatment of trigeminal allodynia. Thus the anti-allodynic action is felt to occur by restoring inhibition that is lost through strychnine-induced glycine receptor antagonism. This study in the mouse, in addition to confirming the ineffectiveness of morphine against dynamic mechanical allodynia in the rat, demonstrates of the effectiveness carbamazepine's equipotent metabolite CBZe in alleviating intracisternal strychnine-induced allodynia (Miraucourt et al., 2009). These findings are consistent with clinical findings that carbamazepine is a first-line treatment (Tomson et al., 1984) and that morphine is ineffective. While this putative model of trigeminal dynamic allodynia in humans does not perfectly replicate the severity or precise pathogenesis of human trigeminal neuralgia, one key justification for translational animal models of human disease is a demonstration of the ability of the model to predict which drugs are effective in the human, which this model achieves very effectively. Since the analgesic isovaline has been shown to be effective in this model and has not yet been tested in humans, it will be one test of the predictive ability of this model. This mouse preparation is simple, reproducible, economical, humane, and permits use of a vast repository transgenic mice for advanced mechanistic studies and may be useful in the development and screening of future drug treatments of clinical trigeminal neuralgia.

4.5 Tables and figures

Relative Strychnine (Str)- induced Allodynia in Mouse Pairs		
Paired condition	**Mouse with greater allodynia**	***p* value**
Str *vs.* aCSF	Str in 6 of 6 pairs	$p = 0.03$
Str *vs.* Str + CBZe	Str in 6 of 6 pairs	$p = 0.03$
Str *vs.* Str + morphine (3 mg/kg s.c.)	Str in 3 of 6 pairs morphine in 3 of 6 pairs	$p = 1.0$
Str *vs.* Str + isovaline 5µl of 4 mM (3 µg)[18]	Str in 8 of 8 pairs[18]	$p = 0.008$

Table 4.1 Relative allodynia for pairs of mice, effect of treatment. Three groups of 6 pairs, (12 mice per comparison), binomial distribution test. One group of 8 pairs (16 mice) isovaline is from previously published data (MacLeod et al., 2010).

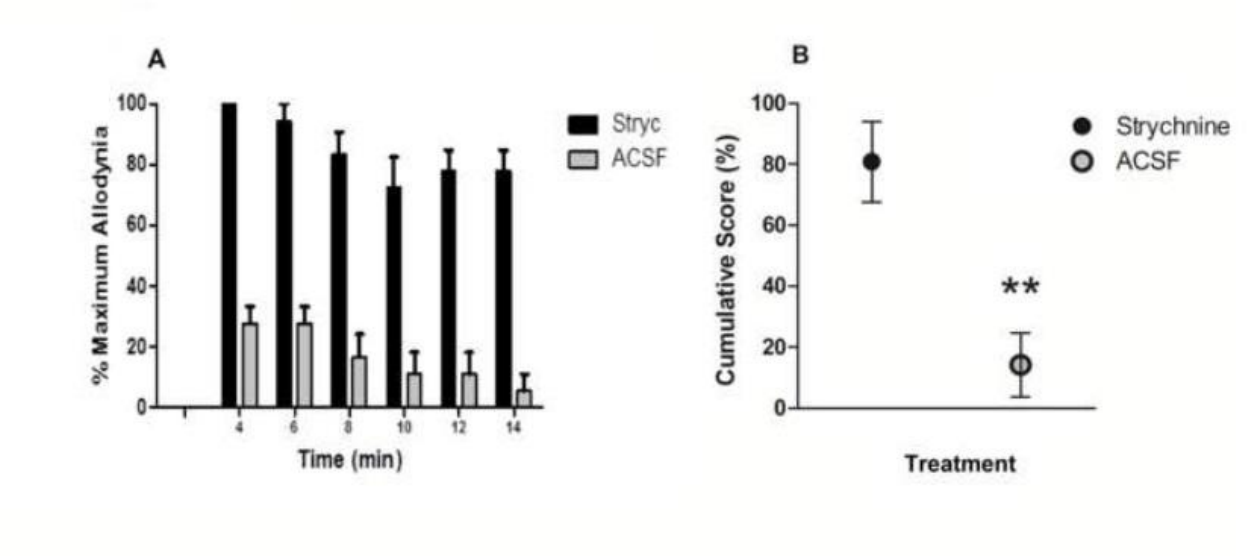

Figure 4.1 Strychnine compared to control (aCSF) allodynia. (A) Time-dependent increase in allodynia score, means ± SD (n = 6). (B) Cumulative allodynia scores between 4 and 14 min, normalized to maximum possible effect, means ± SD (n = 6); ** $p \leq 0.05$.

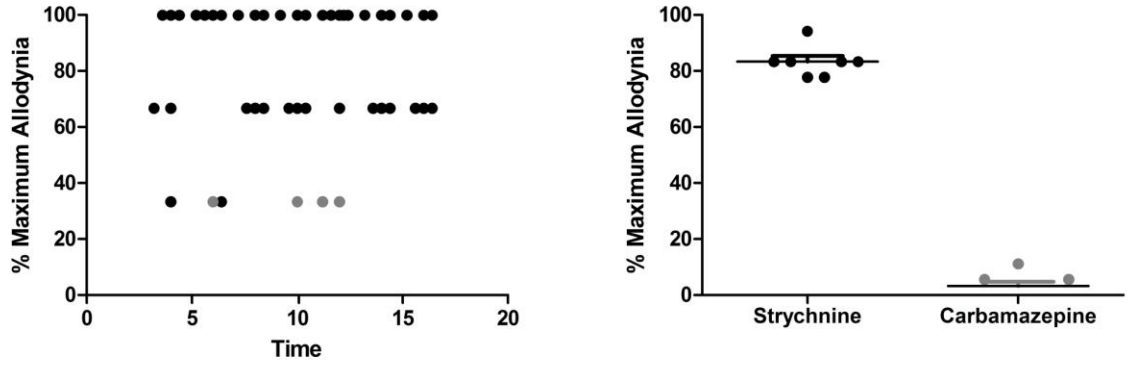

Figure 4.2 Effects of CBZe on strychnine-induced allodynia. A. Time-dependent reduction of allodynia scores, means ± SEM (n = 6). B. Corresponding population cumulative allodynia scores between 4 and 14 min normalized to maximum possible effect means ± SEM (n = 6); ** $p \leq 0.05$.

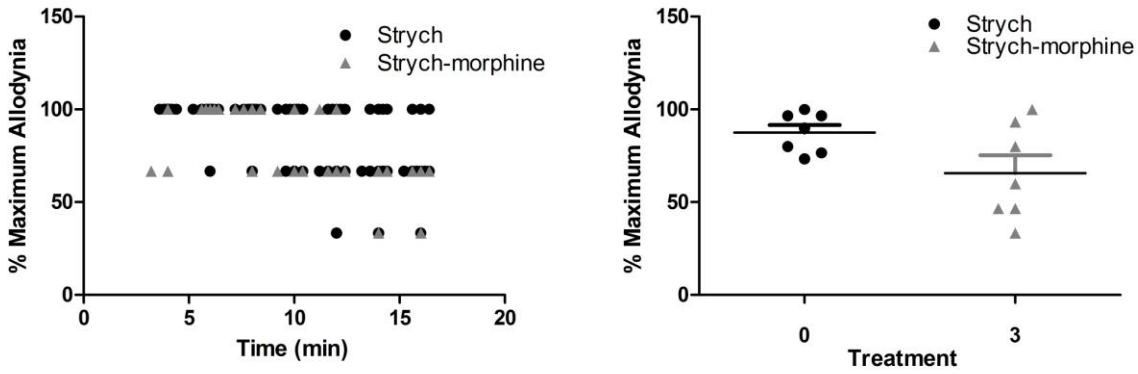

Figure 4.3 Effects of morphine on strychnine-induced allodynia. A. Time-dependent differences in allodynia scores, means ± SD (n = 6). B. Corresponding population cumulative allodynia scores between 4 and 14 min normalized to maximum possible effect means ± SEM (n = 6); ** $p \leq 0.05$.

5 General Discussion

5.1 Introduction

The overarching theme of my graduate work has been based on research pertaining to the mechanisms of action of the novel brain impermeant analgesic, specifically its *in vivo* effects modulated by activation of peripheral $GABA_B$ receptors. The first manuscript in the thesis investigated the peripheral $GABA_B$ receptor-mediated mechanisms of action of isovaline, GABA and baclofen, showing that that selective activation of peripheral $GABA_B$ receptors is sufficient to block heightened cutaneous sensitivity along with arthritis in mice, relevant to the human condition. Furthermore, we speculated that analgesic actions of isovaline and GABA occur predominantly through activation of cutaneously expressed $GABA_{B1}$ and $GABA_{B2}$ receptor subunits, as opposed to those expressed in the dorsal root ganglia.

The third chapter of this thesis assessed the surgical analgesic properties of isovaline in propofol anesthesia. Using Dixon's up-and-down method, we determined that isovaline is produces surgical analgesia in mice as defined by the ability to block purposeful response to a standard alligator tail clip. We found that an anesthetic regimen of isovaline-propofol is capable of producing total surgical anesthesia with a therapeutic index substantially higher than the surgical anesthesia produced by propofol administered with the conventional analgesic adjuvant, fentanyl.

The fourth chapter focused on the development and validation of a mouse model of trigeminal neuralgia based on intracisternal strychnine-induced glycinergic inhibitory dysfunction. We found that when applied into the cisterna magna of a mouse, isovaline is capable of reducing the amount of discomfort produced by a localized injection of the glycine receptor antagonist

strychnine injected into the same area. The clinically ineffective drug morphine did not attenuate allodynia in this model, while an epoxide metabolite of first-line treatment carbamazepine blocked pain, predicting the clinical efficacy in humans. Isovaline was also found to be effective in blocking dynamic allodynia induced by strychnine, suggesting possible utility for screening new drugs.

5.2 Peripheral analgesia by isovaline

The first manuscript in this thesis dealt with assessing the peripheral $GABA_B$ receptor-mediated analgesic properties of isovaline, GABA and baclofen in mice. We found that isovaline, GABA and baclofen produced anti-allodynia which was blocked by the $GABA_B$ receptor antagonist, CGP52432, and amplified by the $GABA_B$ receptor positive allosteric modulator, CGP7930. Using immunohistochemistry on cutaneous tissues at the site of allodynia testing showed expression of both $GABA_{B1}$ and $GABA_{B2}$ receptor subunits, colocalized in keratinocytes, and free nerve endings exhibiting characteristic longitudinal staining pattern used to identify fine afferent neuronal processes (Messlinger, 1996; Radtke et al., 2010). Prior studies demonstrated that radio-labelled isovaline does not penetrate the brain (Shiba et al., 1989). Indeed while GABA has active transporters, cortical slab models of seizures suggest that GABA does not cross the blood-brain barrier in appreciable quantities (Gottesfeld et al., 1971). The observation that baclofen produced substantial sedation while both isovaline and GABA produced no detectable CNS effects strongly corroborates this view. There are currently no peripherally restricted amino acids approved as analgesics; whereas attempts have been unsuccessful to develop peripherally restricted opioid receptor agonists (DeHaven-Hudkins et al., 2004) brain impermeant inhibitory amino acids may comprise a distinct class of analgesics for treatment of pain that completely by-

pass the possibility of adverse CNS effects. While involvement of $GABA_A$ receptors has not been tested *in vivo*, bath application of the $GABA_A$ receptor antagonist bicuculline did not block isovaline-induced conductance increases in nociceptive thalamus, precluding involvement in mediating isovaline's effects (Cooke et al., 2012). We found that while co-localization of $GABA_{B1}$ and $GABA_{B2}$ receptor subunits was observed in both keratinocytes and fine nerve endings, the bulk of confluent $GABA_B$ receptor subunit staining was in keratinocytes (Whitehead et al., 2012). There are several possible explanations for the observed differences in expression intensity. One explanation is that non-neuronal cells such as keratinocytes play a very involved role in endogenous modulation of pain, or that they may regulate expression of inhibitory cannabinoid and $GABA_B$ receptors in response to exposure to pro-inflammatory mediators (Zhang et al., 2010; Radtke et al., 2010). This could be elucidated through patch clamp electrophysiology of keratinocytes excised from glabrous tissue from the distal site of afferent nociception.

Many clinical trials have been undertaken which have demonstrated the efficacy of the prototypical $GABA_B$ receptor agonist, baclofen as an effective antinociceptive agent (Steardo et al., 1984). The observation of equivalent antinociceptive effects of baclofen in mice under wild type and peripheral $GABA_B$ receptor knockdown mice has previously been used to argue that peripheral $GABA_B$ receptors are redundant drug targets for production of $GABA_B$ receptor-mediated analgesia (Gangadharan, 2010). While the authors validated some aspects of a novel peripheral gene knockdown model, their conclusions are hampered by the fact that the only $GABA_B$ agonist tested was baclofen, a drug which readily crosses the blood-brain barrier to produce an indiscernible mixture of peripheral and central analgesia when administered peripherally. The model also fails to account for compensatory changes which occur in response to gene knockdown.

Therefore, the arena of exploration for peripherally restricted $GABA_B$ agonists for treatment of pain remains wide open. Several loci have been identified as promising peripheral tissue drug targets, including keratinocytes, free nerve endings (Zhao et al., 2008) and synoviocytes (Tamura et al., 2009). The efficacy of peripherally administered GABA at $GABA_B$ receptors suggests that structurally analogous compounds in addition to isovaline may be very promising for the alleviation of pain. Indeed, it was also demonstrated in an *in vivo* preparation that isovaline has an unusually long duration of analgesic action (data not shown). These observations may be a result of avid binding to the $GABA_B$ receptor as suggested by molecular models (Dr. R.A. Wall, personal communication), allowing for a longer duration of analgesia, and requiring reduced dosing frequency.

5.2.1 Implications for peripheral $GABA_B$ receptor activity

Identifying the specific peripheral site(s) at which $GABA_B$ receptor activation produces analgesia will have implications for drug development and administration techniques. For example, if keratinocytes and free nerve endings are found to be a principal aspect of $GABA_B$ modulation of pain in the periphery, the development of a cream-based topical preparation of isovaline could be effective (Sawynok, 2003). This would harness local activation of $GABA_B$ receptors specifically at distal sites possibly inducing release of inhibitory substances from keratinocyte cell bodies. Furthermore as isovaline's mechanism of analgesic action is novel from the standpoint of peripheral selectivity, it will also be of interest to examine possible synergistic analgesic interactions between isovaline and peripheral anti-inflammatory/analgesic drugs, including NSAIDs or opioids (Torres-López et al., 2013). Activation of $GABA_B$ receptors has been shown to block morphine-induced behavioural sensitization in rats, indicating a role in blocking morphine sensi-

tization associated with underlying mechanisms of drug abuse and hyperalgesia (Fu et al., 2012). Peripheral analgesia may have implications in CNS for central sensitization and other complications resulting from chronic peripheral pain such as cognitive impairment, which has been shown to result from chronic exposure to pain (May et al., 2008). Recent unpublished laboratory observations indicate that isovaline modulates inhibitory metabotropic glutamate receptors in addition to $GABA_B$ receptors (Asseri et al., Soc. Neurosci. Abstracts, 2013). This suggests that drugs which target heterodimerized receptors consisting of metabotropic glutamate receptors and $GABA_B$ receptors, may be promising candidates for the development of novel analgesics (Pin et al., 2009). While interest has also been expressed in developing analgesics which selectively act upon $GABA_A$ receptors in the context of analgesic drug development (Zeilhofer et al., 2009), possible adverse effects such a pronociception due to K^+-Cl^- gradient reversal under chronic administration due to upregulation of the KCC2 transporter limit the probability of success in clinical trials (Chamma et al., 2012). The discovery and characterization of isovaline's analgesic properties presents a promising means to harness endogenous inhibitory mechanisms to produce analgesia without associated side effects, while keeping cognition intact.

5.2.2 Sites and mechanisms of isovaline action

This thesis has shown that peripherally restricted amino acids, including isovaline, are capable of producing anti-allodynia in mice mediated by metabotropic $GABA_B$ receptors. The anti-allodynic effects of isovaline were dose dependent and long lasting and oral efficacy was shown. The peripheral site(s) of isovaline action resulting in its analgesic properties has yet to be conclusively determined, but are postulated to include direct actions on cutaneous keratinocytes, afferent fine nerve endings, and membrane of synoviocytes along with $GABA_B$ receptors expressed in

the dorsal root ganglia. Central mechanisms of analgesia after local injection are also evident with effects attributable to agonism at $GABA_B$ receptors expressed on the trigeminal root ganglion (Takeda et al., 2004). Blockade of isovaline, GABA and baclofen-induced anti-allodynia by the highly potent selective $GABA_B$ receptor antagonist CGP52432 indicated that isovaline produced anti-allodynia modulated by $GABA_B$ receptors. The observation that GABA anti-allodynia was blocked by the $GABA_B$ receptor antagonist CGP52432 suggests that $GABA_B$ analgesia predominated over $GABA_A$ receptor-mediated effects. The potentiation of analgesia produced by isovaline, GABA and baclofen by the positive allosteric modulator of the $GABA_B$ receptor, CGP7930 further solidifies this mechanism.

Previous studies showed that blockade of isovaline action by non-hydrolysable analogues of GTP indicate that isovaline acts through a G-protein coupled receptor (Cooke et al, 2012). The blockade of isovaline action by a $GABA_B$ antagonist and potentiation by a $GABA_B$ modulator suggests that isovaline is acting in one of two ways: (1) isovaline actions are mediated by cutaneous $GABA_B$ receptors, and/or (2) isovaline activates dorsal root ganglia $GABA_B$ receptors outside the spinal cord. It is also noteworthy that structurally similar amino acids have been shown to be capable of acting via the $GABA_B$ receptor: a handful of L-amino acids, including L-leucine, L-isoleucine and L-valine have been found to potentiate the responses of $GABA_B$ receptors, without direct activation of the receptor (Kerr and Ong, 2003). This was observed in neocortical neurons; application of the aforementioned amino acids alone produced no membrane conductance changes while their co-application with baclofen produced enhanced baclofen-induced membrane hyperpolarization, and significantly prolonged the duration of conductance changes. However it has since been shown that unlike CGP7930, L-leucine, L-isoleucine and L-

valine do not act as true allosteric modulators of $GABA_B$ receptors because they do not increase $GABA_B$-mediated GTP-beta-S binding in native or recombinant cell systems (Urwyler et al., 2004). Speculation has arisen as to the mechanism of the L-amino acid-induced potentiation of baclofen responses observed by Kerr and Ong (2003; cf: Urwyler et al., 2004). Specifically, L-amino acids are hypothesized to alter neural networks by receptors distinct from $GABA_B$, altering the release of GABA. An additional theory proposed by Urwyler et al. (2004) asserted that L-amino acids may modulate downstream intracellular effector molecules such as adenylyl cyclase or cAMP. Indeed, valine has been shown to have effects on spinal neurons (Curtis and Watkins, 1960).

5.3 Separation of peripheral and central components of general anesthesia

This thesis has for the first time demonstrated the *in vivo* efficacy of the amino acid isovaline in producing anesthesia in mice. Co-administered with propofol, isovaline was found to confer total anesthesia with a therapeutic index substantially higher than anesthesia obtained using propofol and fentanyl. This body of work sheds light on the benefits of using a peripherally restricted analgesic during operations and illuminates possible benefits which may arise postoperatively as well from this anesthetic regimen. Future studies will focus predominantly on postoperative incision pain. Isovaline's long duration of action would also be advantageous from a dosing standpoint, requiring less monitoring and facilitating more straightforward dosing regimens.

Isovaline produced substantially higher anesthetic safety than fentanyl, in agreement with previous studies documenting the instability of anesthesia produced by fentanyl-propofol preparations. Furthermore, experimenter observations suggested that while isovaline was non-toxic high

dose fentanyl-injected mice exhibited prohibitive and unexpected hyperactivity – fentanyl was upon first inspection capable of blocking righting reflex but when righted, animals immediately initiated high pace directionless locomotor activity. This may have serious implications for possible hyperlocomotor activity which may occur intraoperatively and postoperatively in humans. While NSAIDs were found to devoid of analgesic efficacy necessary to provide immobility to surgical stimuli (data not shown) it is interesting that the surgical analgesic doses of both fentanyl and isovaline were reduced by co-administrations of hypnotic doses of propofol, suggesting synergism with hypnotic induction via independent mechanisms. Since isovaline and fentanyl produce analgesia via independent mechanisms this raises the possibility that isovaline and fentanyl may be combined to harness differing analgesic mechanisms to produce safer drug combinations for use in surgery. For example, some types of operations may in consist of a stronger stimulus and require enhanced surgical analgesic than available with isovaline alone; this could be achieved via combination dosing with opioids with reduced risk of adverse effects associated with conventional anesthetic preparations.

The next step will be to determine the relative efficacy of isovaline in inhalational anesthesia paradigms such as isoflurane anesthesia. The widely used inhalational anesthetic isoflurane acts in the brain to produce both hypnosis and anesthesia. Interestingly, isoflurane's minimum alveolar concentration for hypnosis was markedly less than its minimum alveolar concentration required to produce immobility (Sonner et al., 1999). It may be hypothesized that the addition of isovaline will allow for surgical anesthesia with a heightened therapeutic index when compared to isoflurane alone. Both animal models and human testing will be required to complete these translational studies.

5.4 Future Studies

5.4.1 Synovial membrane $GABA_B$ receptors

Two novel arthritic mouse models capable of detecting and evaluating analgesics have been recently developed in the Hugill Centre for Anesthesia and Analgesia. These models include a) assessment of the amount of voluntary activity undertaken by mice and b) functional ability as demonstrated by the ability of mice to run under forced exercise, mediated by a low speed rotorod, without falling. As indicated previously, isovaline been shown to be effective in alleviating functional deficits associated with osteoarthritic dysfunction of mouse knee joints (Whitehead et al., 2012). We utilized immunohistochemistry coupled with confocal microscopy to provide evidence that $GABA_B$ receptors are also expressed in glabrous cutaneous tissue excised from mouse hindpaws. Voluntary and forced exercise models of osteoarthritic dysfunction may serve to assist in the development of advanced behavioural models to screen for peripherally acting drugs which could be tailored specifically to the pathophysiology of osteoarthritic pain.

5.4.2 $GABA_B$ receptor knockout mice

Next, it will be of interest to utilize knock-out mice lacking $GABA_B$ receptors altogether, and lacking peripheral $GABA_B$ receptors, to evaluate whether 1) $GABA_B$ receptors are required to mediate the effects of isovaline and 2) there are in fact certain indentifiable peripheral cell types expressing $GABA_{B1}$ and $GABA_{B2}$ receptors which are chiefly responsible for producing the observed analgesic effects of isovaline. It will also be of interest to assess the analgesic properties of isovaline in knockout mice of peripheral metabotropic glutamate receptors as these receptors have been implicated in isovaline's mechanism of action (K. Asseri, B. MacLeod and E. Puil,

personal communication). If mixed receptor effects are in fact due to heterodimerization of $GABA_{B1}$, $GABA_{B2}$ and metabotropic glutamate receptors, we would expect isovaline's effects to be modulated accordingly by the suggested knockdowns.

5.4.3 Intravenous regional analgesia

Without the use of advanced techniques, local injections in most cases result in analgesia not solely attributable to analgesic actions at the site of injection. Based on initial findings using a tourniquet to localize drug action, isovaline produces local cutaneous analgesia in a hot water tail flick model. These experiments involve local application of isovaline under time-sensitive conditions where blood flow has been isolated by a tourniquet applied to the tail or foot. It will be critical to further determine the major sites of action in the periphery, and peripheral mechanisms of action of novel small amino acid analogs of GABA and glycine to further advance towards development of potent peripherally restricted pain drugs.

5.4.4 Assessment of age- and state-dependent $GABA_B$ receptor expression

To identify and characterize endogenous inhibitory control systems mediated by $GABA_B$ receptors in the periphery, it will be necessary to measure expression levels of $GABA_B$ receptors under relevant neuropathies at different time points of human development as well as ambient concentration levels of circulating GABA. This will provide information about the possible roles GABA may play with regard to $GABA_B$ receptors in the periphery and its role in the processing and modulation of pain signaling. For example, we would induce pain using either intra-articular injection of mono-iodoacetate, intraplantar injection of PGE_2 or a paw incision and then take tissue sample at time periods as indicated previously, in order to determine time-dependent expres-

sion levels in subsequent assays. Tissue samples will be taken at post incision day 1, 5, 10, 15 and 20. We will use antibodies against $GABA_B$, $GABA_A$ receptors along with a marker of GABA expression to identify the percentage of increases or decreases in expression levels in all relevant tissues including keratinocytes, fine nerve endings, fibroblasts, astrocyte and microglia. We will also undertake similar studies in newborn mice and postnatal on a daily, weekly and monthly basis. This will allow for detection of developmental state-dependent shifts in $GABA_B$ receptor expression which may be relevant to treatment of pain in infants, children and different ages of adults.

5.5 Limitations

5.5.1 Assessment of blockade of allodynia induced by local PGE_2

PGE_2 injection produces pro-nociceptive effects related to the ability of PGE_2 to sensitize peripheral terminals of small diameter, high threshold primary afferent fibers to thermal, chemical and mechanical stimuli (Kassuya et al., 2007). While co-injection of locally applied analgesics allows for potential assessment of drug action and mechanisms confined to the site of injection, in practice rapid uptake mechanisms exist to induce systemic drug effects making it difficult to pinpoint drug effects in the injected region. One way to overcome this which has been used in pilot studies, is through use of a tourniquet applied to the mouse leg or tail to block off circulation for a brief period of time. Care must be taken in this situation to limit the duration of the study to the time period prior to emergence of ischemia due to loss of blood supply. However, such a technique allows for precise localiztion of drugs to the extremity of interest.

5.5.2 Tail clip and righting reflex ablation as a model of general anesthesia

Use of a standard Haffner tail clip has been previously shown to produce a response in rodents which parallels responsiveness to surgical incision, suggesting that the model is very robust. However, the use of loss of righting reflex as a proxy of unconsciousenss can be problematic. For example, upon first observation administration of high doses of fentanyl were capable of producing unconsciousness according to this definition, yet failed to detect the presence of hyperlocomotion, indicative of the possibility of consciousness. It may be useful to expand the criteria for unconsciousness to include concomitant loss of righting reflex, along with immobility to body pinch.

5.5.3 Glycinergic inhibitory dysfunction as a model of trigeminal neuralgia

While this model predictively validates clinically effective (carbamazepine) and ineffective drugs (morphine), intracisternal strychnine itself replicates only very vaguely the underlying symptomology of clinical trigeminal neuralgia. For example, while human trigeminal neuralgia is characterized by episodes of intense stabbing facial pain, our model only replicates aspects of the dynamic allodynia inherent in the human condition, yet also fails to account for a refractory period between episodes. Furthermore, mechanisms underlying the human condition are probably mediated by many different receptor systems, including but not limited to glycine receptor disinhibition. Use of a standard Haffner tail clip has been shown to produce a response in rodents which parallels responsiveness to surgical incision, suggesting that the model is very robust.

References

Adams CL, Lawrence AJ. CGP7930: a positive allosteric modulator of the $GABA_B$ receptor. CNS Drug Rev 13: 308-316, 2007.

Alkire MT, Miller J. General anesthesia and the neural correlates of consciousness. Progr Brain Res 150: 229-244, 2005.

Alves HC, Valentim AM, Olsson IA, Antunes LM. Intraperitoneal propofol and propofol fentanyl, sufentanil and remifentanil combinations for mouse anaesthesia. Lab Animals 41: 329-36, 2007.

Ameye LG, Young MF. Animal models of osteoarthritis: lessons learned while seeking the "Holy Grail". Curr Opin Rheumatol 18: 537-47, 2006.

Bailey PL, Streisand JB, East KA, East TD, Isern S, Hansen TW, Posthuma EFM, Rozendaal FW, Pace NL, Stanley T. Respiratory depression and analgesia with fentanyl. Anesth Analg 70: 8-15, 1990.

Ballantine RL. Anaesthetic advances in neurosurgery. Proc R Soc Med 63: 821-825, 1970.

Bandelow B. Pregabalin for the treatment of generalized anxiety disorder: a novel pharmacologic intervention. Expert Rev Neurotherap 7: 769-781, 2007.

Basbaum A, Bautista DM, Shcerrer G, Julius D. Neuronal circuitry for pain processing in the dorsal horn. Cell 139: 267-284, 2009.

Becker CM, Hoch W, Betz H. Glycine receptor heterogeneity in rat spinal cord during postnatal development. EMBO J 7: 3717-3726, 1988.

Beecher HK, Todd DP. A study of the deaths associated with anesthesia and surgery. Ann Surg 140: 2-34, 1954.

Bettler B, Kaupmann K, Mossbacher J, Gassmann M. Molecular structure and physiological functions of $GABA_B$ receptors. Physiol Rev 84: 835-867, 2004.

Bettler B, Tiao JY-H. Molecular diversity, trafficking and subcellular localization of $GABA_B$ receptors. Pharmacol Ther 110: 533-543, 2006.

Betz H, Laube B. Glycine receptors: recent insights into their structural organization and functional diversity. J Neurochem 97: 1600-1610, 2006.

Biermann B, Ivankova-Susankova K, Bradaia A, Abdel Aziz, S, Besseyrias V, Kapfhammer JP, Missler M, Gassmann M, Bettler B. The Sushi domains of $GABA_B$ receptors function as axonal targeting signals. J Neurosci 30: 1385-84, 2010.

Bjordal JM, Ljunggren AE, Slordal L. Non-steroidal anti-inflammatory drugs, including cyclo-oxygenase-2 inhibitors, in osteoarthritic knee pain: meta-analysis of randomised placebo controlled trials. BMJ 329: 1317-1323, 2004.

Bonsi P, Cuomo D, De Persis C, Centonze D, Bernardi G, Calabresi P, Pisani A. Modulatory action of metabotropic glutamate receptor (mGluR) 5 on mGluR1 function in striatal cholinergic interneurons. Neuropharmacology 49: 104-113, 2005.

Bormann J, Rundström N, Betz H, Langosch D. Residues within transmembrane segment M2 determine chloride conductance of glycine receptor homo- and hetero-oligomers. EMBO J 12: 3729-3737, 1993.

Bove SE, Calcaterra SL, Brooker RM, Huber CM, Guzman RE, Juneau PL, Schrier DJ, Kilgore KS. Weight bearing as a measure of disease progression and efficacy of anti-inflammatory compounds in a model of monosodium iodoacetate-induced osteoarthritis. Osteoarth Cartilage 11:821–830, 2003.

Bowery NG, Smart TG. GABA and glycine as neurotransmitters: a brief history. Br J Pharmacol 147: S109-S119, 2006.

Bowery NG, Hill DR, Hudson AL, Doble A, Middlemiss DN, Shaw J, Turnbull M. Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor. Nature 283: 92-94, 1980.

Breivik H, Collet B, Ventafridda V, Cohen R, Gallacher D. Survey of chronic pain in Europe: Prevalence, impact on daily life, and treatment. Europ J Pain 10: 287-333, 2006.

Brown DA, Sihra TS. Presynaptic signaling by heterotrimeric G-proteins. Handb Exp Pharmacol 184: 207-260, 2008.

Brückner H, Becker D, Gams W, Degenkolb T. Aib and iva in the biosphere: neither rare nor necessarily extraterrestrial. Chem Biodivers 6: 38-56, 2009.

Busija L, Osborne RH, Roberts C, Buchbinder, R. Systematic review showed measures of individual burden of osteoarthritis poorly capture the patient experience. J Clin Epidemiol 66: 826-837, 2013.

Callaghan B, Haythornthwaite A, Berecki G, Clark RJ, Craik DJ, Adams DJ. Analgesic alpha-conotoxins Vc1.1 and Rg1A inhibit N-type calcium channels in rat sensory neurons via $GABA_B$ receptor activation. J Neurosci 28: 10943-10951, 2008.

Calo G, Rizzi A, Marzola G, Gurrini R, Salvadori S, Beani L, Regoli D, Bianchi C. Pharmacological characterization of the nociceptin receptor mediating hyperalgesia in the mouse tail withdrawal assay. Brit J Pharmacol 125: 373-378, 1998.

Carlton SM, Coggeshall RE. Nociceptive integration: does it have a peripheral component? Pain Forum 7: 71–78, 1998.

Carlton SM, Zhou S, Coggeshall RE. Peripheral GABA(A) receptors: evidence for peripheral primary afferent depolarization. Neuroscience 93: 713–722, 1999.

Catchpole K, Bell MD, Johnson S. Safety in anaesthesia: a study of 12 606 reported incidents from the UK National Reporting and Learning System. Anaesthesia 63: 340-346, 2008.

Chamma I, Chevy Q, Poncer JC, Lévi S. Role of the neuronal K-Cl co-transporter KCC2 in inhibitory and excitatory neurotransmission. Front Cell Neurosci 6: 1-11, 2012.

Christensen HN. Intestinal absorption with special reference to amino acids. Gastroenterology 21: 37-42, 1962.

Clements KM, Ball AD, Jones HB, Brinckmann S, Read SJ, Murray F. Cellular and histopathological changes in the infrapatellar fat pad in the monoiodoacetate model of osteoarthritis pain. Osteoarthr Cartilage 17: 805–812, 2009.

Combe R, Bramwell S, Field M.J. The monosodium iodoacetate model of osteoarthritis: a model of chronic nociceptive pain in rats? Neurosci Lett 370: 236-40, 2004.

Conn V, Hafdahl A, Minor M. Physical Activity Interventions Among Adults with Arthritis: Meta-Analysis of Outcomes. Semin Arthritis Rheu 37: 307-316, 2008.

Cooke JE, Mathers DA, Puil E. Isovaline causes inhibition by increasing potassium conductance in thalamic neurons. Neuroscience 164: 1235-1243, 2009.

Cooke JE, Mathers DA, Puil E. R-isovaline: a subtype-specific agonist at GABA(B)-receptors? Neuroscience 201: 85-95, 2012.

Costigan M, Scholz J, Woolf CJ. Neuropathic pain: a maladaptive response of the nervous system to damage. Annu Rev Neurosci 32: 1-32, 2009.

Couve A, Filippov AK, Connoly CN, Bettler B, Brown DA, Moss SJ. Intracellular retention of recombinant $GABA_B$ receptors. J Biol Chem 273: 26361-26367, 1998.

Crofford LJ, Rowbotham MC, Mease PJ. Pregabalin for the treatment of fibromyalgia syndrome: results of a randomized double-blind, placebo-controlled trial. Arthritis Rheum 52: 1264-1273, 2005.

Cunningham MD, Enna SJ. Evidence for pharmacologically distinct $GABA_B$ receptors associated with cAMP production in rat brain. Brain Res 720: 220-4, 1996.

Curtis DR, Watkins JC. The excitation and depression of spinal neurones by structurally related amino acids. J Neurochem 6: 117-41, 1960.

Dahan A, Aarts L, Smith T. Incidence, reversal and prevention of opioid-induced respiratory depression. Anesthesiology112: 226-238, 2010.

Davidoff RA, Aprison MH, Werman R. The effects of strychnine on the inhibition of interneurons by glycine and gamma-aminobutyric acid. Int J Neuropharmacol. 8: 191-194, 1969.

Davies A, Hendrich J, Van Minh AT, Wratten J, Douglas L, Dolphin AC. Functional biology of the alpha(2)delta subunits of voltage-gated calcium channels. Trends Pharmacol Sci 28: 220-228, 2007.

De Camilli P, Miller PE, Navone F, Theurkauf WE, Vallee RB. Distribution of microtubule-associated protein 2 in the nervous system of the rat studied by immunofluorescence. Neuroscience 11: 817–846, 1984.

DeHaven-Hudkins DL, Dolle RE. Peripherally restricted opioid agonists as novel analgesic agents. Curr Pharm Design 10: 743-57, 2004.

Delacour J. Neurobiology of consciousness: an overview. Behav Brain Res 85: 127-141, 1997

Desarmenien M, Feltz P, Occhipinti G, Santangelo F, Schlichter R. Coexistence of $GABA_A$ and $GABA_B$ receptors on Aδ and C primary afferents. Brit J Pharmacol 81:327-333, 1984.

Devor M. Neuropathic pain and the injured nerve: Peripheral mechanisms. Brit Med Bull 47: 619-630, 1991.

Dickenson AH, Chapman V, Green GM. The pharmacology of excitatory and inhibitory amino acid-mediated events in the transmission and modulation of pain in the spinal cord. Gen Pharmacol 28: 633-638, 1997.

Diversé-Pierluissi M, Remmers AE, Neubig RR, Dunlap K. Novel form of crosstalk between G protein and tyrosine kinase pathways. P Natl Acad Sci USA 94: 5417-21, 1997.

Dixon WJ. The up-and-down method for small samples. J Am Stat Assoc 60: 967-978, 1965.

Drazen JM. Cox-2 Inhibitors – A lesson in unexpected problems. New Engl J Med 352: 1131, 2005.

Drews J. Drug Discovery: A historical perspective. Science 287: 1960-1964, 2000.

Dubin AE, Patapoutian A. Nociceptors: the sensors of the pain pathway. J Clin Invest 120: 3760-3772, 2010.

Eliasmith C, Stewart TC, Choo X, Bekolay T, De Wolf T, Tang Y, Rasmussen D. A large-scale model of the functioning brain. Science 338: 1202-1205, 2012.

Elsila JE, Callahan MP, Glavin DP, Dworkin JP, Bruckner H. Distribution and stable isotopic composition of amino acids from fungal peptaibiotics: assessing the potential for meteoritic contamination. Astrobiology 11: 123–133, 2011.

Enna SJ, McCarson KE. The role of GABA in the mediation and perception of pain. Adv Pharmacol 54: 1-27, 2006.

Erickson MA, Dohi K, Banks, WA. Neuroinflammation: a common pathway in CNS diseases as mediated at the blood–brain barrier. Neuroimmunomodulat 19: 121–130, 2012.

Evers AS, Crowder M, Balser JR. General Anesthetics. *Goodman & Gilman's The Pharmacological Basis of Therapeutics.* 11th Edition, New York, McGraw-Hill, 2006.

Ezici M, Bestas A, Erhan OL. As an analgesic ketamine versus fentnayl for total intravenous anesthesia. Saudi J Anaesth 7: 107-108, 2013.

Fadool DA, Holmes TC, Berman K, Dagan D, Levitan IB. Tyrosine phosphorylation modulates current amplitude and kinetics of a neuronal voltage-gated potassium channel. J Neurophysiol 78: 1563-1573, 1997.

Fernihough J, Gentry C, Malcangio M, Fox A, Rediske J, Pellas T, Kidd B, Bevan S, Winter J. Pain related behaviour in two models of osteoarthritis in the rat knee. Pain 112: 83-93, 2004.

Fowler CE, Aryal P, Suen KF, Slesinger PA. Evidence for association of GABA(B) receptors with Kir3 channels and regulators of G protein signalling (RGS4) proteins. J Physiol Sci 580: 51-65, 2007.

Fromm GH, Terrence CF. Comparison of L-baclofen and racemic baclofen in trigeminal neuralgia. Neurology37: 1712-1728, 1987.

Fu Z, Yang H, Xiao Y, Zhao G, Huang H. The ɤ-aminobutyric acid type B $GABA_B$ receptor agonist baclofen inhibits morphine sensitization by decreasing the dopamine level in rat nucleus accumbens. Behav Brain Funct 8: 1-10, 2012.

Galvez T, Parmentier ML, Joly C, Malitschek B, Kaupmann K, Kuhn R, Bittiger H, Froestl W, Bettler B, Pin JP. Mutagenesis and modeling of the $GABA_B$ receptor extracellular domain support a Venus flytap mechanism for ligand binding. J Biol Chem 274: 13362-13369, 1999.

Galvez T, Prezeau L, Milioti G, Franek M, Joly C, Froestl W, Bettler B, Bertrand HO, Blahos J, Pin JP. Mapping the agonist-binding site of $GABA_B$ type 1 subunit sheds light on the activation process of $GABA_B$ receptors. J Biol Chem 275: 41166-41174, 2000.

Galvez T, Urwyler S, Prezeau L, Mosbacher J, Joly C, Malischek B, Heid J, Brabet I, Froestl W, Bettler B, Kaupmann K, Pin JP. Ca^{2+} requirement for high-affinity ɤ-aminobutyric acid (GABA) binding at $GABA_B$ receptors: involvement of serine 269 of the $GABA_{B1}$ subunit. Mol Pharmacol 57: 419-426, 2000.

Gangadharan V, Agarwal N, Brugger S, Tegeder I, Bettler B, Kuner R, Kurejova M. Conditional gene deletion reveals functional redundancy of $GABA_B$ receptors in peripheral nociceptors *in vivo*. Mol Pain 5: 68, 2009.

Garcia, S. Neuroscience Online http://neuroscience.uth.tmc.edu/index.htm, 2009.

Gaskin, DJ, Richard P. The Economic Costs of Pain in the United States. J Pain 13: 715-724, 2012.

Gelgor L, Phillips S, Mitchell D. Hyperalgesia following ischaemia of the rat's tail. Pain 24: 251-257, 1986.

Ghavanini AA, Mathers DA, Kim HS, Puil E. Distinctive glycinergic currents with fast and slow kinetics in thalamus. J Neurophysiol 95: 3438-3448, 2006.

Ghavanini AA, Mathers DA, Puil E. Glycinergic inhibition in thalamus revealed by synaptic receptor blockade. Neuropharmacology 49: 338-349, 2005.

Gielen M, Stienstra R. Tourniquet hypertension and its prevention: a review. Region Anesth Pain M. 16: 191-194, 1991.

Gold MS, Gebhart G.F. Nociceptor sensitization in pain pathogenesis. Nat Med 16: 1248–1257, 2010.

Gottesfeld Z, Krnjevic K, Reiffenstein RJ. Penetration of circulating GABA into long-isolated cortical slabs. Can J Physiol Pharm 49: 70-78, 1971.

Greenberg MF, Pollard ZF. Adult strabismus surgery under propofol sedation with local versus general anesthesia. J AAPOS 2: 116-120, 2003.

Grossman ML. Afferent and efferent connections of the rat tail flick reflex (a model used to analyze pain control mechanisms). J Comp Neurol 206: 9-16, 1982.

Harvey RJ, Depner UB, Wässle H, Ahmadi S, Heindl C, Reinold H, Smart TG, Harvey K, Schütz B, Abo-Salem OM, Zimmer A, Poisbeau P, Welzl H, Wolfer DP, Betz H, Zeilhofer HU, Müller U. GlyR alpha3: an essential target for spinal PGE_2-mediated inflammatory pain sensitization. Science 304: 884-7, 2004.

Harvey RJ, Schmieden V, Von Holst A, Laube B, Rohrer H, Betz H. Glycine receptors containing the alpha4 subunit in the embryonic sympathetic nervous system, spinal cord and male genital ridge. Eur J Neurosci 12: 994-1001, 2000.

Harvey VL, Dickenson AH. Behavioural and electrophysiological characterisation of experimentally induced osteoarthritis and neuropathy in C57Bl/6 mice. Mol Pain 5: 18-26, 2009.

Hasbargen T, Ahmed MM, Miranpuri G, Li L, Kahle KT, Resnick D, Sun D. Role of NKCC1 and KCC2 in the development of chronic neuropathic pain following spinal cord injury. Ann NY Acad Sci 1198: 168-172, 2010.

Hayes M, Tyers MB. Determination of receptors that mediate opiate side effects in the mouse. Br J Pharmac 79: 731-736, 1983.

Heal DJ, Akagi H, Bowdler JM, Green AR. Repeated electroconvulsive shock attenuates clonidine-induced hypoactivity in rodents. Eur J Pharmacol 75: 231–237, 1981.

Hill DR. $GABA_B$ receptor modulation of adenylate cyclase activity in rat brain slices. Brit J Pharmacol 84: 249-57, 1985.

Holtman Jr. JR, Buller AL, Hamosh P, Da Silva AMT, Gillis RA. Respiratory depression produced by glycine injected into the cisterna magna of cats. Neuropharmacology 11: 1223-1225, 1982.

Holtman Jr. JR, Jellish WS. Opioid-induced hyperalgesia and burn pain. J Burn Care Res 33: 692-701, 2012.

Hooper L, Brown TJ, Elliot R, Payne K, Roberts C. The effectiveness of five strategies for the prevention of gastrointestinal toxicity induced by non-steroidal anti-inflammatory drugs: systematic review. Brit Med J 239: 948-956, 2004.

Hudetz AG. General anesthesia and human brain connectivity. Brain Connect 2: 291-302, 2012.

Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30: 103-114, 1987.

Hunter JC, Singh L. Role of excitatory amino acid receptors in the mediation of the nociceptive response to formalin in the rat. Neurosci Lett 174: 217-221, 1994.

Issberner U, Reeh PW, Steen KH. Pain due to tissue acidosis: a mechanism for inflammatory and ischemic myalgia? Neurosci Lett 208: 191-194, 1996.

Ito K, Tanaka K, Nishibe Y, Hasegawa J, Ueno H. GABAsynthesizing enzyme, GAD67, from dermal fibroblasts: evidence for a new skin function. Biochim Biophys Acta 1770: 291-296, 2007.

Janson W, Stein C. Peripheral opioid analgesia. Curr Pharm Biotechnol 4: 270-274, 2003.

Jasmin L, Wu MV, Ohara PT. GABA puts a stop to pain. Curr Drug Targets CNS Neurol Disord 3: 487-505, 2004.

Jensen ML, Timmermann DB, Johansen TH, Schousboe A, Varming T, Ahring PK. The beta subunit determines the ion selectivity of the $GABA_A$ receptor. J Biol Chem 277: 41438-41447, 2002.

Jessel TM, Kandel ER, Schartz, J.H. *Principles of Neural Science.* New York: McGraw-Hill. ISBN 0-8385-7701-6, 2000.

Jia F, Pignataro L, Schofield CM, Yue M, Harrison NL, Goldstein PA. An extrasynaptic $GABA_A$ receptor mediates tonic inhibition in thalamic VB neurons. J Neurophysiol 94: 4491-4501, 2005.

Juni A, Klein G, Kest B. Morphine hyperalgesia in mice is unrelated to opioid activity, analgesia, or tolerance: evidence for multiple diverse hyperalgesic systems. Brain Res 1070: 35-44, 2006.

Kakee A, Tetsuya T, Mikihiko N, Takashi T, Sugiyama Y. Efflux of a suppressive neurotransmitter, GABA, across the blood–brain barrier. J Neurochem 79: 110-118, 2008.

Kassuya CA, Ferreira J, Claudino RF, Calixto JB. Intraplantar PGE_2 causes nociceptive behaviour and mechanical allodynia: the role of prostanoid E receptors and protein kinases. Br J Pharmacol 150: 727–737, 2007.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B. GABA(B)-receptor subtypes assemble into functional heteromeric complexes. Nature 396: 683-687, 1998.

Keefe FJ, Brown GK, Wallston KA, Caldwell DS. Coping with rheumatoid arthritis pain: catastrophizing as a maladaptive strategy. Pain 37: 51-56, 1989.

Kerr DI, Ong J. Potentiation of metabotropic $GABA_B$ receptors by L-amino acids and dipeptides in rat neocortex. Eur J Pharmacol 468: 103-108, 2003.

Kirsch J, Kuhse J, Betz H. Targeting of glycine receptor subunits to gephyrin-rich domains in transfected human embryonic kidney cells. Mol Cell Neurosci 6: 450-461, 1995.

Kirsh K, Peppin J, Coleman J. Characterization of prescription opioid abuse in the United States: focus on route of administration. J Pain Palliat Care Pharmacother 26: 348-61, 2012.

Kitt CA, Gruber K, Davis M, Woolf C, Levine J.D. Trigeminal mechanical allodynia: opportunities for research and treatment. Pain 85: 3-10, 2000.

Knabl J, Witschi R, Hosl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy M, Rudolph U, Mohler H, Zeilhofer HU. Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes. Nature 451: 330-334, 2008.

Konttinen Y, Kemppinen P, Segerberg M, Hukkanen M. Peripheral and spinal neural mechanisms in arthritis, with particular reference to treatment of inflammation and pain. Arthritis Rheum 37: 965-82, 1994.

Kvenvolden KA, Lawless J, Pering K, Peterson E, Flores J, Ponnamperuma C, Kaplan I, Moore C. Evidence for extraterrestrial amino-acids and hydrocarbons in the Murchison meteorite. Nature 228: 923-926, 1970.

Lagasse R. Anesthesia safety: model or myth? A review of the published literature and analysis of current original data. Anesthesiology 97: 1609-1617, 2002.

Langford D. Coding of facial expressions of pain in the laboratory mouse. Nat Methods 7: 447-449, 2010.

Lanza M, Fassio A, Gemignani A, Bonanno G., Raiteri, M. CGP52432: a novel potent and selective $GABA_B$ autoreceptor antagonist in rat cerebral cortex. Eur J Pharmacol 237:191-195, 1993.

Lasso-de-la-Vega MC, Zapater P, Such J, Perez-Mateo M, Horga JF. Gabapentin-associated hepatotoxicity. Am J Gastroenterol 96: 3460-3462, 2001.

Lauria G, Motta M, Procacci P, Trovato AE, Bettler B. Altered peripheral myelination in mice lacking $GABA_B$ receptors. Mol Cell Neurosci 37: 599–609, 2008.

Le Bars D, Gozarui M, Cadden SW. Animal models of nociception. Pharmacol Rev 53: 597-652, 2001.

Lee IO, Son JK, Lim ES, Kim YS. Pharmacology of intracisternal or intrathecal glycine, muscimol, and baclofen in strychnine-induced thermal hyperalgesia of mice. J Korean Med Sci 26: 1371-1377, 2011.

Levy G, Gibaldi M. Pharmacokinetics of drug action. Annu Rev Pharmacolog 12: 85-98, 1971.

Lewis T. Experiments relating to cutaneous hyperalgesia and its spread through somatic nerves. Clin Sci 2: 373–421, 1936.

Liu XB, Jones EG, Huntley GW, Molinari M. Tachykinin immunoreactivity in terminals of trigeminal afferent fibers in adult and fetal monkey thalamus. Exp Brain Res 78: 479-88, 1989.

Luscher C, Jan LY, Stoffel M, Malenka RC, Nicoll RA. G protein-coupled inwardly rectifying K^+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. Neuron 19: 687-695, 1997.

Lynch JW. Molecular structure and function of the glycine receptor chloride channel. Physiol Rev 84: 1051-1095, 2004.

MacLeod BA, Wang JT, Chung CC, Ries CR, Schwarz SK, Puil E. Analgesic properties of the novel amino acid, isovaline. Anesth Analg 110: 1206-1214, 2010.

Magnaghi V. GABA and neuroactive steroid interactions in the glia: new roles for old players? Curr Neuropharmacol 5: 47–64, 2007.

Malosio ML, Marqueze-Pouey B, Kuhse J, Betz H. Widespread expression of glycine receptor subunit mRNAs in the adult and developing ratbrain. EMBO J 10: 2401-2409, 1991.

Manchikanti L, Pampati V, Damron KS, Beyer CD, Barnhill RC, Fellows B. Prevalence of prescription drug abuse and dependency in patients with chronic pain in western Kentucky. J Kentucky Med Assoc 101: 511-517, 2003.

May A. Chronic pain may change the structure of the brain. Pain 137: 7-15, 2008

McDougall JJ. Arthritis and pain: neurogenic origin of joint pain. Arthritis Res Ther 110:1206-1214, 2006.

Messlinger K. Functional morphology of nociceptive and other fine sensory endings (free nerve endings) in different tissues. Prog Brain Res 113: 273–298, 1996.

Michael JWP, Schlüter-Brust KU, Eysel P. The epidemiology, etiology, diagnosis, and treatment of osteoarthritis of the knee. Pharmakologie 42: 109-11F8, 2010.

Micheva KD, Taylor CP, Smith SJ. Pregabain reduces the release of synaptic vesicles from cultured hippocampal neurons. Mol Pharmacol 70: 467-476, 2006.

Mintz IM, Bean BP. $GABA_B$ receptor inhibition of P-type Ca2+ channels in central neurons. Neuron 10: 889-98, 1993.

Miraucourt LS, Dallel R, Voisin DL. Glycine inhibitory dysfunction turns touch into pain through PKCgamma interneurons. PLoS One e1116, 2007.

Miraucourt LS, Moisset X, Dallel R, Voisin DL. Glycine inhibitory dysfunction induces a selectively dynamic, morphine-resistant, and neurokinin 1 receptor-independent mechanical allodynia. J Neurosci 29: 2519-2525, 2009.

Misra A, Ganesh S, Shahiwala A, Shah SP. Drug delivery to the central nervous system: a review. J Pharm Pharm Sci 6: 252-73, 2003.

Missner A, Pohl P. 110 years of the Meyer-Overton rule: predicting membrane permeability of gases and other small compounds. Chemphyschem 10: 1405-1414, 2009.

Miu P, Puil E. Isoflurane-induced impairment of synaptic transmission in hippocampal neurons. Exp Brain Res 75: 354-360, 1989.

Nishikawa K, Kubo K, Obata H, Yanagawa Y, Saito Shigeru. The influence of manipulations to alter ambient GABA concentrations on the hypnotic and immobilizing actions produced by sevoflurane, propofol, and midazolam. Neuropharmacology 61: 172-180, 2011.

Nordskog M, Hautala AW. *The Opposite of Cold: the Northwoods Finnish Sauna Tradition*. Minneapolis: University of Minnesota Press, 2010.

Norojan G, Clive D. Cyclo-oxygenase-2 inhibitors and the kidney. Drug Safety 25: 165-172, 2002.

Okeson JP. *The Clinical Management of Orofacial Pain. Bell's Orofacial Pains: the Clinical Management of Orofacial Pain.* Quintessence Publishing Corp: Chapter 14, 2005.

Pau CH. The development and evaluation of tourniquet-induced post-ischaemic allodynia: a method for investigating the mechanism of and treatment for chronic pain. The University of British Columbia Master's Thesis in the Department of Anesthesiology, Pharmacology & Therapeutics, 2009.

Pin JP, Comps-Agrar L, Maurel D, Monnier C, Rives ML, Trinquet E, Kniazeff J, Rondard P, Prézeau L. G-protein-couple receptor oligomers: two or more for what? Lessons from mGlu and $GABA_B$ receptors. J Physiol 587: 5337-5344, 2009.

Pizzarello S, Cronin JR. Non-racemic amino acids in the Murray and Murchison meteorites. Geochim Cosmochim Act 64: 329-338, 2000.

Pontier SM, Lahaie N, Ginham R, St-Gelais F, Bonin H, Bell DJ, Flynn H, Trudeau LE, McIlhinney J, White JH, Bouvier M. Coordinated action of NSF and PKC regulates $GABA_B$ receptor signaling efficacy. EMBO J 25: 2698-709, 2006.

Puri J, Bellinger LL, Kramer PR. Estrogen in cycling rats alters gene expression in the temporomandibular joint, trigeminal ganglia and trigeminal subnucleus caudalis/upper cervical cord junction. J Cell Physiol 226: 3169-80, 2011.

Raap J, Erkelens K, Ogrel A, Skladnev DA, Brückner H. Fungal biosynthesis of non-ribosomal peptide antibiotics and alpha, alpha-dialkylated amino acid constituents. J Pept Sci 11: 331-8, 2005.

Radtke C, Vogt PM, Devor M, Kocsis JD. Keratinocytes acting on injured afferents induce extreme neuronal hyperexcitability and chronic pain. Pain 148: 94-102, 2010.

Ramachandran VS. Phantom limbs and neural plasticity. Arch Neurol 57: 1317-1320, 2000.

Reis GM, Duarte ID. Baclofen, an agonist at peripheral $GABA_B$ receptors, induces antinociception via activation of TEA-sensitive potassium channels. Br J Pharmacol 149: 733-73, 2006.

Richards N, McMahon SB. Targeting novel peripheral mediators for the treatment of chronic pain. Br J Anaesth 111: 46-51, 2013.

Ries CR, Puil E. Ionic mechanism of isoflurane's actions on thalamocortical neurons. J Neurophysiol 81: 1802-1809, 1999.

Roberts LA, Connor M. TRPV1 antagonists as a potential treatment of hyperalgesia. Recent Pat CNS Drug Discov 1: 65-76, 2006.

Rodriguez G, Luis A, Varas C, Patrona C. Differential effects of aspirin and non-aspirin nonsteroidal anti-inflammatory drugs in the primary prevention of myocardial infarction in postmenopausal women. Epidemiology 11: 382-387, 2000.

Saegusa Y, Tabata H. Usefulness of infrared thermometry in determining body temperature in mice. J Vet Med Sci 65: 1365–1367, 2003.

Saint D, Thomas T, Gage PW. $GABA_B$ agonists modulate a transient potassium current in cultured mammalian hippocampal neurons. Neurosci Lett 118: 9-13, 1990.

Sanders RD, Tononi G, Laureys S, Sleigh JW. Unresponsiveness [not equal to] unconsciousness. Anesthesiology 116: 946-959, 2012.

Sawynok J. Topical and peripherally acting analgesics. Pharmacol Rev 55: 1-20, 2003.

Schug SA, Gandham N. Opioids: Clinical Use. *Textbook of pain.* 5th ed. London: Elsevier, 2007.

Searle JR. How to study consciousness scientifically. Philos Trans R Soc Lond B Biol Sci 353: 1935-1942, 1998.

Shafer A, Doze VA, Shafer SL. White PF. Pharmacokinetics and pharmacodynamics of propofol infusions during general anesthesia. Anesthesiology 69: 348-356, 1988.

Shiba K, Mori H, Hisada K. Analogues of alpha-aminoisobutyric acid with various alkyl groups as potential tumour imaging agents, effects of chain length on tumour and normal tissue specificity. Nucl Med Commun 10: 751-758, 1989.

Sinclair RJ, Sathian K, Burton H. Neuronal responses in ventroposterolateral nucleus of thalamus in monkeys (Macaca mulatta) during active touch of gratings. Somatosens Mot Res 8: 293-300, 1991.

Smits A, Jin Z, Elsir T, Pedder H, Nister M, Alafuzoff I, Dimberg A, Edqvist PH, Ponten F. $GABA_A$ channel subunit expression in human glioma correlates with tumor histology and clinical outcome. PLoS One 7: e37041, 2012.

Sneddon LU, Braithwaite VS, Gentle MJ. Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. Proc Biol Sci 270: 115-12, 2003.

Sonner JM, Gong D, Li J, Eger EI, Laster MJ. Mouse strain modestly influences minimum alveolar anesthetic concentration and convulsivity of inhaled compounds. Anesth Analg 89: 1030-1034, 1999.

Sonner JM, Antognini JF, Dutton RC, Flood P, Gray AT, Harris RA, Homanics GE, Kendig J. Inhaled anesthetics and immobility: mechanisms, mysteries and minimum alveolar anesthetic concentration. Anesth Analg 97: 718-740, 2004.

Spanswick CC, Main CJ. *Pain Management: an Interdisciplinary Approach.* Edinburgh: Churchill Livingstone ISBN 0-443-05683-8: 93-100, 2000.

Spencer PS. Animal models for screening new agents. Br J Clin Pharmacol 3: 5-12, 1976.

Steardo L, Leo A, Marano E. Efficacy of baclofen in trigeminal neuralgia and some other painful conditions. A clinical trial. Eur Neurol 23: 51-5,1984.

Stoelting RS, Hillier SC. *Handbook of Pharmacology and Physiology in Anesthetic Practice.* 2006.

Takeda M, Tanimoto T, Ikeda M, Kadoi J, Matsumoto S. Activation of $GABA_B$ receptor inhibits the excitability of rat small diameter trigeminal root ganglion neurons. Neuroscience 123: 491-505, 2004.

Tallarida RJ. Drug synergism: its detection and applications. J Pharmacol Exp Ther 298: 865-72, 2001.

Tamura S, Watanabe M, Kanbara K, Yanagawa T, Watanabe K, Otsuki Y, Kinoshia M. Expression and distribution of GABAergic system in rat knee join synovial membrane. Histol Histopathol 24: 1009-1019, 2009.

Terrence CF, Sax M, Fromm GH, Chang CH, Yoo CS. Effect of baclofen enantiomorphs on the spinal trigeminal nucleus and steric similarities of carbamazepine. Pharmacology 27: 85-94, 1983.

Theodorsson A, Holm L, Theodorsson E. Modern anesthesia and peroperative monitoring methods reduce pre- and postoperative mortality during transient occlusion of the middle cerebral artery in rats. Brain Res Brain Res Protoc 14: 181-190, 2005.

Todd AJ, Neuronal circuitry for pain processing in the dorsal horn. Nat Rev Neurosci 11: 823-836, 2010.

Tomson T, Bertilsson L. Potent therapeutic effect of carbamazepine-10-11-epoxide in trigeminal mechanical allodynia. Arch Neurol 41: 598-601, 1984.

Torres-López JE, Carmona-Díaz E, Cortés-Peñaloza JL, Guzmán-Priego CG, Rocha-González HI. Antinociceptive synergy between diclofenac and morphine after local injection into the inflamed site. Pharmacol Rep 65: 358-67, 2013.

Urwyler S, Gjoni T, Kaupmann K, Pozza MF, Mosbacher J. Selected amino acids, dipeptides and arylalkylamine derivatives do not act as allosteric modulators at $GABA_B$ receptors. Eur J Pharmacol 483: 147-153, 2004.

van Gelder NM, Elliot KAC. Disposition of ɤ-aminobutyric acid administered to mammals. J Neurochem 3: 139-143,1958.

Van Kleef M, van Genderen WE, Narouze S, Nurmikko TJ, van Zundert J, Geurts JW, Mekhail N. Trigeminal mechanical allodynia. Pain Pract 9: 252-259, 2009.

Vonkeman HE, van de Laar MA. Nonsteroidal anti-inflammatory drugs: adverse effects and their prevention. Semin Arthritis Rheum 39: 294-3122, 2010.

Vos BP, Strassman AM, Maciewicz RJ. Behavioral evidence of trigeminal neuropathic pain following chronic constriction injury to the rat's infraorbital nerve. J Neurosci 14: 2708-23, 1994.

Wang P, Slaughter M. Effects of GABA receptor antagonists on retinal glycine Receptors and on homomeric glycine receptor alpha subunits. J Neurophysiol 92: 3120-3126, 2005.

Watanabe M, Maemura K, Kanbara K, Tamayama T, Hayasaki H. GABA and GABA receptors in the central nervous system and other organs. Int Rev Cytol 213: 1-47, 2002.

Whitehead RA, Puil E, Ries CR, Schwarz SK, Wall RA, Cooke JE, Putrenko I, Sallam NA, MacLeod BA. GABA(B) receptor-mediated selective peripheral analgesia by the non-proteingogenic amino acid, isovaline. Neuroscience 213: 154-60, 2012.

WHO Scientific Group on the Burden of Musculoskeletal Conditions at the Start of the New Millenium. The burden of musculoskeletal conditions at the start of the new millennium. World Health Organ Tech Rep Ser 919: 1-218, 2003.

Woolf C, Doubell T. The pathophysiology of chronic pain – increased sensitivity to low threshold Aß-fibre inputs. Curr Opin Neurobiol 4: 525-534, 1994.

Woolf CJ. Central sensitization: Implications for the diagnosis and treatment of pain. Pain 152: S2-S15, 2011.

Woolf CJ, Mitchell M. Mechanism-based Pain Diagnosis: Isues for Analgesic Drug Development. Anesthesiology 95: 241-249, 2001.

Yamakura T, Bertaccini E, Trudell JR, Harris RA. Anesthetics and ion channels: molecular models and sites of action. Ann Rev Pharmacol Toxicol 41: 23-51, 2001.

Yu L, Ran B, Li M, Shi Z. Gabapentin and pregabalin in the management of postoperative pain after lumbar spinal surgery: a systematic review and meta-analysis. Spine [Epub ahead of print], 2013

Zarbin MA, Wamsley JK, Kuhar MJ. Glycine receptor: light microscopic autoradiographic localization with [3H] strychnine. J Neurosci 1: 532-547, 1981.

Zeilhofer HU, Witschi R, Hösl K. Subtype-selective $GABA_A$ receptor mimetics--novel antihyperalgesic agents? J Mol Med (Ber) 87: 465-469, 2009.

Zhang J. Chen L, Su T, Cao F, Meng X, Pei L, Shi J, Pan HL, Li M. Electroacupuncture increases CB2 receptor expression on keratinocytes and infiltrating inflammatory cells in inflamed skin tissues of rats. J Pain 11: 1250-1258, 2010.

Zhao P, Barr TP, Houc Q, Dib-Hajja SD, Blacka JA, Albrechtc PJ, Petersend K, Eisenberge EF. Voltage-gated sodium channel expression in rat and human epidermal keratinocytes: Evidence for a role in pain. Pain 1: 90-105, 2008.

Zollner C, Schafer M. [Opioids in Anesthesia]. Anaesthesist 57: 729-740, 2008.

Appendix I

Appendix A: animal usage approval

A10-0196 Peripheral GABA B Analgesic activity: Approved

Last Name	First Name	Employer	Rank	Online Training	Practical Training
Whitehead	Ryan A.	Anesthesiology, Pharmacology & Therapeutics	Graduate Student	2722-08	RBH-145-11
Kaye	Julian A.	Medicine, Department of	M&P Staff	3879-09	RBH-173-07,RA-21-07
Sallam	Nada	Anesthesiology, Pharmacology & Therapeutics	Post Doctoral Fellow	1831-06	RBH-204-07, RSx-109-12
Wang	Jeff Yun Fan	Anesthesiology, Pharmacology & Therapeutics	Undergraduate Student	4309-10	
Asseri	Khalid A	Anesthesiology, Pharmacology & Therapeutics	Graduate Student	2587-08	RBH-185-11

*Please contact UBC Researcher Information Services (http://rise.ubc.ca) for more information on this and related protocols.

Appendix B: isovaline use as a wartime analgesic

Another area of critical interest is the development of isovaline and related amino acids for treatment of Warfighter analgesia in such a way that avoid unacceptable cognitive deficits for soldiers engaged in battle. This would facilitate rapid removal of injured soldiers from the battlefield. This is an urgent need as maintaining a comptetitive advantage over enemy nations in battlefield cognition is essential. The development of isovaline for warfare analgesia also fits in well for management of pain associated with traumatic or war-related injuries. Specific needs identified by the US Army include: Development of alternatives to current opioid analgesics for severe pain management by the medic/corpsman on the battlefield/remote locations, development of strategies for management of chronic pain under the care of a clinician in non-deployed settings, identification of pain generators, development of strategies for acute pain management in deployed locations, including battlefield and resource-limited environments, development of strategies for identifying and addressing biopsychosocial aspects of pain, development of strategies for management of acute pain under the care of a clinician in non-deployed settings, development of strategies for chronic pain management in deployed locations, including battlefield and resource-limited environments, and development of substance misuse and abuse assessments and treatments in pain management. Isovaline is also a promising agent for reducing the possibility of addiction, which is currently a high risk outcome of postoperative pain control using opioids.