EFFECTS OF ISONALINE AND ACBC
ON TONIC AND ACUTE NOCICEPTIVE MODELS IN RODENTS

by

Cheryl C.W. Chung

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ABSTRACT

Chronic pain is a prevalent problem in society and is inadequately managed using current analgesics. Central disinhibition, produced by dysregulation of excitatory or inhibitory signaling, is responsible for most, if not all, chronic pain states. In particular, an impairment of glycinergic and GABAergic pathways is associated with hypersensitivity and allodynia. Selectively restoration of such inhibitory signaling will likely be of therapeutic value. We explored this hypothesis by characterizing in vivo effects of novel analgesics isovaline and 1-amino-cyclobutane carboxylic acid (ACBC), using a broad spectrum of acute, tonic and chronic pain models.

At all doses tested, neither isovaline nor ACBC have appreciable effects on heart rate, breathing rate or behaviour in rodents. They decreased both formalin-induced phase I and II paw licking response. The effect of isovaline and ACBC on monosodium iodoacetate- (MIA-) induced osteoarthritis in rats could not be evaluated due to large interanimal variability. However, behavioural assays for mouse osteoarthritis were examined; of these, the inverted wire mesh was the most promising. A substantial decrease of hind paw grip duration was observed in MIA-treated animals which lasted for over 21 days. The grip impairment was reversed by morphine, but not diclofenac, on day 14 only.

Isovaline and ACBC are effective in heat-induced acute nociceptive models in mice. Both compounds increased tail flick latency in the hot water tail immersion assay with a non-sigmoidal dose-response relationship, in which middle, but not higher doses were efficacious. A
similar pattern of dose-response was observed in the hotplate model for ACBC. High dose isovaline did not change latency to paw licking on the hotplate.

The effects of intraplantar administration of glutamate in mice have also been examined. Peripheral glutamate elicited paw licking and allodynia in a dose-dependent fashion. Paw licking response was blocked by co-administration of ACBC, while isovaline was not effective.

These results indicate both compounds have antinociceptive properties without obvious side effects. They likely have mixed effects on different receptor systems. Further exploration is required to elucidate their mechanisms of action. However restoration of central inhibition appears to be a route for the discovery of new analgesics.
# TABLE OF CONTENTS

**ABSTRACT** ................................................................................................................................... ii

**TABLE OF CONTENTS** ............................................................................................................ iv

**LIST OF FIGURES** ..................................................................................................................... ix

**LIST OF ABBREVIATIONS** .................................................................................................... xii

**ACKNOWLEDGEMENTS** ...................................................................................................... xiii

**Chapter 1. Introduction** ............................................................................................................... 1

1.1. History of Analgesia................................................................................................................ 1
1.2. Pain as a Sensation .................................................................................................................... 4
1.3. Evaluation of Pain ...................................................................................................................... 5
1.4. Types of Pain.............................................................................................................................. 6
1.5. Acute Pain ................................................................................................................................ 6
1.6. Animal Models of Acute Pain.................................................................................................. 7
   1.6.1. Tail-Flick Assay .................................................................................................................. 7
   1.6.2. Hot Plate Assay ................................................................................................................ 8
1.7. Chronic Pain.............................................................................................................................. 9
1.8. Animal Models of Chronic Pain.............................................................................................. 10
1.9. Osteoarthritis (OA) ................................................................................................................ 11
   1.9.1. Animal Models of OA ....................................................................................................... 12
   1.9.2. Treatment & Interventions for OA .................................................................................. 13
1.10. The Pain Pathways .................................................................................................................. 14
   1.10.1. The Neospinothalamic Tract ......................................................................................... 16
   1.10.2. The Paleospinothalamic Tract ...................................................................................... 16
   1.10.3. The Archispinothalamic Tract ...................................................................................... 17
1.11. Neurotransmitters Involved in the Regulation of Pain........................................................... 17
1.12. Glutamate ............................................................................................................................... 17
1.13. NMDA Receptors

1.13.1. NMDA Receptors and Pain

1.13.2. NMDA Antagonists

1.14. GABA Receptors

1.14.1. GABA Receptors and Pain

1.15. Glycine Receptors

1.15.1. Glycine Receptors and Pain

1.16. Potential Drug Candidates

1.16.1. Study of Small Amino Acids

1.16.2. Isovaline & ACBC

1.16.3. Chemical Structures of Isovaline & ACBC

1.16.4. Previous Studies of Isovaline & ACBC

1.17. Rationale

Chapter 2. Materials, Equipment & Methods

2.1. Animals

2.2. Drugs

2.3. Equipment

2.3.1. Anesthesia

2.3.2. Hamilton Syringe Injections

2.3.3. Mouse Restrainers for Assaying Effects of Subcutaneous Injections and Tail Immersion

2.3.4. Observation Chambers

2.3.5. Video and Data Capture Setup

2.3.6. Incapacitance Tester for the Rat Osteoarthritis Model

2.3.7. Alldynia Testing for the Rat Osteoarthritis Model

2.3.8. Rotarod

2.3.9. Hot Plate

2.4. Methods

2.4.1. Randomization & Blinding

2.4.2. Isovaline Maximum Tolerated Dose (MTD) in Rats

2.4.3. Dose Finding Studies for Isovaline & ACBC
2.5. Osteoarthritis Model in Rats ................................................................. 37
  2.5.1. Induction of Osteoarthritis ............................................................... 37
  2.5.2. Behavioural Assays of Osteoarthritis ........................................... 37
  2.5.3. Knee Joint Diameter Measurements ............................................ 38
  2.5.4. Utilizing the Incapacitance Tester (Measuring Change in Body Weight Distribution) ................................................................. 38
  2.5.5. Von Frey Hair Stimulation (Static Plantar Allodynia) .................. 38
  2.5.6. Pharmacological Testing ............................................................... 39

2.6. Osteoarthritis Model in Mice .............................................................. 39
  2.6.1. Induction of Osteoarthritis ............................................................... 39
  2.6.2. Behavioural Assays of Osteoarthritis ........................................... 39
  2.6.3. Inverted Wire Mesh Assay ............................................................... 40
  2.6.4. Paw Grip on a 70° Incline Plane ................................................... 40
  2.6.5. Rotarod Test .................................................................................. 40

2.7. Heat-induced Models of Nociception ................................................ 41
  2.7.1. Hot Water Tail Immersion Test ..................................................... 41
  2.7.2. Hot Plate Test ............................................................................... 42

2.8. Glutamate-induced Paw Licking in Mice .......................................... 43

2.9. Glutamate-induced Allodynia in Mice .............................................. 44

2.10. Statistical Analysis ............................................................................ 45

Chapter 3. Results ......................................................................................... 46

3.1. Isovaline Maximum Tolerated Dose (MTD) in Rats ......................... 46

3.2. Dose Finding Studies for Isovaline & ACBC ..................................... 48

3.3. Osteoarthritis Model in Rats ............................................................... 50
  3.3.1. Pilot Study ..................................................................................... 50
  3.3.2. Randomized Double-blinded Study .............................................. 52
  3.3.3. Changes in Knee Joint Diameter ................................................ 53
  3.3.4. Incapacitance Testing (Change in Body Weight Distribution) ........ 54
  3.3.5. Effects of Drugs on Body Weight Distribution ............................ 55
  3.3.6. Von Frey Hair Stimulation (Static Plantar Allodynia) .................. 57

3.4. Osteoarthritis Model in Mice ............................................................. 59
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4.1. Inverted Wire Mesh Assay</td>
<td>59</td>
</tr>
<tr>
<td>3.4.2. Paw Grip on a 70° Incline Plane</td>
<td>64</td>
</tr>
<tr>
<td>3.4.3. Rotarod test</td>
<td>66</td>
</tr>
<tr>
<td>3.5. Heat-induced Models of Nociception</td>
<td>67</td>
</tr>
<tr>
<td>3.5.1. Hot Water Tail Immersion Test</td>
<td>67</td>
</tr>
<tr>
<td>3.5.2. Hot Plate Test</td>
<td>70</td>
</tr>
<tr>
<td>3.6. Glutamate-induced Paw Licking in Mice</td>
<td>72</td>
</tr>
<tr>
<td>3.7. Glutamate-induced Alloodynia in Mice</td>
<td>73</td>
</tr>
<tr>
<td><strong>Chapter 4. Discussion</strong></td>
<td>76</td>
</tr>
<tr>
<td>4.1. Isovaline Maximum Tolerated Dose (MTD) in Rats</td>
<td>76</td>
</tr>
<tr>
<td>4.2. Dose Finding Studies for Isovaline &amp; ACBC</td>
<td>77</td>
</tr>
<tr>
<td>4.3. Dixon’s Up-and-Down Method</td>
<td>79</td>
</tr>
<tr>
<td>4.3.1. Randomized, Blinded 2-Staircase Up-and-Down Method</td>
<td>81</td>
</tr>
<tr>
<td>4.4. Osteoarthritis Model in Rats</td>
<td>81</td>
</tr>
<tr>
<td>4.4.1. Knee Joint Diameter Measurements</td>
<td>82</td>
</tr>
<tr>
<td>4.4.2. Incapacitance Tester (Change in Body Weight Distribution)</td>
<td>82</td>
</tr>
<tr>
<td>4.4.3. Von Frey Hair Stimulation (Static Plantar Alloodynia)</td>
<td>83</td>
</tr>
<tr>
<td>4.5. Osteoarthritis Model in Mice</td>
<td>84</td>
</tr>
<tr>
<td>4.5.1. Inverted Wire Mesh Assay</td>
<td>85</td>
</tr>
<tr>
<td>4.5.2. Paw Grip on a 70° Incline Plane</td>
<td>86</td>
</tr>
<tr>
<td>4.5.3. Rotarod Test</td>
<td>87</td>
</tr>
<tr>
<td>4.6. Heat-induced Models of Nociception</td>
<td>88</td>
</tr>
<tr>
<td>4.6.1. Hot Water Tail Immersion Test</td>
<td>88</td>
</tr>
<tr>
<td>4.6.2. Hot Plate Test</td>
<td>90</td>
</tr>
<tr>
<td>4.7. Mechanisms of Action of Isovaline &amp; ACBC</td>
<td>90</td>
</tr>
<tr>
<td>4.7.1. Glutamate-induced Paw Licking and Alloodynia in Mice</td>
<td>91</td>
</tr>
<tr>
<td>4.7.2. Evidence from Electrophysiology and Receptor Modelling</td>
<td>92</td>
</tr>
<tr>
<td>4.7.3. Structure Activity Relationships</td>
<td>94</td>
</tr>
<tr>
<td>4.8. Towards Better Analgesics Testing</td>
<td>94</td>
</tr>
<tr>
<td>4.8.1. Development of Better Animal Pain Models</td>
<td>95</td>
</tr>
<tr>
<td>4.8.2. Importance of Translational Studies</td>
<td>96</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. A simple schematic depicting the three nociceptive pathways............................... 15

Figure 2. Schematic representations of the NMDA receptor complex. ................................. 19

Figure 3. Schematic representations of the GABA\textsubscript{A} receptor complex. ......................... 22

Figure 4. Chemical structures for (A) glycine, (B) isovaline, (C) \(\gamma\)-aminobutyric acid (GABA) and (D) 1-amino-cyclobutane carboxylic acid (ACBC). ............................................. 26

Figure 5. A schematic of the custom mouse restrainer. .......................................................... 31

Figure 6. The incapacitance tester. .......................................................................................... 33

Figure 7. Time course for the dose finding studies. ................................................................. 36

Figure 8. Time course for the hot water tail immersion assay. .................................................. 42

Figure 9. Time course for the hot plate assay.......................................................................... 43

Figure 10. Sample polygraph trace of a rat administered 2000 mg/kg (s.c.) isovaline.......... 47

Figure 11. Determination of the ED\textsubscript{50}s of isovaline and ACBC for inhibition of phase II formalin-induced paw licking response using the up-and-down method .................. 49

Figure 12. Changes in knee diameter upon intra-articular administration of MIA in the pilot study................................................................................................................. 51

Figure 13. Changes in body weight distribution upon intra-articular administration of MIA as measured by the incapacitance tester in the pilot study........................................... 51

Figure 14. Changes in knee diameter upon intra-articular administration of MIA in the randomized blinded study................................................................. 53

Figure 15. Changes in body weight distribution of rats in (A) acute and (B) chronic stages of the MIA-induced osteoarthritic model as measured by the incapacitance tester in the randomized blinded study................................................................. 54

Figure 16. Effects of saline on body weight distribution of rats in the MIA-induced osteoarthritis model.................................................................................................................. 55
Figure 17. Effects of isovaline and ACBC on the capacitance tester compared to saline, morphine and sodium diclofenac control................................................................. 56

Figure 18. Changes in hind paw withdrawal threshold (PWT) of rats in (A) acute and (B) chronic stages of the MIA-induced osteoarthritic model as measured by the von Frey hair stimulation in the randomized blinded study................................................................. 57

Figure 19. Effects of saline, morphine, diclofenac, isovaline and ACBC on static allodynic responses in the (A) acute (Days 2, 3, 4) and (B) chronic (Days 28, 29, 30) stages of the MIA-induced osteoarthritis model................................................................. 58

Figure 20. Effects of intra-articular administration of MIA in mice over time......................... 59

Figure 21. Effects of morphine on the (A) acute and (B) chronic stages of osteoarthritis in the inverted wire mesh assay. .............................................................................................................. 61

Figure 22. Effects of diclofenac on the (A) acute and (B) chronic stages of osteoarthritis in the inverted wire mesh assay. .............................................................................................................. 62

Figure 23. Effects of ACBC on the (A) acute and (B) chronic stages of osteoarthritis in the inverted wire mesh assay. .............................................................................................................. 63

Figure 24. Effects of intra-articular administration of MIA in mice over time......................... 64

Figure 25. Effects of morphine, diclofenac and ACBC on the ability of mice to perform the 70° incline plane test in the acute stages of the MIA-induced osteoarthritis model. ....... 65

Figure 26. Effects of diclofenac on rotarod performance in the acute stage of the MIA-induced osteoarthritis model......................................................................................... 66

Figure 27. Effects of morphine on rotarod performance in the (A) acute and (B) chronic stages of the MIA-induced osteoarthritis model............................................................. 67

Figure 28. Saline vehicle control had no effects on mice tail flick response. ......................... 68

Figure 29. Effects of isovaline on the hot water tail immersion test........................................ 69

Figure 30. Effects of ACBC and 82 mM NaCl (osmolarity control) on the hot water tail immersion test................................................................. 70

Figure 31. Effects of ACBC on the hot plate test and spontaneous breathing rate............... 71
Figure 32. Effects of isovaline on the hot plate test. ................................................................. 72

Figure 33. Effects of ACBC on the glutamate-induced paw licking response in mice ............... 73

Figure 34. Determination of the EC$_{50}$ of intraplantar glutamate necessary to produce allodynia in mice using the randomized, blinded, double staircase up-and-down method. ........ 74

Figure 35. Isovaline had no effects on the glutamate-induced allodynia model in mice. .......... 75

Figure 36. A visual description of Dixon’s up-and-down protocol. ......................................... 80
LIST OF ABBREVIATIONS

ACBCH 1-amino-cyclobutane carboxylic acid
AMPA α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ASICs acid-sensing ion channels
CMRH Certified Medical Representatives Institute
CNS central nervous system
EC$_{50}$ effective concentration 50
ECG electrocardiogram
ED$_{50}$ effective dose 50
GABA γ-aminobutyric acid
IASP International Association for the Study of Pain
IL intralaminar
i.v. intravenous
mGlu metabotropic glutamatergic
MIA monosodium iodoacetate
MTD maximum tolerated dose
NGFs neurotrophic factors
NMDA N-methyl D-asparate
NSAID non-steroidal anti-inflammatory drug
OA osteoarthritis
PWT paw withdrawal threshold
RM-ANOVA repeat measures analysis of variance
S1 primary somatosensory cortex
S2 secondary somatosensory cortex
s.c. subcutaneous
SD standard deviation
SEM standard error of the mean
TRPV1 transient receptor potential channels
VPL ventroposterolateral
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And so we have reached the end of this chapter and it is time to start anew. Yet our memories and these pages stand witness, our passage. I offer, once again, my thanks. It has been an unforgettable experience. I will treasure these memories, always.
Chapter 1. Introduction

1.1. History of Analgesia

Pharmacological methods for pain relief have been used by man for over 6000 years. The opium poppy, for example, is the oldest known analgesic and was discovered in Sumerian artifacts dated to 4000BC. The juice of the opium poppy was used to treat numerous ailments, from asthma to internal diseases and women’s illnesses. One of its several active components, morphine, was isolated in 1805 by the German pharmacist Friedrich Sertürner. Willow bark tea is another ancient pain remedy. In 400BC, Hippocrates indicated the use of the powder of the bark and leaves of the willow tree to relieve headaches and other pains. Willow bark has also been used for centuries in China and North America. The Reverend Edward Stone in 1763 isolated salicylic acid from dried willow bark which gave rise to Aspirin, the first nonsteroidal anti-inflammatory drug (NSAID).

Since then, many other NSAIDs have been discovered and synthesized. Although these agents are useful in the management of pain, they vary in effectiveness and have many undesirable side effects, which limit their use. NSAIDs and related compounds, such as acetaminophen, are the mainstay for the treatment of mild to moderate pain. The adverse effects of NSAIDs include gastrointestinal ulceration, bleeding, and renal impairment due to nonspecific down-regulation of COX-1 enzyme activities. The newer COX-2 selective inhibitors (e.g. celecoxib and rofecoxib) were designed to reduce these side effects by selective targeting of inflammatory enzymes. However they were shown to be no better at
preventing gastric bleeding than a non-selective NSAID while increasing the risk of myocardial infarctions and thrombotic cardiovascular events (Therapeutics Initiative, 2002; Wright et al., 2001).

Opioid analgesics are used in cases of severe pain. They are associated with many, undesirable side effects such as nausea, vomiting, sedation and respiratory depression. Patients also develop tolerance to the analgesic effects of opioids. The drugs may lose effectiveness after prolonged use, which is of course necessary for effective management of chronic pain. Therefore, there is a great need for novel analgesics for treatment of intractable chronic pain with fewer side effects.

Current research on analgesics has been focused on the search for drugs with mechanisms of action that differ from that of opioids and NSAIDs. So far N-methyl D-aspartate (NMDA) receptor antagonists (e.g. ketamine) and gabapentin have not proven to be potent standalone painkillers, and as such are primarily used as adjuvant analgesics administered in conjunction with conventional agents. Also problematic is accumulating evidence for significant systemic toxicity and corresponding side-effects associated with use of such agents.

Other nociceptive systems currently under investigation include serotonergic / dopaminergic system, cannabinoids, neurotrophic factors (NGFs), substance P, transient receptor potential channels (TRPV1) and acid-sensing ion channels (ASICs) (For a brief review of modulators of the pain system, refer to (Woolf and Salter, 2000) and
In particular, peripheral pain-related receptors garnered great interest as potential new therapeutic targets. For example, the P2X3 subtype of ATP receptors elicit painful burning sensation when activated in humans and produce thermal hyperalgesia and nocifensive behaviour in rats (Hamilton et al., 2000). Likewise, the TRPV1 channels, found predominantly in small diameter nociceptive afferents, are activated by heat, protons and capsaicin (the component in chili peppers giving rise to burning sensations). Antagonists of TRPV1 receptors exhibit antinociceptive activities in rodent inflammatory pain models and hyperalgesia models (Gavva et al., 2005; Honore et al., 2005). They are currently under various stages of Phase I and II clinical studies (reviewed in Gunthorpe and Chizh, 2009). However, the focus of our current research is on agents that affect central inhibition pathways and its role in the regulation of pain.

At the Hugill Centre for Anesthesia and Analgesia Research in the University of British Columbia, Department of Anesthesiology, Pharmacology & Therapeutics, recent electrophysiological findings and putative ligand-receptor chemical modeling has led to investigation of the analgesic effects of glycine receptor analogues. Work by Drs. Puil and Mathers demonstrated the presence of functional glycine receptors within the thalamus in nociceptive nuclei (Ghavanini et al., 2005; Ghavanini et al., 2006). The thalamus modulates many sensory inputs, including pain, before projecting the signal to the cortex. Therefore, inhibitory glycinereric transmission could modulate pain pathways. This work led to T. Wang’s study of glycine and its analogues’s effects on allodynic and nociceptive models in mice (Wang, 2008). From his work, isovaline and its cyclic
analogue, 1-amino-cyclobutane carboxylic acid (ACBC) were identified as promising analgesic candidates. This prompted the following research thesis to further examine the analgesic properties of isovaline and ACBC in other acute and chronic pain models, along with establishing a new model for assessment of osteoarthritic pain in mice.

1.2. Pain as a Sensation

To examine analgesia, we must first address pain. The International Association for the Study of Pain (IASP) defined pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (International Association for the Study of Pain Sub-Committee on Taxonomy, 1979). Pain consists of two components: 1) nociception, the activation, transmission and processing of nerve impulses generated by noxious stimuli and 2) emotional affect, the accompanying unpleasant psychological response to the stimuli. The experience of pain is by definition subjective. There is, furthermore, a large variation in the pain threshold and intensity of pain perceived by different individuals to a given noxious stimulus. Therefore, objective comparison of the effectiveness of analgesics in pain management is difficult.

Hypersensitivity is enhancement of pain and nociceptive responses. Allodynia is the perception of pain to a normally innocuous stimulus. Both hypersensitivity and allodynia can arise from modification to the nociceptive pathways. It is thought that repeated stimulation of nociceptors could lead to peripheral sensitization (Woolf and Salter, 2000). Inflammatory mediators released during tissue insult also heighten pain sensation.
1.3. Evaluation of Pain

Clinically, pain is measured using a subjective pain scale, an ordinal scale typically ranging from 0 (no pain) to 10 (the worst pain imaginable). Patients are asked to indicate the intensity of pain they experienced by pointing along this scale (Downie et al., 1978). These ratings are then used to compare the effectiveness of pain management between treatment groups. However, this pain scale has inherent problems. First, it is completely subjective, ruling out a psychophysical model of stimulus versus response. Second, there are no definite indicators for different levels on the scale. For example, what qualifies as “moderate pain” varies between individuals. Moreover, not all patients are equally adept at defining the limits of pain; a higher intensity of pain than previously imaginable is nearly always possible.

Evaluation of pain and analgesia in animals is even more challenging since animals cannot provide a verbal report of the degree of pain they are experiencing. Therefore biochemical, electrophysiological and behavioral measurements are used as surrogates for pain. Of these, behavioral responses are the only reliable indicators for unpleasant sensations (Le Bars, D Gozariu, M Cadden, S W., 2001). These responses are, however, nonspecific; there is no guarantee that the response pertains to a noxious stimuli. For example an avoidance response could be due to fear as opposed to pain. Similarly, a lack of response could be due to an inability to respond to the pain (ataxia, sedation) instead of analgesia. Thus, preclinical assessment of candidate analgesic compounds is full of challenges. A broad array of assays (e.g. acute, inflammatory and neuropathic pain
models, CNS toxicity models and pharmacokinetic studies) must be used to confirm drug activities.

The predictive value of animal pain models to therapeutics in human is also rather poor (B. A. Chizh et al., 2009). For example, disease states such as post-herpetic neuralgia are difficult to replicate in animals. Likewise, how does inhibition of a nociceptive reflex in rodents translates into analgesia in humans? Nevertheless, as stated by Whiteside and colleagues, “even if animal models are only 10% predictive they are still essential tools in the drug discovery repertoire.” (Whiteside et al., 2008). Despite the current limitations in animal pain models, they remain our best strategy for identifying drug candidates for new analgesics.

1.4. Types of Pain

Pain is an evolutionary mechanism to limit or prevent potential tissue damage. Pain can be classified into acute pain and chronic pain.

1.5. Acute Pain

Acute pain lasts for a short duration (less than 3 months), is self-limiting and is important for preventing tissue damage. Acute pain is experienced when a finger is nicked with a knife or a hand is placed on a hot stove. We withdraw our hand upon touching a hot stove to prevent burns. Similarly, pain from a knife cut stops us from further injuring ourselves.
1.6. Animal Models of Acute Pain

Animal models for acute nociception have been studied since the 1920s (Le Bars, D Gozariu, M Cadden, S W., 2001). Assays measured changes in the rate, frequency or intensity of the withdrawal response or other nocifensive behavior of animals in response to an acute noxious stimulus. Of these assays, tail-flick and hot plate tests in rodents are considered to be gold standards.

1.6.1. Tail-Flick Assay

In the tail-flick test, the tip of a rat or a mouse’s tail is either placed beneath a radiant heat source or submerged in a hot water bath. The latency to the tail withdrawal response (tail flick latency) is measured. The tail withdrawal is considered to be a nociceptive spinal reflex. Section or cold block of the upper spinal cord cannot abolish the tail flick response (Bonnycastle et al., 1953; Irwin et al., 1951; Sinclair et al., 1988).

Douglas and Carstens (1997) suggested a pathway for heat activation of nociceptors innervating the tail. The nociceptive afferent fibers enter the spinal cord over sacral (S4) to coccygeal (Co3) segments, which relay the impulse to sacral dorsal horn interneurons. The interneurons then synapse with motor neurons located in segments lumbar (L4) to coccygeal (Co3) to innervate three sets of back muscles which control tail movements.

Increases in neuronal responses parallel motor outputs (Douglass and Carstens, 1997) - at ~36°C, neuronal responses are first detected, while the tail flick response requires a temperature of 40°C. Intensity of response increases linearly up to 48-54°C, whereupon
stimulus-saturation is reached. Morphine (1 & 2 mg/kg) directly suppressed sacral spinal neuron activity and attenuated the tail flick response (Douglass and Carstens, 1997).

The hot water tail immersion test is considered to be the most robust and consistent of tail-flick assays (Kruger, 2001). It provides uniform heating of the tail and rapidly raises cutaneous temperature in a linear fashion. It is relatively insensitive to tail pigmentation, precise positioning of the heat source and is reproducible over time (Kruger, 2001; Le Bars, D Gozariu, M Cadden, S W., 2001; Sewell and Spencer, 1976). Traditionally a 55°C water bath is used for the detection of opioid analgesia. The temperature of the bath can be lowered to identify partial agonists ((Luttinger, 1985; Sewell and Spencer, 1976). This test, however, is not sensitive to non-opioid analgesics; NSAIDs (aspirin, ibuprofen, indomethacin) and acetaminophen have little or no significant effect on this model (Luttinger, 1985; Sewell and Spencer, 1976).

1.6.2. Hot Plate Assay

Compared to the tail-flick test, the hot plate assay is a less robust model of heat-induced nociception (Kruger, 2001; Le Bars, D Gozariu, M Cadden, S W., 2001). In this test, a mouse or a rat is placed into a chamber with a metallic floor heated by a thermode or circulating hot liquid maintained at a constant temperature. The four paws and perhaps a portion of the tail of the animal are stimulated. A mouse generally displays two behavioural responses: paw licking and jumping, both of which are considered to be supraspinal responses (Le Bars, D Gozariu, M Cadden, S W., 2001). The reaction time of the animal is measured. Delay of nocifensive behaviour is indicative of nociceptive blockade. The hot plate test is the most sensitive to opioid analgesics, although inhibitory
effects of acetaminophen and aspirin can be detected at lower temperatures ($< 50^\circ$C) (Ankier, 1974). The behavioral responses in rats are more complex. Twenty behavioural patterns have been recorded; the animal may sniff and lick its forepaws or hindpaws, straighten up, stamp its feet or groom (Espejo and Mir, 1993). Due to the large variety of responses exhibited by rats, response time measurements in this model are difficult. There is also a strong learning component in this model. Animals display learned avoidance behaviours. Repeated testing leads to a shortening of the animal’s response time (Kruger, 2001). Paw licking behaviour also decreases and is replaced by jumping escape responses (Le Bars, D Gozariu, M Cadden, S W., 2001).

1.7. Chronic Pain

Chronic pain is simply defined as pain that persists beyond the natural time course for healing. Epidemiological studies suggest chronic pain is a major public health concern. In an American survey, 42% of respondents indicated they have experienced pain on a daily basis (Manchikanti et al., 2003). Chronic pain also affects close to one in five adults in Australia (Beirith et al., 2002). Chronic pain negatively impacts quality of life and poses a greater economic burden than cancer and cardiovascular diseases combined (Turk and Rudy, 1988).

The origin of chronic pain is not well understood. However, 0.05-1.5% of patients have chronic pain postoperatively. Clinical research has identified several risks factors for developing persistent post-surgical pain (Shipton and Tait, 2005). This list includes: preoperative distress and pain, reoperation, sex (young females are more susceptible),
severe postoperative pain and postoperative complications (e.g. infection, organ rupture and bleeding).

Chronic pain can also arise from central pain states, where the CNS becomes sensitized and activates without external stimuli (Woolf and Salter, 2000). For example, phantom limb pain can occur where a patient experiences pain that appears to originate from an amputated limb. Trauma to afferent nociceptive pathways is thought to cause abnormal activities in interneurons resulting in pain perception in the absence of nociceptive stimulation. Conventional analgesics and sedatives are ineffective in these conditions.

**1.8. Animal Models of Chronic Pain**

Tonic nociceptive models typically involve peripheral administration of algogenic substances to produce inflammatory response. Animals display vigorous licking, grooming or writhing behaviour depending on the site of administration. These are not true models of chronic pain since their responses abate in tens of minutes.

The formalin-induced paw licking model is most commonly used. Formalin is a 37% formaldehyde solution. Usually a 0.5 – 15% formalin solution is injected subcutaneously into the paw of a mouse or a rat (Le Bars, D Gozariu, M Cadden, S W., 2001). Pain behaviour is either scored by posture, frequency or duration of response. Posture can be scored using a 4-level qualitative scale: 0, normal posture; 1, paw resting on ground but non-weight bearing; 2, paw elevated; 3, active licking, biting or shaking of paw. Responses are measured continuously over time or in regular time intervals (Le Bars, D Gozariu, M Cadden, S W., 2001; Negus et al., 2006; Tjolsen et al., 1992). Rodents treated
with formalin typically display a biphasic pattern of paw licking and flinching. The early (phase I) nociceptive response lasts for ~5 min and is followed by a quiescent period between 10 to 15 min. The second phase (phase II) of paw licking response initiates at 20 min and continues for 60 to 90 min post formalin administration (Hunskaar and Hole, 1987; Negus et al., 2006; Sevostianova et al., 2003; Tjolsen et al., 1992). The two phases can be selectively inhibited by different drugs, suggesting that they are mediated by different mechanisms. Phase I licking response can be attributed primarily to direct activation of C-fibers by peripheral formalin, while prostaglandin induced inflammatory processes mediate the phase II licking response (Hunskaar and Hole, 1987). Opioid analgesics (morphine, codeine) effectively inhibit both phase I and II responses, whereas NSAIDs (indomethacin, naproxen) decreased phase II but not phase I responses (Hunskaar and Hole, 1987). NBQX, an α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor antagonist, selectively suppresses phase I response at doses that do not producing motor impairment (Hunter, 1994). In contrast, the NMDA receptor antagonist AP5 preferentially inhibits rat dorsal horn neuron activities in the second phase (Haley et al., 1990).

1.9. **Osteoarthritis (OA)**

Another cause of chronic pain is musculoskeletal damage. The prevalence of aging-associated disorders of joints is on the rise. Osteoarthritis (OA) is a degenerative joint disease affecting over 80% of the population above age 75 (Scott, 2006). OA commonly develops in weight-bearing joints such as the knees and the hip. From x-ray radiography, OA is characterized by a loss of articular cartilage, subchondral remodeling and joint misalignment. As the disease progresses, flexibility of the joint is impaired until
replacement surgery is required to maintain mobility (Fernihough et al., 2004). The most debilitating aspect in OA is the accompanying pain, typically localized to the afflicted joint (Fernihough et al., 2004). The World Health Organization estimated that about 10% of the world’s population over age 60 suffers from OA pain (WHO Scientific Group on the Burden of Musculoskeletal Conditions at the Start of the New Millennium, 2003).

Peripheral sensitization is believed to be the cause of OA pain (McDougall, 2006a). Peripheral and central neuronal pathways undergo plastic changes during inflammation which give rise to hyperalgesia and allodynia. A group of normally quiescent afferent neurons known as “silent nociceptors” become active upon the induction of inflammation and tissue injury. The recruitment of these receptors contributes to peripheral sensitization. Additionally, the activation threshold of normal joint nociceptors is reduced. The mechanoreceptors become hypersensitized to joint movements. Afferent firing rate significantly increases in response to both normal (innocuous) and hyper-rotation (noxious) of the knee in arthritic animals (McDougall, 2006b). The CNS is thought to interpret heightened discharge from afferent fibers as joint pain.

1.9.1. Animal Models of OA

Many models of OA in animals are currently available, ranging from spontaneous models in aging animals and in genetically modified mice as well as experimentally-induced models using surgery, enzymes and other chemicals. In the past, research has focused on histopathology and chemical mediators in OA in attempts to understand the generation and progression of OA joint degeneration (Ameye and Young, 2006). The pain aspect of OA has been largely neglected until recently. It was found that administration of
monosodium iodoacetate (MIA) in the knee joints of rats results in pain behaviour and biochemical changes to the joint similar to that of human OA (Bove et al., 2003; Combe et al., 2004; Fernihough et al., 2004; Kobayashi et al., 2003; Pomonis et al., 2005). Iodoacetate is a potent metabolic inhibitor of glyceraldehyde-3-phosphate dehydrogenase. Injection of MIA in to the intraarticular space disturbs glycolysis and results in death of the surrounding chondrocytes. Cell death leads to articular cartilage degeneration resembling that of human OA (Fernihough et al., 2004; Guingamp et al., 1997; Pomonis et al., 2005). Two phases of pain response occur in the model. An acute inflammatory pain response occurs in the first week after MIA administration. The chronic stage of the model occurs after 14 days and lasts for over 60 days (Combe et al., 2004). The chronic stage is not associated with inflammation. Rats also display mechanical hyperalgesia, tactile allodynia and changes to hind paw weight distribution in both stages which are reversible by administration of conventional analgesics (Bove et al., 2003; Combe et al., 2004; Fernihough et al., 2004; Pomonis et al., 2005).

1.9.2. Treatment & Interventions for OA

Currently there are no disease-altering therapies for OA. Treatments are centered towards pain management. Analgesics on the market do not effectively relieve OA pain (McDougall, 2006b). Patients on these medications still report debilitating pain. Unwanted side effects of conventional agents also preclude their long-term use. Hence there is a pressing need for new analgesics to relieve pain in OA and other chronic pain conditions, preferably based on novel mechanisms of action.
1.10. The Pain Pathways

In the search for new analgesics, we should first examine pain pathways and neurotransmitters involved in the regulation of pain. Figure 1 shows a simple schematic for nociceptive pathways. Under normal conditions, noxious stimuli (e.g. a pin prick) activate peripheral nociceptors. Nociceptors then transmit the signal to primary afferent fibers. Nociceptive fibers are classified into 2 types (Aδ and C) based on their structure, diameter and conduction velocity. Aδ fibers are intermediate sized fibers (3 – 8 µm in diameter) with thin myelination. They conduct fast “sharp” discriminatory pain and cold thermal signals at 10 – 30 m/s. The C fibers are thin (0.2 – 1.5 µm in diameter), unmyelinated, slow conducting (0.5 – 2.5 m/s) fibers. C fibers have unspecialized nerve endings sensitive to heat, slow “dull” non-discriminatory pain and the presence of algogenic substances like K+, acetylcholine, proteolytic enzymes, serotonin, prostaglandins, substance P and histamine.
Figure 1. A simple schematic depicting the three nociceptive pathways (modified from Garcia, 2009).

Nociceptive signals are propagated along the primary afferent fiber to the dorsal horn region of the spinal cord (Garcia, 2009). The primary afferent synapses with a dorsal horn interneuron which in turn synapses with spinothalamic neurons that project to the brain. Several different nociceptive pathways are used depending on the type and location of nociceptive stimuli.
1.10.1. The Neospinothalamic Tract

The neospinothalamic pathway is shown in Figure 1 in blue. Fast pain travels along the A\textit{\delta} fibers to synapse at the dorsal horn, predominant in the lamina I region, with dendrites of spinothalamic neurons. Axons of the second-order neurons decussate in the anterior white commissure to ascend contralaterally along the lateral spinothalamic tract (Garcia, 2009). They terminate at the ventroposterolateral (VPL) and ventroposteroinferior nuclei of the thalamus. The VPL nucleus is thought to act as a relay center, and processes discriminatory pain information before transmitting the signal to the primary somatosensory cortex (S1).

First-order A\textit{\delta} nociceptive afferents from the head and face originate from the trigeminal ganglion (Garcia, 2009). These trigeminal fibers descend to the medulla to synapse at the spinal trigeminal nucleus. The second-order tract neuron crosses the midline, and ascends along the trigeminothalamic tract to synapse at the ventroposteromedial nucleus of the thalamus. The thalamic neuron then projects to S1. The C-type fibers along the trigeminal distribution synapse at the intralaminar (IL) nuclei of the thalamus. IL neurons then carry non-specific nociceptive signals to the secondary somatosensory cortex (S2).

1.10.2. The Paleospinothalamic Tract

The paleospinothalamic tract (illustrated in red within Figure 1) is a more “primitive” nociceptive pathway which 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. The second-order interneurons are multireceptive and also receive input from mechanoreceptors and thermoreceptors. They
synapse with tract neurons in laminae IV to VIII. These neurons ascend bilaterally along 3 tracts: spinomesencephalic / spinoreticular tract (terminates at the mesencephalic reticular formation), spinotectal tract (terminates at the periaqueductal gray and hypothalamus) and spinohypothalamic tract (terminates at the intralaminar (IL) thalamus). The tracts are collectively referred to as the anterospinothalamic tract. The paleospinothalamic neurons innervate the affective centers (limbic system, cingulated cortex, hypothalamus, balsa ganglia, insular cortex and S2).

1.10.3. The Archispinothalamic Tract

The archispinothalamic tract (shown in green within Figure 1) is the oldest system, which carries diffused nociceptive impulses (Garcia, 2009). Dorsal root afferents synapse at the substantia gelantinosa. Interneurons in turn synapse at laminae region IV to VII with spinal tract neurons. Nociceptive impulses then ascend bilaterally along the multisynaptic tract to the mesencephalic reticular formation, periaqueductal gray, thalamus, hypothalamus and the limbic system.

1.11. Neurotransmitters Involved in the Regulation of Pain

The three predominant neurotransmitters are glutamate, glycine and \( \gamma \)-aminobutyric acid (GABA). Neuropeptides such as neurokinins, opioids, substance P, somatostatin and galanin also modulate nociceptive transmissions (Dickenson, 1995).

1.12. Glutamate

Glutamate is the predominant excitatory neurotransmitter in the CNS. Enhancement of glutamatergic pathways is suggested to be involved in the generation and maintenance of
persistent pain. Following intense nociceptive stimulation, tissue injury and neuron
damage, glutamatergic synaptic transmission in the spinal cord dorsal horn neurons is
greatly elevated (Bleakman et al., 2006). Central sensitization of glycineergic pathways in
supraspinal nociceptive regions has also been documented (Huang et al., 2006). Central
sensitization induces hyperalgesia and allodynia by increasing signal intensity and
decreasing activation threshold of dorsal horn neurons. It also increases pain sensitivity
of nearby non-injured tissues. Glutamate acts on both ionotropic (AMPA, NMDA,
kainate) receptors and metabotropic (mGlu1-8) receptors to produce its excitatory effects
(Bleakman et al., 2006). The balance of excitatory and inhibitory signals is important in
maintaining normal physiological sensation of pain, while pathophysiological pain states
can arise from an imbalance of inputs. For example upregulation of glutamatergic
neurotransmission is a major component of inflammatory and neuropathic pain (reviewed
in (Woolf and Salter, 2000)). Similarly a deficiency of inhibitory neurotransmission leads
to abnormal pain sensitivity (allodynia) by decreasing thresholds of nociceptive pathways
(Sivilotti and Woolf, 1994).

1.13. NMDA Receptors

N-methyl D-aspartate (NMDA) receptors are non-selective cationic receptors (refer to
Figure 2A for the structure and binding sites). They are heterotetrameric channels usually
consisting of two NR1 and two NR2 subunits. Genes for seven subunits has been
discovered (GLU_N1, GLU_N2A, GLU_N2B, GLU_N2C, GLU_N2D, GLU_N3A and GLU_N3B);
multiple splice variants exist for each subunit (Bleakman et al., 2006). Figure 2B depicts
a schematic representation of the NR1 and NR2 subunits. Each subunit contains four
transmembrane domains (M 1 – 4). The intermembrane pore forming P loop exists
between M1 and M2. A large extracellular region between M3 and M4 contains the ligand binding site. Glutamate binds to the NR2 subunit while the modulatory co-agonist site (glycine$_B$ site) is found on the NR1 subunit. The NR3 subunit is inhibitory.

Figure 2. Schematic representations of the NMDA receptor complex. (A) Structural model of the NMDA receptor complete with its binding sites. (B) Structure of the NR1 and NR2 subunits. Figures adopted from (Anaesthesia UK, 2009).
Normally, extracellular $\text{Mg}^{2+}$ blocks the NMDA channel. Upon membrane depolarization, the $\text{Mg}^{2+}$ block is removed. Binding of glutamate then induces an excitatory postsynaptic current. The $\text{Mg}^{2+}$ blockade can also be removed by binding of glycine at the co-agonist site. Binding of protons at the polyamine modulatory site stabilizes the low conductive state, which can be blocked by competitive binding of endogenous polyamines (spermine and spermidine) (Bleakman et al., 2006). In pain states, NMDA receptors become phosphorylated, enhancing channel conductivity.

1.13.1. NMDA Receptors and Pain

The NMDA receptor is thought to mediate many spinal responses to noxious stimuli. High frequency stimulation of nociceptive fibers induces a “wind-up” phenomenon (Bleakman et al., 2006). Channel conductivity is enhanced upon membrane depolarization, leading to long term potentiation of synapses. NMDA receptors are found to be localized in nociceptive pathways. These receptors also undergo upregulation in neuropathic and inflammatory pain states.

1.13.2. NMDA Antagonists

Numerous NMDA receptor antagonists have been discovered and they primarily interact with the glutamate site on the $\text{GLU}_{2\text{NB}}$ subunit (Bleakman et al., 2006). However these agents are not useful as standalone therapies due to low efficacy and undesirable side effects. Clinical use of MK801 (dizocipine) and ketamine do not seem to inhibit peripheral “wind-up” (Bleakman et al., 2006). They also have strong hallucinogenic and sedative properties due to central nervous system activities.
1.14. GABA Receptors

\(\gamma\)-Aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in supraspinal CNS. GABAergic interneurons can also be found in the substantia gelatinosa within the spinal dorsal horn. Three subtypes of GABA receptors have been identified, GABA\(\text{A}\), GABA\(\text{B}\) and GABA\(\text{C}\) (renamed GABA\(\text{A} - \rho\) by the International Union of Basic and Clinical Pharmacology due to close homology to GABA\(\text{A}\) receptors, (Olsen and Sieghart, 2008)) receptors. GABA\(\text{A}\) and GABA\(\text{A} - \rho\) are ionophores that predominantly gate chloride ions. The chloride conductance increase induces hyperpolarization, decreasing membrane excitability and input resistance.

GABA\(\text{A}\) receptors are heteropentameric channels most commonly formed by two \(\alpha\), two \(\beta\) and one \(\gamma\) subunits (Figure 3). Each subunit has four membrane spanning domains (M1 – M4), with M2 forming the channel pore. The GABA\(\text{A}\) receptor has two binding sites for GABA situated at the interface between the \(\alpha\) and \(\beta\) subunits. The receptor also has a barbiturate modulatory site in the \(\beta\) subunit and a benzodiazepine binding site at the \(\alpha - \gamma\) interface. The GABA\(\text{A}\) receptor is activated by muscimol and inhibited by bicuculline and picrotoxin. The GABA\(\text{A} - \rho\) subtype contains \(\rho\) subunits instead of \(\gamma\) subunits as in GABA\(\text{A}\) receptors. Its distribution is limited to the visual pathways and the substantia gelatinosa of the spinal cord (Johnston, 1996). The \(\rho\) receptor has higher affinity for GABA. It is also insensitive to bicuculline, barbiturates and benzodiapines. The GABA\(\text{B}\) receptor is G-protein coupled. Activation enhances \(K^+\) efflux or suppresses \(Ca^{2+}\) influx. Baclofen is an agonist at the receptor while phaclofen and saclofen are antagonists.
Figure 3. Schematic representations of the GABA$_A$ receptor complex. (A) Left diagram shows the structure of a single subunit. Right diagram depicts the receptor complex formed by a symmetric arrangement of 5 subunits. The orange cylinders, representing the M2 domains, form the channel pore. (B) The subunit composition of a typical GABA$_A$ receptor along with its binding sites. BDZ, benzodiazepine; BAR, barbiturate.
1.14.1. GABA Receptors and Pain

GABAergic neurons are widely distributed in the CNS. Around 30% of interneurons within laminae I-IV of the spinal dorsal horn possess GABA receptors (Bowery et al., 1987). Blockade of rat spinal GABA receptors enhanced C-fiber evoked responses of dorsal horn neurons (Sokal and Chapman, 2001) and caused mechanical allodynia (Hao et al., 1994). In peripheral nerve injury, GABAergic transmission in the superficial dorsal horn is impaired, resulting in thermal and mechanical allodynia that is reversed by intrathecal GABA administration in rats (Eaton et al., 1999). In inflammation, synaptic inhibition by glycine also decreases (Harvey et al., 2004; Muller et al., 2003).

1.15. Glycine Receptors

Glycine is a simple amino acid that acts on two types of ionotropic receptors. They are the conventional strychnine sensitive glycine receptor (glycineA receptor) as well as the strychnine insensitive glycineB binding site on the NR1 subunit of the NMDA receptor.

The glycineA receptor is pentameric in structure and permeable to chloride and bicarbonate ions. It is comprised of two types of subunits (α, β) linked to the anchoring protein gephyrin, which allows for postsynaptic clustering of glycine and GABA_A receptor subtypes (Betz and Laube, 2006). GlycineA subunits share structural similarities with ionotropic GABA_A subunits. Each subunit has a large extracellular N-terminus, four transmembrane domain segments (M1 – M4), a long intracellular P-loop between M3 and M4 and a short extracellular C-terminus. The transmembrane domains along with the N-terminus cysteine-bound motif are highly conserved among glycineA subunits. Four α subunit genes (α1 – 4) and one gene for the β subunit have been discovered. However,
regions of the N-terminus and intracellular loop undergo exon splicing, giving rise to a large heterogeneity of glycine$_A$ receptors. Both $\alpha$ and $\beta$ subunits are thought to be involved in ligand binding, but strychnine has a higher affinity for the $\beta\alpha$ interface.

Expression of $\alpha_1 – 4$ subunits is regionally and developmentally regulated. Glycine$_A$ receptor $\alpha_1$ subunit has been localized in the spinal cord, brainstem and colliculi of adult rodents, whereas $\alpha_2$ is a neonatal subunit only expressed in low level within adult hippocampal, cerebral cortical and thalamic neurons (Sato et al., 1992). Moderate levels of the $\alpha_3$ subunit mRNA are transcribed in the spinal cord, cerebellum and the olfactory bulb (Malosio et al., 1991). A splice variant of the $\alpha_3$ subunit (substitution of proline by leucine at position 185) confers higher agonist affinity and is postulated to exist within extrasynaptic receptors (Meier et al., 2005). Thus far, mRNA expression of the $\alpha_4$ subunit has yet to be localized within the mammalian CNS (Harvey et al., 2000).

1.15.1. Glycine$_A$ Receptors and Pain

Glycine receptors have been identified within interneurons of the superficial laminae I and II (substantia gelatinosa) of the spinal cord dorsal horn. These interneurons are thought to regulate nociceptive inputs from the primary nociceptive afferents. Inhibition of spinal glycine$_A$ receptor by strychnine produces allodynia and hyperalgesia possibly due to interaction with this system. Glycinergic inhibition within dorsal neurons has been shown to induce central sensitization to inflammatory pain (Ahmadi et al., 2002). In particular, the $\alpha_3$ subunit is found to be localized in distinct populations within the superficial laminae of the dorsal horn. Therefore agonists that selectively target glycine
α3 subform of receptors may provide analgesia. Isoform specific glycinergic agents have yet to be discovered.

1.16. Potential Drug Candidates

Central disinhibition of glycinergic and GABAergic neurotransmission has been implicated in inflammatory and neuropathic pain (Sherman et al., 1997 Aug; Sivilotti and Woolf, 1994). Due to reduced tonic inhibition, previously subthreshold stimuli can be perceived as painful. Therefore, a pharmacological approach for treatment of chronic pain is to elevate inhibitory neurotransmission.

1.16.1. Study of Small Amino Acids

Administration of glycine and GABA results in toxicity which precludes their use as therapeutic agents (Ballanyi et al., 1999; Holtman et al., 1982). Thus, past research in our lab focused on examining the analgesic effects of glycine- and GABA-like amino acids. From these studies, two compounds, isovaline and 1-amino-cyclobutane carboxylic acid (ACBC) have shown potential as analgesics.

1.16.2. Isovaline & ACBC

Isovaline is an α-amino acid that closely resembles glycine and GABA. It is a non-biogenic amino acid that is, surprisingly, only found naturally in carbonaceous meteorites (Pizzarello and Weber, 2004). The Murchison meteorite is one of the most well studied meteorites of this group due its large size. Isovaline was found within the meteorite in relatively large quantities and in enantiomeric excess (Glavin and Dworkin, 2009). Having survived entry to Earth, isovaline is likely a very stable molecule, possibly resistant to isomerization. Its activities at glycine and GABA receptors are currently
under investigation. 1-amino-cyclobutane carboxylic acid (ACBC) is structurally very similar to isovaline. It is a partial agonist selective for the glycine\textsubscript{B} modulatory site of the NMDA receptor (Hood et al., 1989).

1.16.3. Chemical Structures of Isovaline & ACBC

Isovaline (2-amino-2-methylbutanoic acid) has a methyl and ethyl substitution on the \( \alpha \)-carbon of glycine (see Figure 4). Its single chiral center gives rise to two enantiomers R- and S-isovaline. A two-carbon extension on the amino acid backbone of glycine results in GABA. Isovaline shares the four carbon backbone with GABA (indicated in pink within figure). The methyl and ethyl groups of isovaline can be connected to form a cyclic butane ring (highlighted in green within figure). The resulting amino acid is ACBC (1-amino-cyclobutane carboxylic acid).

![Chemical structures](image)

Figure 4. Chemical structures for (A) glycine, (B) isovaline, (C) \( \gamma \)-aminobutyric acid (GABA) and (D) 1-amino-cyclobutane carboxylic acid (ACBC).
1.16.4. Previous Studies of Isovaline & ACBC

Previous work in the lab examined the effects of isovaline on a strychnine-induced allodynia model and the formalin foot assay. Strychnine administered in the cisterna magna, or in the lumbar intrathecal space, produced dynamic allodynia in mice (Wang, 2008). The animals displayed avoidance and vigorous grooming behaviour when stroked with a von Frey filament. These nocifensive responses were eliminated when isovaline was co-administered with strychnine, suggesting that isovaline may be an agonist at the glycineA receptor.

In the formalin foot assay, lumbar intrathecal administration of RS-isovaline in mice compared to vehicle control produced a dose-dependent decrease to the duration of paw licking in both phase I and phase II (Wang, 2008). No significant difference was observed in formalin-induced responses between R and S-isomers (Wang, 2008). Similarly, preliminary study suggested that intrathecal ACBC also decreases phase I and II paw licking response (Wang, 2008). Intravenous isovaline produced a dose-dependent decrease in the second phase of the formalin foot test. For phase I, we obtained a non-sigmoidal dose response curve. Low doses of intravenous isovaline, but not high dose, reduced the phase I paw licking duration.

There was no observable respiratory depression or sedation in isovaline and ACBC treated mice. The lack of motor impairment after isovaline administration was confirmed using the rotarod test. There were no significant changes to the time mice spent on the
rotarod between intravenous saline control group and intravenous R and S-isovaline (500 mg/kg) groups (Wang, 2008).

1.17. Rationale

Research on central disinhibition has suggested potential therapeutic benefits in the restoration of GABA and glycineergic neurotransmission for the treatment of chronic pain. As mentioned previously, past experiments in our lab have explored the analgesic effects of various glycine- and GABA-like amino acids in two pain models. Of these compounds, isovaline and ACBC were the most promising. They demonstrated anti-nociceptive and antiallodynic properties without observable systemic toxicity in mice. The next step is to assess their effects in other acute and chronic pain models and to determine their mechanisms of action. Since drug effects may be influenced by the specific characteristics of the model, a comprehensive assessment of the dose-dependent effects of isovaline and ACBC across a range of models is desirable. This research also provides additional support for the hypothesis that agents which enhance inhibitory neurotransmission and or suppress excitatory transmission are therapeutic in pain states.
Chapter 2. Materials, Equipment & Methods

2.1. Animals

Studies were conducted with approval from the Animal Care Committee of the University of British Columbia. Rats (Sprague-Dawley, female, 200-300g) and mice (CD-1, female, 20-30g) were used in the experiments. Animals were kept in environmentally controlled conditions: at an ambient room temperature of 21 ± 1 °C, a relative humidity of 55 ± 5% and a 12h light – 12h dark cycle with lights on at 07:00 h. Animals were group-housed (rats 2-4 per cage, mice 12 per cage) with free access to standard laboratory chow and water.

2.2. Drugs

All solutions were prepared in saline (0.9% NaCl in distilled water) unless otherwise noted. Solutions made from drugs in hydrochloride salts were buffered with NaOH to pH 7.2 - 7.5. Strychnine, formalin, MIA, glutamate, sodium diclofenac and ACBC were purchased from Sigma Chemical Company (St. Louis MO, USA). Morphine hydrochloride was purchased from MacFarlan Smith Ltd (Edinburgh, Great Britain). RS-isovaline hydrochloride and base were synthesized by Biofine International (Vancouver, BC Canada). RS-isovaline monohydrate was purchased from ACROS Organics (Geel, Belgium). Isoflurane was obtained from Baxter Corporation (Mississauga, ON Canada).

For the formalin-induced paw licking experiments, 20% formalin stock solution was diluted with distilled water to 5%. Formalin was injected intraplantarly at a volume of 20
μl in rats and 5 μl in mice. For MIA-induced osteoarthritis experiments, MIA was prepared for rats at 80 mg/ml, 25 μl was injected intraarticularly into the right hind knee. 5% MIA (7.5 μl per knee) was administered to mice to induce osteoarthritis. For glutamate paw licking and allodynia experiments, 49 mM to 3 M glutamate solution was prepared with 0.3 mM KCl dissolved in saline and titrated to pH 8.2 using NaOH.

2.3. Equipment

2.3.1. Anesthesia

Surgeries for the Maximum Tolerated Dose (MTD) study and induction of osteoarthritis using MIA were conducted under isoflurane anesthesia. An Ohio vaporizer (Madison WI) with an oxygen flow rate of 2 l/ min was connected to a Mapleson E circuit (Miller, 2005). A T-piece connected the nose mask to the fresh gas limb and the low-resistance expiratory limb to prevent rebreathing. Animals were first placed in an induction chamber, and exposed to 5% isoflurane. Then 1.75 – 2.5% isoflurane was delivered through the nose mask to maintain anesthesia. Animals were euthanized with an overdose of isoflurane promptly followed by cervical dislocation.

2.3.2. Hamilton Syringe Injections

Small volume precise injections were done using 25 and 50 μl Hamilton microliter gas tight syringes with Luer lock fittings (Hamilton Co., Reno, NV). The syringes were attached to disposable 30 G 1” needles (BD, Franklin Lakes, NJ).
2.3.3. Mouse Restrainers for Assaying Effects of Subcutaneous Injections and Tail Immersion

Mouse restrainers were custom-built using 60 ml plastic disposable syringes (Figure 5). With the plunger removed, the syringe was cut at the 45 ml mark, leaving a housing connected to a Luer-lock opening. Small ventilation holes were drilled into the housing around the Luer-lock along with an opening for subcutaneous injection at the top of the housing. The rubber stopper portion of the plunger was subsequently pulled out. A hole-puncher was used to punch a hole through the center of the rubber piece. The restrainer was then covered to encourage the mouse to crawl inside. The animal’s tail was subsequently slotted through the rubber piece and pushed into the syringe housing. This enclosed the animal while exposing the tail for the tail immersion assay.

Figure 5. A schematic of the custom mouse restrainer.
2.3.4. Observation Chambers

Transparent plastic cages were used as observation chambers. A 30 cm by 15 cm by 15 cm cage was used for mice. The chamber was divided into two halves using a black internal wall to allow for observation of two animals simultaneously. A 45 cm by 30 cm by 20 cm cage was used for observation of rats.

2.3.5. Video and Data Capture Setup

With the exception of allodynia testing, all experiments were captured using an overhead video camera (Canon, Mississauga, ON). For allodynia studies, the video camera was positioned beneath a raised platform to better capture paw movements. The video camera was connected to an analogue to digital video capture system (Q-See, Anaheim, CA).

Blood pressure and ECG recordings in the MTD study were captured by a data acquisition system, PowerLab 8/30 (AD Research, Colorado Spring, CO) and visualized using Chart 5 (AD Research, Colorado Spring, CO). All video and data were recorded and stored on a computer hard drive.

2.3.6. Incapacitance Tester for the Rat Osteoarthritis Model

Two pocket-size digital balances (My Weigh, Vancouver, BC) were positioned side-by-side. The rat was placed in a plexiglass holder set at a 45° incline. The bottom is open to allow the animal to stand with its hind paws resting, one on each balance. A photograph of the device is shown in Figure 6. The difference in weight measured by the pads was recorded using the video camera system.
2.3.7. **Allodynia Testing for the Rat Osteoarthritis Model**

A rat holder was made by cutting off the bottom a 3” plexiglass tube to leave a tube with a semi-circular opening on both ends. A wire mesh was attached to the bottom of the tube and slots were cut close to both ends of the tube such that two pieces of plexiglass dividers could be dropped through the slots to confine the animal within the holder.

The rat was then placed into this holder with a mesh bottom. This allowed for the access of the plantar surface of its paws to test for allodynia. Paw withdrawal thresholds were determined using a Touch Test Sensory Evaluator Kit (Stoelting Co, Wood Dale, IL).
2.3.8. Rotarod

A rotating drum (4 cm in diameter) was attached to a motor set at 20 rpm. The drum is partitioned with opaque plexiglass to create 4 chambers such that 4 mice can be tested at the same time.

2.3.9. Hot Plate

A 38 cm by 31 cm by 18 cm chamber was built with a heated metal bottom. A hot water bath circulated water to the chamber to maintain the hot plate at 52 ± 1 °C.

2.4. Methods

2.4.1. Randomization & Blinding

Unless otherwise indicated as a pilot study, the experiments were conducted in a randomized and double-blinded fashion in groups of 4 – 7 animals per line. Treatment groups were randomized using a randomization program (http://www.randomizer.org). The randomization code was only broken after completion of the line of experiment.

2.4.2. Isovaline Maximum Tolerated Dose (MTD) in Rats

We determined the Maximum Tolerated Dose (MTD) of isovaline in rats (n = 6) using the up-and-down (staircase) method (Dixon, 1970). The first rat was administered isovaline (s.c. injection, dissolved in saline, injection volume < 1 ml) at the 500 mg/kg (estimated ED<sub>50</sub> based on mouse data). Its heart rate, blood pressure, ECG, respiratory rate and gross behavior were monitored for up to two hours upon injection. A change of 40% from baseline of any measured parameters was considered as a positive sign of toxicity. Based on the presence or absence of toxicity, the dose in the next animal was
increased or decreased in steps of 500 mg/kg according to the up and down method. MTD testing stopped when an N of 5 was reached or if no toxicity was observed at the upper limit.

The rat was first anesthetized with isoflurane for surgery. The left carotid artery was isolated and a saline filled-PE50 indwelling catheter inserted into the artery and tied down with thread. The end of the catheter was then tunneled beneath the skin to emerge at the nape of the neck. For ECG recording, two 75 micron Teflon-coated steel wires were placed beneath the skin: one at the right forelimb and the second at the left rear limb. These wires were also tunneled under the skin to emerge at the neck. The animal was allowed to recover from anesthesia. The leads were then attached to a data acquisition system PowerLab 8/30 (AD Research, Colorado Spring, CO) and visualized with Chart 5 (AD Research, Colorado Spring, CO). An overhead video camera system was also used to record behavioral responses.

2.4.3. Dose Finding Studies for Isovaline & ACBC

The ED$_{50}$s of isovaline (in rats) and ACBC (in mice) were determined in the formalin-induced paw licking assay using Dixon’s up-and-down method (Dixon, 1970). Refer to Figure 7 for a time course of the studies. Rats (n = 7) and mice (n = 10) were given subcutaneous injections of isovaline (injection volume of 0.3 ml) and ACBC (injection volume of 0.1 ml) respectively. The first rat was given an initial dose at 500 mg/kg of isovaline, while the first mouse received 94 mg/kg of ACBC. The animal was then placed into the observation chamber and videotaped. Five minutes after drug administration, the animal was restrained and we injected 5% formalin (20 µL in rats, 5 µL in mice)
subcutaneously into the plantar surface of the right hind paw. The animal was returned to the observation chamber. The amount of time the animal spent licking the injected paw was timed using a stopwatch and recorded in 5 minute periods for a total of 40 minutes. The licking time was compared to that of the control saline treated animal. The dose of the next animal was stepped up (doubled) or stepped down (halved) depending on whether the licking time in the phase II response decreased from the control by more than 60% as according to the up-and-down method.

Figure 7. Time course for the dose finding studies.
2.5. Osteoarthritis Model in Rats

2.5.1. Induction of Osteoarthritis

Osteoarthritis was chemically induced in rats by intra-articular injection of monosodium iodoacetate (MIA) using a Hamilton syringe. The rat was first anesthetized with isoflurane, and then placed in a supine position. The animal’s right hind knee was elevated and immobilized with the left hand and the right index finger was used to palpate the space beneath the patella. Upon location of the space, the needle was inserted through the infrapatella ligament into the right knee joint. The needle was then gently pushed from side to side, with the requirement that the knee should freely turn from left to right with movement of the needle. Upon confirmation of the location, 2 mg of MIA in 25 µl of saline solution was administered into the joint. In the pilot experiment (n = 6), the left hind knee was also injected with saline control. Upon injection, animals were allowed to recover from anesthesia and returned to their cages.

2.5.2. Behavioural Assays of Osteoarthritis

The progression of osteoarthritis in rats was monitored using 3 different assays: 1) measurement of knee joint diameters, 2) measurement of body weight distribution using the incapacitance tester and 3) measurement of allodynia using von Frey hairs. In the pilot, measurements were taken prior to induction of osteoarthritis (Day -1) and after induction on Day 1, 3, 7, 14, 21 and 28. In the experiment, pharmacological testing occurred on the acute stage (Day 2, 3, 4) and the chronic stage (Day 28, 29, 30).
2.5.3. Knee Joint Diameter Measurements

The inflammation of the joint was monitored by comparing the difference in joint diameter between the right (arthritic) and left (non-arthritic) knees. The animal was restrained with a towel with the knee flexed at a 90° angle. We then measured knee diameter using a digital caliper.

2.5.4. Utilizing the Incapacitance Tester (Measuring Change in Body Weight Distribution)

The rat was placed into the incapacitance tester with its hind paw on the two force transducers and allowed to acclimatize for 1 minute. Then the difference in weight measured on the pads was recorded using a video camera for a 1 minute period. Readings of the two scales every second were manually recorded at a later time by video playback. The difference in body weight distribution between the left and right limb was determined using the mean of 60 data points within the minute.

2.5.5. Von Frey Hair Stimulation (Static Plantar Allodynia)

The rat was placed into the custom holder with a wire mesh bottom set on an elevated platform. The Von Frey filament was gently applied to the plantar surface of the hind paw until the filament bends (6 trials per hair). Hairs of increasing thickness were used until a paw lift or paw licking response was observed in all 6 trials. The paw withdrawal threshold (PWT) was determined as the minimum pressure required to elicit a response in all 6 trials. The PWTs of the right and left paws were compared.
2.5.6. Pharmacological Testing

Drug testing took place in both acute (Day 2, 3, 4) and chronic (Day 28, 29, 30) stages of the osteoarthritis rat model. Saline, morphine (6 mg / kg), diclofenac (30 mg / kg), isoaline (200, 500, 1000 mg / kg) and ACBC (30, 60, 120 mg / kg) were randomized in lines of 6 or 7 and administered subcutaneously (injection volume ~0.8 ml). Behavioural responses 30 min after drug administration were compared to pre-treatment values.

2.6. Osteoarthritis Model in Mice

2.6.1. Induction of Osteoarthritis

A procedure similar to that used in the OA rats was undertaken. The mouse was anesthetized and its knee flexed, upon which 7.5 µl of 5% MIA was injected into the intraarticular space using a 30 gauge needle attached to a 25 µl Hamilton syringe. The depth of the needle penetration was less than 3 mm. Both right and left hind knees received MIA injections. The animal was monitored for at least 30 minutes after recovery from anesthesia before it was returned to its cage.

2.6.2. Behavioural Assays of Osteoarthritis

Three different assays were examined in the mouse model: 1) inverted wire mesh assay, 2) paw grip on a 70° incline plane and 3) the rotarod assay. Osteoarthritic progression of the animals was monitored for 21 days after MIA injection. The effects of subcutaneous morphine (6 mg/kg), diclofenac (30 mg/kg) and ACBC (60, 120 mg/kg) on these assays were examined on Day 3 (acute phrase) and Day 14 (chronic phrase).
2.6.3. Inverted Wire Mesh Assay

Each mouse was placed on to a wire mesh, and the mesh was subsequently inverted. A stopwatch was used to measure the duration the mouse maintained its grip on the mesh while suspended upside down. Two parameters were studied: the grip duration (the duration where all four paws grip the wire mesh) and the duration on the mesh (the time it takes for the animal to fall off the mesh). An arbitrary selected 40 s cutoff was imposed.

2.6.4. Paw Grip on a 70° Incline Plane

A 30 cm by 15 cm by 15 cm plastic cage was tilted at a 70° angle from the horizon. The bottom of the cage was lined with a dense foam pad. The mouse was placed onto the center of the incline. We observed its ability to grip and climb the incline. Its performance was qualitatively evaluated using an impairment scale from 0 – 3. A score of 0 indicated no motor impairment; the animal readily climbed up the incline. A score of 1 indicated minor motor impairment; there was slight slippage on one hind paw. A score of 2 indicated moderate motor impairment; although its overall position was maintained, there was continual slippage with both hind paws. A score of 3 indicated pronounced motor impairment; the animal was unable to maintain grip and slid down the incline.

2.6.5. Rotarod Test

The animal was placed on a rotating drum and was therefore compelled to walk to remain on the rod. A stopwatch was used to record its duration on the rod, with an arbitrary cutoff time of 240 s. Naïve mice were trained twice a day on the apparatus for three days
prior to osteoarthritis induction. They were retested on Day 3 and Day 14 before and 30 min after drug administration.

2.7. *Heat-induced Models of Nociception*

We examined the efficacy and pharmacokinetics of isovaline and ACBC using conventional acute models of nociception in mice. The two models we used were the hot water tail immersion test and the hot plate test.

2.7.1. *Hot Water Tail Immersion Test*

In this assay, the mouse was placed into a custom-made restrainer with its tail exposed outside the holder. The distal 1.5 cm of its tail was dipped into a hot water bath set at 49.0 ± 0.2 °C as outlined in the time course in Figure 8. We used a stopwatch to record the latency to its tail flick response. A cutoff of 15 s was imposed to prevent thermal injury. Tail flick response was measured in duplicates per time point. Its latency time was taken as the average between the two values. The animal’s tail was wiped dry with a paper towel between measurements.

Prior to drug administration, the animal entered a pre-screening process. First its tail was pre-heated by a single dip into the water bath at time -20 min. Then tail flick response was measured at time -15 and -1 min. The mouse was included into the study if its latency time was less than 6 s.
At time 0 min, the mouse was given a subcutaneous injection of one of saline control, RS-isovaline (125, 250, 500, 1000 mg/kg) or ACBC (30, 60, 120 mg/kg). We had also conducted an additional unblinded experiment using 82 mM NaCl (with approximately the same osmolarity as high dose ACBC) as an osmolarity control. Volume of injection was ~0.15 ml. After drug administration, tail flick latency was measured at 3, 5, 10, 15, 20, 30, 45 and 60 min. Since each animal’s latency time rapidly returned to baseline, testing period of later studies was shortened to 20 min. Tail flick latencies were compared to pre-dose values.

![Diagram](image)

Figure 8. Time course for the hot water tail immersion assay.

### 2.7.2. Hot Plate Test

The mouse was released in the center of the hot plate chamber with the floor heated to 52 ± 1°C. We used a stopwatch to measure the latency to hind paw licking. Animals were pre-tested 5 minutes prior to drug treatment (Figure 9). A breathing rate measurement was also taken. The mouse was then placed onto a platform and the number of breaths (taken in 15 seconds) was recorded. At time 0 min, saline, isovaline (1000 mg / kg) or
ACBC (30, 60, 120 mg / kg) was injected subcutaneously. Latency to hot plate response was tested 10, 20, 30 and 60 min after drug administration. Breathing rate measurements were taken post 15 and 25 min. Breathing rates and hot plate latencies were compared to saline controls at individual time points.

Figure 9. Time course for the hot plate assay.

2.8. Glutamate-induced Paw Licking in Mice

The effects of ACBC on glutamate-induced paw licking were studied. The mouse was restrained in a holder and its right hind paw immobilized with a pair of fine tweezers. ACBC (3.06 mg / 20 µl) or saline (20 µl) was administered intraplantarly into the right paw using a 30 G 1” needle attached to a Hamilton syringe. Glutamate (3 M, 20 µl) was administered immediately afterwards into the same paw. We observed paw licking response in the animal over 15 min in 5 min bins. The duration of paw licking within the 15 min period between ACBC and saline groups was compared.
2.9. *Glutamate-induced Allodynia in Mice*

We first established the EC$_{50}$ of glutamate for allodynia using the randomized, blinded, double staircase up-and-down method (Cornsweet, 1962). A positive allodynic response was defined as a decrease in the PWT of the right paw by more than 50%. The mouse was placed on an elevated platform with a wire mesh bottom. We determined the paw withdrawal threshold (PWT) of its left and right hind paws using von Frey filaments in a similar fashion as in the procedure for rats. Hairs of increasing thickness were applied to the plantar surface of the paw until a paw lift or paw licking response was obtained. Animals were pre-tested and then given an intraplantar injection of glutamate (20 µl; in varying concentrations to the right hind paw) according to the double staircase up-and-down method. Animals were retested 5 min after glutamate injection. A total of 17 animals were tested with concentrations ranging from 47 mM to 3 M. The effect of intraplantar strychnine (20 µl) on PWT was similarly examined using the up-and-down method. Strychnine concentration was increased up to the seizure threshold.

A concentration-response curve for RS-isovaline was obtained using the glutamate-induced allodynia model. Animals were pre-tested 5 min prior to treatment. At time 0 min, 20 µl of saline or isovaline (0.5 mg, 1 mg or 2 mg, dissolved in 20 µl saline) was injected into the right hind paw, immediately followed by an intraplantar injection of 750 mM glutamate (20 µl) into the same paw. The mice were returned to the elevated platform and observed for 5 min. After 5 min, PWT was reassessed. The percentage change in PWT for treatment groups was compared to that of the saline control.
2.10. **Statistical Analysis**

We assigned the level of statistical significance ($\alpha$) to be 5% and analyzed the data using GraphPad Prism 5.0 (San Diego, CA). Data from the up-and-down studies were analyzed using statistics procedures as outlined by Dixon (1970). For all other behavioural data, the D’Agostino-Pearson normality test was used to confirm data were normally distributed. Comparisons of pre and post drug values restricted to two means were analyzed using the two-tailed paired Student’s T-test with the exception of the impairment scores from the 70° incline plane assay. Impairment scores were non-parametric and thus were analyzed using Wilcoxon’s matched pairs test. Repeat measures analysis of variance (RM-ANOVA) was used for the remaining data. The Bonferroni multiple comparison test was performed if the ANOVA was significant. All results were plotted and referred to as mean ± SEM unless otherwise noted.
Chapter 3. Results

3.1. Isovaline Maximum Tolerated Dose (MTD) in Rats

We determined the MTD in female Sprague-Dawley rats using the up-and-down method. Rats were administered isovaline at a dose of 519 mg/kg up to 2000 mg/kg. At all test doses, no significant changes in heart rate, blood pressure, respiratory rate or the ECG were observed (see Figure 10). No animal exhibited any obvious changes in behaviour. Isovaline at 2000 mg/kg was the highest possible dose deliverable by s.c. injection to the rat. As such, it was demonstrated that subcutaneous administration of isovaline is well tolerated in the rat up to a dose of 2000 mg/kg.
Figure 10. Sample polygraph trace of a rat administered 2000 mg/kg (s.c.) isovaline.
3.2. *Dose Finding Studies for Isovaline & ACBC*

The ED$_{50}$s of isovaline in rats and ACBC in mice were determined using the formalin-induced paw licking model by employing the up-and-down method. A typical biphasic paw licking response was obtained in both rat and mouse controls. Phase I paw licking response occurred 0 – 5 min post formalin administration, which was followed by a quiescent period from 5 – 20 min. The second phase of paw licking response was observed initially at 20 min and was sustained to the end of the observational period at 40 min. The cumulative durations of phase I licking for rats and mice were 182 s and 92 s respectively, while phase II cumulative licking durations were 293 s and 363 s respectively. These values fall within the 95% confidence interval of response times from historical controls (Sevostianova et al., 2003; Wang, 2008). Figure 11 displays the data for the dose-finding studies. The ED$_{50}$s and SDs of isovaline and ACBC for more than 60% inhibition of paw licking response in the second phase were determined to be 740 ± 20 mg/kg and 88 ± 9 mg/kg, respectively.
Figure 11. Determination of the ED$_{50}$s of isovaline and ACBC for inhibition of phase II formalin-induced paw licking response by more than 60% as compared to control, using the up-and-down method.
3.3. Osteoarthritis Model in Rats

3.3.1. Pilot Study

A group of six rats (Sprague-Dawley, female, 200 – 225 g) were given intra-articular injections of MIA (2 mg in 20 µl saline) into the right knee joint and saline control (20 µl) into the left knee. Overall, MIA injections were well tolerated. Animals displayed both normal feeding behaviour and rate of weight gain. Elevated right hind paws and hopping gaits were observed in 3 out of 6 animals (the rest placed less weight on their right hind paw while walking). Significant swelling of the right knee was observed for the first seven days after MIA administration (Figure 12) (measured as the diameter difference between Day 0 and Day 3 -3.0 ± 0.6 mm, P < 0.001; difference between Day 0 and Day 7 -2.4 ± 0.2 mm, P < 0.001; n = 6). Knee swelling subsided by Day 14 (difference between Day 0 and Day 14 -0.2 ± 0.2 mm, P > 0.05; n =6). Significant changes in body weight distribution were observed (Figure 13). Non-arthritic animals distributed body weight equally between left and right hind paws, while MIA treated animals placed less weight on the right limb (difference in weight shift MIA Day 3 group 45 ± 14 g vs. non-arthritic group 1 ± 0.75 g; P < 0.05; n = 6).
Figure 12. Changes in knee diameter upon intra-articular administration of MIA (2 mg in 20 µl of saline) to the right knee in the pilot study. Repeat Measures One-Way ANOVA with Bonferroni’s multiple comparison post-test, *** P < 0.001; n = 6 per time point.

Figure 13. Changes in body weight distribution upon intra-articular administration of MIA (2 mg in 20 µl of saline) to the right knee as measured by the capacitance tester in the pilot study. Repeat Measures One-Way ANOVA with Bonferroni’s multiple comparison post-test, * P < 0.05; n = 6 per time point.
3.3.2. Randomized Double-blinded Study

Rats (Sprague-Dawley, female, 200 – 225 g) were randomly assigned to one of control, isovaline or ACBC treatment groups with 6 to 7 animals per line for 4 lines (n = 9 per group). The right knee joints were injected with MIA (2 mg) and behavioural testing of the animals occurred in acute (Day 2, 3, 4) and chronic (Day 28, 29, 30) stages of this model. The dose of the subsequent day was increased to obtain a cumulative dose-response relationship.
3.3.3. Changes in Knee Joint Diameter

Changes in knee joint diameter after MIA administration in the randomized blinded study were comparable to pilot data. The animals’ right knees were significantly swollen in the acute stage (Figure 14) (pre-MIA -0.02 ± 0.07 mm vs acute stage (Day 2, 3 and 4) 2.4 ± 0.2, 1.8 ± 0.2 and 1.3 ± 0.3 mm respectively; n ≥ 9 per time point; P < 0.001 for each comparison to control). No difference in knee joint diameter was observed between saline, isovaline or ACBC- treated animals in the acute stage. Inflammation of the right knee was not apparent in the chronic stage (Day 28 0.2 ± 0.3 mm; Day 29 -0.1 ± 0.2 mm; Day 30 0.0 ± 0.2 mm).

![Knee diameter of MIA treated rats](image)

Figure 14. Changes in knee diameter upon intra-articular administration of MIA (2 mg in 20 μl of saline) in the randomized blinded study. Repeat Measures One-Way ANOVA with Bonferroni’s multiple comparison post-test, *** P < 0.001; n ≥ 9 per time point.
3.3.4. Incapacitance Testing (Change in Body Weight Distribution)

The shift in body weight distribution prior to drug treatment in the randomized blinded study was similar to that in the pilot (Figure 15). The arthritic rats in the acute stage (Day 2, 3, 4) placed significantly less weight on their right hind paw compared to non-arthritic animals (acute stage Day 2, 3 and 4 $40 \pm 6$, $41 \pm 10$ and $38 \pm 8$ g respectively vs pre-MIA $-6 \pm 2$ g; $P < 0.001$ for every comparison; n > 10). In the chronic stage, there were significant changes in body weight distribution before drug treatment on Day 29 and 30 ($32 \pm 10$ and $26 \pm 10$ g respectively vs pre-MIA $-6 \pm 6$ g; $P < 0.01$ and 0.05; n = 9 per group) but not on Day 28 ($3.6 \pm 12$ g; $P > 0.05$; n = 9 per group).

![Body weight distribution for MIA-treated rats](image)

Figure 15. Changes in body weight distribution of rats in (A) acute and (B) chronic stages of the MIA-induced osteoarthritic model as measured by the incapacitance tester in the randomized blinded study. MIA (2 mg in 20 μl of saline) was administered into the right knee joint at Day 0. Weights were taken prior to drug treatment. Repeat Measures One-Way ANOVA with Bonferroni’s multiple comparison post-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n = 13 per time point (acute stage) and 9 per time point (chronic stage).
3.3.5. Effects of Drugs on Body Weight Distribution

Unblinded saline administration (s.c. ~0.8 ml) had no significant effect on the capacitance test results (Figure 16) \((P >> 0.05; n = 6 \text{ per time point})\). However there was large variability in responses \((\text{SEM} > 50\%)\).

![Figure 16](image_url)

Figure 16. Effects of saline (~0.8 ml s.c.) on body weight distribution of rats in the MIA-induced osteoarthritis model. Post-dose values were taken 30 minutes after saline administration. \(n = 6\) per time point.

Large interanimal variability was also observed upon administration of isoaline, ACBC, morphine and diclofenac in the randomized and blinded study (Figure 17). The results were unblinded after two lines. Overall, no group was significantly different than the saline control \((P >> 0.05)\).
Figure 17. Effects of isovaline (200 – 1000 mg/kg s.c.) and ACBC (30 – 120 mg/kg s.c.) on the incapacitance tester compared to saline, morphine (6 mg/kg s.c.) and sodium diclofenac (30 mg/kg s.c.) control. Individual data points are plotted along with means ± SDs.
3.3.6. Von Frey Hair Stimulation (Static Plantar Allodynia)

Allodynia testing was only conducted in the randomized, blinded study. Animals became significantly more sensitive to stimulation on the plantar surface of their right hind paw in both acute and chronic stages of the MIA model as compared to pre-MIA treatment (P < 0.001, refer to Figure 18 for exact values). Animals typically displayed a paw lift response when touched with the von Frey filament as oppose to a flinch or licking response.

![Static alldonyia for MIA-treated rats](image)

Figure 18. Changes in hind paw withdrawal threshold (PWT) of rats in (A) acute and (B) chronic stages of the MIA-induced osteoarthritic model as measured by the von Frey hair stimulation in the randomized blinded study. Measurements were taken prior to drug treatment. Repeat Measures One-Way ANOVA with Bonferroni’s multiple comparison post-test, *** P < 0.001; n = 27 per time point (acute stage) and 20 per time point (chronic stage).

Morphine (6 mg/kg s.c.) and sodium diclofenac (30 mg/kg s.c.) reversed alldynic responses in the acute stage (Days 2, 3 and 4) of the osteoarthritis model (morphine 56 ± 12%, diclofenac 33 ± 11%, saline -13 ± 16%; P < 0.01 and P < 0.05 respectively; n > 12 per group), while isoalvaine (200 – 1000 mg/kg s.c.) and ACBC (30 – 120 mg/kg s.c.) were ineffective (Figure 19). In the
chronic stage (Days 28, 29, 30), neither morphine (6 mg/kg s.c.) nor diclofenac (30 mg/kg s.c.) showed a significant effect in reversing alldynic responses in an unblinded study (morphine 48 ± 18%, diclofenac 18 ± 30% and saline 27 ± 10%, n ≥ 6 per group). In addition, 3 out of 8 chronic animals could not be tested due to lack of alldynia or adverse reactions to drugs. One third of the diclofenac group died possibly due to internal bleeding. We decided not to continue testing with isovaline and ACBC in this model because of large interanimal variance along with the high attrition rate. We also observed sedation in the morphine group and vigorous scratching at the site of injection (indicative of local irritation) upon diclofenac administration. Sedation and local irritation were not observed in saline, isovaline and ACBC treated animals.

Figure 19. Effects of saline, morphine (6 mg/kg s.c.), diclofenac (30 mg/kg s.c.), isovaline (200 – 1000 mg/kg s.c.) and ACBC (30 – 120 mg/kg s.c.) on static alldynic responses in the (A) acute (Days 2, 3, 4) and (B) chronic (Days 28, 29, 30) stages of the MIA-induced osteoarthritis model. Percent reversal of paw withdrawal threshold (PWT) is defined as the percent difference between pre-dose and 30 min post-drug administration. One-Way ANOVA with Bonferroni’s multiple comparison post-test, * P < 0.05, ** P < 0.01; n > 12 (acute) and 6 (chronic) per group.
3.4. Osteoarthritis Model in Mice

One in five mice developed acute toxicity 10 min after recovery from MIA (5%, 7.5 µl in both hind knees) administration. Animals developed respiratory depression and responded sluggishly to touch. These effects were reversed after 60-120 minutes. One in fifteen animals displayed seizure-like activities (abrupt jumping and running within cage) the day after osteoarthritis induction.

3.4.1. Inverted Wire Mesh Assay

The time a mouse can maintain its grip and stay on an inverted wire mesh was recorded. Non-arthritic animals typically maintained their grip and stayed on the mesh for greater than 40 s. Substantial grip impairment was seen in MIA-treated animals from Day 1 and persisted for over 21 days (Figure 20).

Figure 20. Effects of intra-articular administration of MIA (5% in 7.5 µl saline, both hind knees) in mice over time. Paw grip duration was defined as the period that all four paws grip the mesh. An arbitrary 40 s cutoff was imposed. (n ≥ 10 per time point).
All results for grip duration and duration the animal remained on the wire mesh were similar and will be described together as grip strength. Morphine (6 mg/kg s.c.) was effective in increasing grip strength in the chronic but not acute stage (Figure 21). Morphine had no effect on non-arthritic animals. Diclofenac (30 mg/kg s.c.) had no effect on grip strength in either acute and chronic stage (Figure 22). A small pilot experiment with ACBC (60 and 120 mg/kg s.c., n = 5 per group) did not reverse grip impairment in the acute or chronic stage (Figure 23). Sedation was not observed in morphine treated animals; however mice displayed Straub tails 60 min after drug administration.
A) Effects of morphine (6 mg/kg s.c.)
acute stage

Wire mesh grip duration

Duration on wire mesh before falling

B) Effects of morphine (6 mg/kg s.c.)
chronic stage

Wire mesh grip duration

Duration on wire mesh before falling

Figure 21. Effects of morphine (6 mg/kg s.c.) on the (A) acute (Day 3) and (B) chronic (Day 14) stages of osteoarthritis in the inverted wire mesh assay. Paired T-test comparisons between pre-dose and post-dose for arthritic and non-arthritic animals. * P < 0.05; n = 10 per group.
Figure 22. Effects of diclofenac (30 mg/kg s.c.) on the (A) acute (Day 3) and (B) chronic (Day 14) stages of osteoarthritis in the inverted wire mesh assay. n = 6 (acute) and 8 (chronic) per group.
Figure 23. Effects of ACBC (60, 120 mg/kg s.c.) on the (A) acute (Day 3) and (B) chronic (Day 14) stages of osteoarthritis in the inverted wire mesh assay. n = 5 per group.
3.4.2. Paw Grip on a 70° Incline Plane

Non-arthritic animals were all able to climb the incline plane with ease. MIA administration substantially impaired the animal’s grip and climbing abilities in the acute phrase (Day 1 – 7). This impairment spontaneously decreased after Day 10. A near complete reversal was seen on Day 21 (Figure 24). Morphine (6 mg/kg s.c.), diclofenac (30 mg/kg s.c.) and ACBC (120 mg/kg) had no effect in reversing grip impairment in the acute phrase of this assay (Figure 25).

![Impairment of grip on 70° incline plane](image)

Figure 24. Effects of intra-articular administration of MIA (5% in 7.5 μl saline, both hind knees) in mice over time. A qualitative grip impairment scale was used from 0 (no impairment) to 3 (severe impairment). n ≥ 10 per time point.
Effects of drugs on the 70° incline plane assay (acute stage)

Figure 25. Effects of morphine (6 mg/kg s.c.), diclofenac (30 mg/kg s.c.) and ACBC (120 mg/kg s.c.) on the ability of mice to perform the 70° incline plane test in the acute (Day 3) stages of the MIA-induced osteoarthritis model. n = 9 (morphine, diclofenac) and 6 (ACBC) per group.
3.4.3. Rotarod test

Osteoarthritis produced by MIA (5%) administration in mice did not significantly alter the animal’s ability to stay on the rotarod. Morphine (6 mg/kg) and diclofenac (30 mg/kg) did not alter rotarod times in the acute or chronic stages of the model (Figure 26 and Figure 27).

![Effect of diclofenac (30 mg/kg s.c.) on rotarod performance (Acute stage)](image)

Figure 26. Effects of diclofenac (30 mg/kg s.c.) on rotarod performance in the acute (Day 3) stage of the MIA-induced osteoarthritis model. n = 6 per group.
Effects of morphine (6 mg/kg s.c.) on rotarod performance

Figure 27. Effects of morphine (6 mg/kg s.c.) on rotarod performance in the (A) acute (Day 3) and (B) chronic (Day 14) stages of the MIA-induced osteoarthritis model. n = 9 (acute) and 8 (chronic) mice per group.

3.5. Heat-induced Models of Nociception

3.5.1. Hot Water Tail Immersion Test

All mice were placed into darkened plastic restrainers and their tails were preheated and pre-tested in a 49 ± 0.2 °C water bath at t = -20, -15, and -1 min. Pre-test values were determined as the mean of two replicates at t = -1 min. One in twenty mice failed to meet the pre-screening latency time of less than 6 s and was removed from the study. The saline vehicle control had no effect on tail flick latency (Figure 28). Latency times were stable over 60 min of testing.
Figure 28. Saline vehicle control (~0.8 ml s.c.) had no effects on mice tail flick response. n = 9.

Isovaline increased tail flick latency time 5 min post injection at 250 mg/kg (P < 0.05) and 500 mg/kg (P < 0.01) doses but not at 125 and 1000 mg/kg (Figure 29). Similarly, ACBC increased 5 min post injection latency time at 30 mg/kg (P < 0.05) and 60 mg/kg (P < 0.05) but not at the high dose of 120 mg/kg (Figure 30). The osmolarity control (82 mM NaCl) significantly decreased the tail flick latency 15 min post-injection (P < 0.05) (Panel D in Figure 30).
Figure 29. Effects of isovaline (125, 250, 500, 1000 mg/kg s.c.) on the hot water tail immersion test. Repeat Measures One-Way ANOVA with Bonferroni’s multiple comparison post-test, ** P < 0.01, * P < 0.05; n = 6 per group.
3.5.2. Hot Plate Test

ACBC administered subcutaneously at 60 mg/kg significantly increased the paw licking latency of mice at 20 and 30 min (Figure 31) (P < 0.001 and <0.05 respectively). ACBC was ineffective at the low dose (30 mg/kg) and high dose (120 mg/kg). No changes to breathing rate were seen.
in either 15 or 25 min time points for any dose of ACBC. The high dose (120 mg/kg) of ACBC was compared to saline control group in panel D of Figure 31. Isovaline (1000 mg/kg s.c.) had no significant effect on the hot plate test (Figure 32). We did not observe sedation or local irritation in ACBC or isovaline-treated animals under any dose.

Figure 31. Effects of ACBC (30, 60 and 120 mg/kg s.c.) on the hot plate test and spontaneous breathing rate. Two-Way ANOVA with Bonferroni’s multiple comparison post-test, * P < 0.05, *** P < 0.001; n = 6 (30 mg/kg) and 8 (60, 120 mg/kg) animals per group.
3.6. *Glutamate-induced Paw Licking in Mice*

Intraplantar glutamate (20 µl of a 3 M solution) administered into the right hind paw of the mouse elicited a vigorous paw licking response which lasted for 15 min (Figure 33). Concurrent administration of ACBC (3.1 mg in 20 µl; maximum soluble dose) attenuated this licking response. The effect of ACBC was most apparent in the 5 to 15 min period. A single outlier was identified and removed from the ACBC group; its value was greater than three standard deviations away from the mean.
Figure 33. Effects of ACBC (3.1 mg in 20 μl) on the glutamate-induced paw licking response in mice. Glutamate (20 μl, 3 M) was administered concurrently with saline or ACBC into the right hind paw. The duration of licking was measured in 5 min bins for 15 min. Effects of saline and ACBC for the entire 15 min period were compared using the unpaired Student’s T-test. ** P < 0.01; n = 6 (saline) and 5 (ACBC). A single outlier was removed from the ACBC group.

### 3.7. Glutamate-induced Allodynia in Mice

The EC$_{50}$ for glutamate-induced allodynia was determined using the randomized, blinded, double staircase up-and-down method. The EC$_{50}$ and SD required to elicit an allodynic response (as defined by a change in PWT by more than 50%) was 350 ± 50 mM (Figure 34; Dixon up-and-down statistics). From the up-and-down test, we selected the glutamate concentration for testing to be 750 mM. Isovaline (0.5, 1.0, 2.0 mg in 20 μl saline) did not significantly reverse allodynia in this model as compared to saline control (Figure 35). Administration of strychnine (0.2 – 4 mM) did not produce allodynia or paw licking behaviour. Higher strychnine concentrations elicited tonic convulsion in mice.
Glutamate allodynia response using the up-and-down method

Figure 34. Determination of the EC$_{50}$ of intraplantar glutamate (20 μl) necessary to produce allodynia in mice using the randomized, blinded, double staircase up-and-down method. Allodynia was defined as a change in the paw withdrawal threshold (PWT) by more than 50%. A “yes” response was allodynic while a “no” response was not.
Effects of isovaline on glutamate-induced allodynia

Figure 35. Isovaline (intraplantar administration in 20 µl saline) had no effects on the glutamate-induced allodynia model in mice. Glutamate (750 mM, 20 µl) was co-administered with isovaline. Paw withdrawal threshold (PWT) was measured based on response to von Frey filaments on the plantar surface of the insulted paw. n = 6 animals per group.
Chapter 4. Discussion

Previous research in our lab has identified anti-allodynic and analgesic effects of isovaline. This led to our current investigation on the effects of isovaline and its cyclic analogue, ACBC. Here, we characterized their analgesic effects on different acute and chronic nociceptive models and allude to their mechanisms of action. The primary objective was to establish their efficacy in the rat MIA-induced osteoarthritis model of chronic pain. However, animals varied significantly in their responses to drug treatments. Overall, large intra-group variability prevented effective drug comparisons. Therefore we redirected attention to the development of a mouse osteoarthritis model. Both amino acids demonstrated antinociceptive properties without observable CNS and respiratory toxicity, further supporting our hypothesis that pharmacologic enhancement of inhibitory neurotransmission has therapeutic value in pain management.

4.1. Isovaline Maximum Tolerated Dose (MTD) in Rats

The MTD study represents our first examination of the side effects and toxicity of isovaline in rats. We selected our initial testing dose based on mouse data. Mice could be safely treated with 1000 mg/kg isovaline (s.c. or i.v.) (unpublished results). Based on body surface area to body weight ratio, the allometric scaling between rat and mouse is 1 to 2 (Fletcher, 2002). An equivalent dose in rats would be 500 mg/kg. This was our starting dose in the up-and-down method for the MTD study.

Subcutaneous injection of 500 - 2000 mg/kg isovaline had no major effects on cardiovascular and respiratory parameters (heart rate, blood pressure, breathing rate, ECG) during our 1 h observational period. We also did not observe any signs of sedation in the animals; they readily
displayed startle behaviour in response to sudden noise. There were only transient increases in heart rate, blood pressure and breathing rate immediately following drug administration, presumably due to sympathetic activation from the restraint and injection procedures. Since 2000 mg/kg (1 ml injection volume) was the highest administrable dose deliverable by subcutaneous administration, isovaline was well-tolerated in rats up to this maximum soluble dose. These results parallel our previous findings in mice (Wang, 2008) that systemic administration of isovaline did not interfere with rotarod performance, a classical assay for CNS toxicity. Overall, isovaline and ACBC appear to be less toxic than glycine and GABA which cause respiratory depression when administered centrally (Ballanyi et al., 1999; Holtman et al., 1982).

4.2. Dose Finding Studies for Isovaline & ACBC

We determined the ED$_{50}$s of isovaline and ACBC in rat and mouse respectively using Dixon’s up-and-down method. The formalin-induced paw licking model was chosen due to our familiarity with this model. Previously, effects of a single high intravenous dose of isovaline on the formalin-induced paw licking model have been studied in mice (Wang, 2008). We expanded upon this work, exploring the dose-response relationships of isovaline and ACBC in formalin-induced licking response. The up-and-down method was chosen to reduce the number of animals required for these studies.

We observed a dose-response relationship for the inhibition of phase II paw licking response. Although isovaline and ACBC were administered to rat and mouse respectively, ACBC appears to be 10 fold more potent than isovaline. Since the equivalent dose in rats is typically half of that in mice, the actual difference in potency is likely to be more dramatic.
Even though phase II response was the objective of this study, we also measured phase I paw licking responses. Both ACBC and isovaline appeared to have modest effects in reducing phase I responses (ACBC 59 ± 8 s vs control, 92.5 s; isovaline, 70 ± 9 s vs control, 137 s). However there were no clear dose-response relationships. Additional experiments in our lab with intravenous isovaline in this model also demonstrated a non-sigmoidal dose-response relationship in the phase I response (manuscript in preparation). Low doses (50 and 250 mg/kg) of isovaline decreased phase I paw licking but a high dose (500 mg/kg) was ineffective.

The second phase of the formalin-induced paw licking model is also sometimes referred to as the “chronic” phase since drug efficacy on reducing phase II response is strongly correlated with clinical drug efficacy in the management of chronic pain (Negus et al., 2006; Sevostianova et al., 2003). In particular, a phase II response has been implicated in inflammatory processes with involvement of histamine, serotonin, prostaglandin and bradykinin (Shibata et al., 1989). NSAIDs and opioids readily inhibit phase II paw licking behaviour (Alreja et al., 1984; Dubuisson and Dennis, 1977 Dec; Hunskaar and Hole, 1987; Sevostianova et al., 2003). However inflammation alone is insufficient in eliciting pain responses since other inflammatory stimuli (such as yeast and carrageenan), which induce even greater degree of inflammation, produced little nocifensive behaviour in rats (Brown et al., 1968; Wheeler-Aceto et al., 1990 Feb). This suggests that the mechanism of phase II nociceptive behaviour is more complex than just a peripheral inflammatory response. Selective blockade of phase II paw licking has also been demonstrated with NMDA receptor antagonists (MK-801, ACEA-1011) and an NK1 receptor antagonist (CP-96345) (Vaccarino et al., 1993; Yamamoto and Yaksh, 1991). Therefore, it is difficult to attribute the effects of isovaline and ACBC to a particular mechanism of action in this
model. Nonetheless, antinociceptive activities of isovaline and ACBC on phase II response strongly suggest these agents will provide analgesia in chronic pain.

### 4.3. Dixon’s Up-and-Down Method

The up-and-down method, also known as the staircase-method, was proposed by Dixon and Mood (1970) as an alternative approach for examining quantal dose-response as opposed to traditional fixed-sample-size method (Dixon, 1970). The up-and-down method provides the means to estimate $ED_{50}$ values for a given yes-no response using a significantly lower number of animals than traditional dose-response approaches without sacrificing accuracy. $ED_{50}$ estimates between the two methods show a correlation of 0.99 (Lichtman, 1998). For this reason, the Animal Care Committee recommends the use of the up-and-down method in experiments whenever possible.

To use the up-and-down method, we must first establish a cutoff point for our response. Individual animals are scored with or without response, depending on whether they meet the predefined cutoff value. For example, in a lethality study, a “yes”-response is obtained if the animal dies from a given dose, and “no”- response is obtained if the animal survives. In our MTD study, we defined toxicity to be a change in any of the measured baseline parameters (heart rate, blood pressure, breathing rate) by 40% from control values. The second criteria required for the method is the slope of the dose-response curve. The slope is used to determine the step size (the size of dose increment) in the method. For a traditional dose-response with an approximate 1:1 drug-to-receptor binding (Hill slope of 1), the dose increment ratio is $\log (2)$ (Dixon, 1970).
Next, we selected a starting dose that estimated to be close to the ED$_{50}$ value. Figure 36 illustrates the up-and-down protocol. The first trial tests a single animal at our starting dose and we observe it for a quantal response. If no response was observed (denoted as “No” or ○), the dose for the second trial was increased by our step size. If a response was observed (denoted as “Yes” or ●), the dose for the next trial was decreased by our step size. A single animal was tested in the second trial and based on its response, the dose was stepped either up or down. The process continued until an acceptable nominal sample size was reached.

Figure 36. A visual description of Dixon’s up-and-down protocol. T1 – T5 indicates trial 1 to 5. (+) in red denotes a “yes” response and (-) in blue denotes a “no” response. The pink arrows show the progression of the protocol.
The nominal sample size (N) of the up-and-down sequence was the number of trials following the first reversal of response. A reversal was a change from a “yes” to a “no” response or vice versa. An N of 5 or 6 is generally recommended. Statistical power is best gained by running several up-and-down sequences. The ED$_{50}$ value can then be calculated as outlined by Dixon’s statistics (Dixon, 1970).

4.3.1. Randomized, Blinded 2-Staircase Up-and-Down Method

With the normal up-and-down method, dose blinding is not possible since the experimenter is aware of the response of the preceding animal and thus can guess the relative dose of the current animal. This issue was resolved by running two sequences simultaneously. An ascending sequence (initial dose below estimated ED$_{50}$) was used for one staircase while a descending sequence (initial dose above estimated ED$_{50}$) was used for the second. By randomizing the order of presentation of the two sequences, the experimenter was effectively blinded to the trend of the responses (Cornsweet, 1962).

4.4. Osteoarthritis Model in Rats

Despite the fact that the MIA-induced osteoarthritis model has been presented in the literature as an effective and reliable method for investigation of novel analgesics (Combe et al., 2004; Fernihough et al., 2004; Kobayashi et al., 2003; Pomonis et al., 2005), we were unable to replicate literature reportes of consistency in measurements of animal behaviour. We observed the distinct acute and chronic phases of this model which should allow for the assessment of inflammatory and non-inflammatory chronic pain. NSAID (diclofenac) was reported to be effective only in the acute phase of the model while opioids (morphine) reversed nociceptive behaviour in both acute and chronic phases (Fernihough et al., 2004). In this study, the animal’s
responsiveness to drug treatment was too variable for meaningful group comparisons. Increased variability in drug responses could have been due to the use of a repeat dosing regimen over a three-day period, as opposed to the typical single dose testing protocol. High doses were also used which could have promoted the development of drug tolerance.

4.4.1. Knee Joint Diameter Measurements

Inflammation of the right knee was consistently observed in all MIA-administered animals in both the pilot and main study. This inflammation lasted for 7 days and was most prominent on Day 2. The observation was consistent with findings in the literature (Fernihough et al., 2004; Pomonis et al., 2005; van der Kraan et al., 1989). Injection of similar doses of MIA was reported to cause patella displacement and rough-edge formation on the tibia and femur, an indication of bone lysis and inflammation of the knee (Pomonis et al., 2005). The inflammation was accompanied by a significant decrease in bone mineral content and density, which progressively worsened over the course of 21 days (Fernihough et al., 2004). The degree of inflammation of the joint did not seem to be correlated to the severity of later nociceptive behaviours (incapacitance measures or paw withdrawal thresholds to von Frey hair stimulation) in the chronic phase. This observation is similar to clinical osteoarthritis in which radiographically defined histopathology of the knee correlates poorly to joint pain (Scott, 2006). The inability of diclofenac to decrease knee swelling was also noted by Fernihough and colleagues (Fernihough et al., 2004).

4.4.2. Incapacitance Tester (Change in Body Weight Distribution)

Animals displayed a significant shift in their body weight distribution upon MIA induction of osteoarthritis. This shift was the most prominent in the acute phase of the model (≈40 g from pre-
treatment values). From the pilot, body weight distribution measurements at Day 28 (~30 g) were greater than those at Day 14 (~ 13 g). Therefore chronic testing in the main study was conducted on Days 28, 29 and 30. The large variance between measurements before and after saline injection was unexpected. This later translated to insignificant results for drug treatment groups. Animals were not equally sensitive to treatments. We made attempts to reduce variance by modifying the testing protocol but they were largely unsuccessful. Modification to hour of testing, lighting conditions, animal training and acclimatization had little effect in reducing error.

4.4.3. Von Frey Hair Stimulation (Static Plantar Alloodynia)

MIA-induced knee osteoarthritis has been reported to induce allodynia in the paw of the afflicted limb represented by sharp withdrawal response when the plantar surface of the paw was stimulated with calibrated von Frey filaments or with a digital von Frey (Combe et al., 2004; Fernihough et al., 2004). This response was observable from Day 3 to Day 63, although the allodynia was more pronounced in the chronic stage (Day 14 onwards). A sharply delineated flinch response was not observed in our study. Instead, the rats simply placed less weight on the arthritic limb and therefore required less force to elicit a paw lift response. The animals were also more sensitive in the acute stage as compared to the chronic stage. The difference in response could have been due to a difference in the apparatus setup. A wire mesh was used as the flooring in our experiment instead of a conventional smooth metal grid. The wire mesh may have stimulated their paws and resulted in desensitization. Therefore the assay could have been measuring the amount of weight placed on the paw, as opposed to true allodynia. Nonetheless, morphine and diclofenac significantly increased PWT in the acute stage, while isovaline and ACBC were not effective. However, three separate doses of each drug (200, 500 and 1000 mg/kg for isovaline; 30, 60, 120 mg/kg for ACBC) were combined for the drug comparison to increase
sample size. We cannot exclude the possibility that individual doses would be effective. In particular, there was a trend for low dose isovaline (200 mg/kg) to increase PWT. However the sample size was too small for statistical comparison (n = 3).

4.5. Osteoarthritis Model in Mice

The development of MIA-induced osteoarthritis (OA) in mice mirrors that in rats (van der Kraan et al., 1989). Intraarticular MIA leads to dose-dependent joint swelling one day after administration. Inflammation was not present in later stages. Histological changes to the joint (chondrocyte death, cartilage thinning, loss of proteoglycan staining, osteophyte formation and synovial infiltration) were similar to those in rats. Of note, osteophytes, bone edema and synovitis are clinically associated with joint pain (Bollet, 2001; Creamer et al., 1999; Felson et al., 2001). In previous studies, experimentally-induced arthritic pain had chiefly focused on rats; the present results are unique in the establishment of an OA pain model in mice. The use of mice opens the avenue for genetic manipulation in the study of OA pain.

In comparison to rats, mice are more sensitive to intraarticular MIA administration, requiring half the concentration (mice 5% vs rats 10% MIA) to elicit reproducible pain-like behaviour. Increased sensitivity to MIA, a cellular glycolytic inhibitor, was unsurprising given that mice have a higher metabolic rate than rats. As a result of their smaller size and higher sensitivity, the depth of injection in mice must be carefully regulated to prevent systemic toxicities (sedation and respiratory depression). In a pilot experiment using Evans Blue dye, staining of the underlying muscle was apparent when the needle was positioned at a depth greater than 3 mm. A depth guard, or simply withdrawing the needle prior to injection, effectively decreased the rate of acute toxicity.
Three behavioural assays (inverted wire mesh, paw grip on 70° incline, rotarod) tested in this model were selected based on precedence in mouse and rat inflammatory arthritis and OA models. Of the three, only the inverted wire mesh test reliably detected a drug-reversible deficit in MIA-induced OA mice.

4.5.1. Inverted Wire Mesh Assay

Osteoarthritic (OA) pain is best characterized as movement-induced pain which radiates from the afflicted joint. A bilateral hind limb compressive grip force assay in rats has recently been described (Chandran et al., 2009). The rat was gently restrained and its hind paw permitted to grasp a wire mesh frame attached to a strain gauge. The grip force was measured by moving the animal in a rostral-to-caudal direction until the grip was broken. Monoarthritis of the right knee induced by MIA administration produced hind limb grip impairment, which was reversed by tramadol, diclofenac, celecoxib and gabapentin. Morphine and indomethacin had minor effects.

The inverted wire mesh protocol is an adaptation of the compressive grip force assay. Due to the small size and relatively weaker grip force of mice, paw grip is difficult to measure using a strain gauge. Instead, we opted to measure paw grip by the duration the animal can maintain its grip on an inverted wire mesh. The two measured parameters, paw grip duration (in which all four paws grip the mesh) and duration before falling (total duration on the mesh), gave comparable results. The paw grip duration appeared to be slightly more sensitive in detecting weakness in a single hind paw; however the fall was more prominent and therefore was easier to measure.
A dramatic impairment in grip duration was observed in MIA-induced mice from Day 1 to the end of the observational period on Day 21. Similar to findings by Chandran et al. (2009) in the rat, morphine significantly improved grip duration in the chronic stage of OA in mice. Our results were contradictory with regard to the effects of diclofenac. Diclofenac had no effect on paw grip in mice despite precedent in rats. The route of administration (current experiment, s.c. vs. (Chandran et al., 2009), p.o.) could have contributed to the difference. Mixed results have also been reported on the efficacy of NSAIDs in the incapacitance test in the chronic stage in rats (Bove et al., 2003; Fernihough et al., 2004; Pomonis et al., 2005). While Chandran et al. (2009) did not study pharmacological interventions in the acute stage on rat paw grip strength, we observed that neither morphine nor diclofenac were effective in mice on Day 3. In a preliminary study, there was a trend for ACBC (60 mg/kg) to increase grip duration in the chronic stage. However a larger sample size will be required to elucidate significance.

4.5.2. Paw Grip on a 70° Incline Plane

The strength of paw grip in mice has also been established by qualitative assessment on an incline plane for inflammatory arthritis (Williams et al., 1993). The authors focused on functional assessment of arthritis and did not examine the effects of pharmacological interventions. We found this test to be less sensitive and statistically less powerful than the inverted wire mesh assay. First, osteoarthritis was localized to the hind knee as opposed to generalized arthritis in the original protocol. Thus hind limb weakness can be counterbalanced by forepaw grip on the incline plane, as opposed to the inverted wire mesh. Compensation by the forepaws may have contributed to the recovery of grip impairment in the chronic stage. Second, nonparametric statistical tests necessary for ordinal impairment scores are inherently less
sensitive than parametric statistics, making slight improvements in grip strength difficult to detect. Indeed, no significant changes were seen for any drug treatment groups.

4.5.3. Rotarod Test

The rotarod test had previously been administered to determine grip strength of rats with adjuvant-induced arthritis (Perrine and Takesue, 1968). Intradermal injection of Complete Freund’s Adjuvant (CFA) in the distal region of the tail produced inflammatory polyarthritis, initiating at the site of injection and subsequently spreading to all joints (Wilson et al., 2006). Treated animals had a propensity to fall off the rotarod (2.5” in diameter rotating at 15 rpm) over a 30 s interval 10 days after adjuvant administration and progressively worsened over time. The percentage increases of rats failing the rotarod test correlates to the percentage increase in hind paw inflammation. Acute administration of anti-inflammatory drugs (indomethacin, phenylbutazone and hydrocortisone) significantly improved success rate on the rotarod compared to control without decreasing paw inflammation.

Progressive worsening in rotarod performance was not observed in MIA-induced OA mice; on the contrary, the animals compensated for the disability with practice. Improvement in rotarod times was seen over a series of three same-day replicates, which led to an overall non-significant difference compared to non-arthritic animals. Systemic CFA-induced arthritis is associated with a delayed T-cell mediated hypersensitivity reaction in addition to multiple-joint arthritis (Pearson and Wood, 1959), while MIA-induced arthritis is strictly localized to the knee joints (van der Kraan et al., 1989). Unsurprisingly, the rotarod test does not readily detect motor impairment in MIA-treated animals as compared to those treated with CFA.
The rotarod is also a classical test for gross motor and CNS impairment. Drugs with sedative properties worsen rotarod performance (Seguin et al., 1995). Perrine and Takesue (1968) also reported that agents such as morphine and chlorpromazine at doses with marked sedative effects failed to enhance rotarod performance in arthritis rats. In the current study, morphine (6 mg/kg) did not produce noticeable sedation in mice. However it also failed to improve rotarod performance in both acute and chronic stages. Similarly, diclofenac had no effect on this assay. Therefore the rotarod test is, overall, not useful in the assessment of analgesia in OA animals.

4.6. Heat-induced Models of Nociception

In attempts to characterize the pharmacokinetics and dose response of isovaline and ACBC, we studied their effects on two phasic models of pain. Unusual dose-response relationships were obtained from both tail-immersion and hot plate assays, in which middle but not higher doses were efficacious. This pattern of response was consistent for isovaline and ACBC.

4.6.1. Hot Water Tail Immersion Test

Administration of opioid analgesics leads to prominent increase of latency times in the tail flick assay, which persist for over an hour (Luttinger, 1985). It was highly puzzling that isovaline and ACBC consistently increased latency time only at 5 minutes. A short duration of action normally indicates rapid metabolism or elimination of the drug from system. Yet, systematic (i.v. and s.c.) administration of isovaline and ACBC clearly inhibited phase II formalin-induced paw licking response, which suggests the drugs remained in circulation for at least 40 min.

The tail flick response is also influenced by multiple physiological factors mainly associated with vasomotor tone (Le Bars, D Gozariu, M Cadden, S W., 2001). Changes in cutaneous and
basal temperature influence the thermal heating of the tail. Activation of nociceptors is ultimately dependent on basal temperature of the tail; the delay for the tail to reach threshold temperature is proportional to the difference in temperature between the tail and bath (Le Bars, D Gozariu, M Cadden, S W., 2001). Since the tail is a thermoregulatory organ in rodents, changes in tail temperature by vasomotor tone will affect latency times. For example, vasodilation leads to decreased cutaneous temperature and translates into an increase in latency. Similarly, vasoconstriction decreases heat loss and results in a decreased in response time. Interaction between pathways that convey nociceptive signals and those that regulate blood pressure can result in homeostatic-like modulation of tail flick responses, which could explain the brevity of isovaline and ACBC’s activities.

Furthermore, the relatively low potency of isovaline and ACBC resulted in a need for highly concentrated solutions to levels that had never been previously tested. These solutions greatly exceed physiological osmolarity and can lead to cellular damage at the site of injection. Although the site of injection (upper back) was far away from the site of testing (distal portion of tail), hypertonicity could still potentially affect tail flick responses. Indeed, an 82 mM NaCl high osmolarity control was found to induce hyperalgesia. There was significant decrease in tail flick latency 15 min after subcutaneous administration of the osmolarity control. The trend to decrease latency time post 15 min was also present at the high dose (1000 mg/kg) isovaline group; however this decrease was not significant. One can speculate that the observed tail flick response is due to an interaction of two processes: (1) the analgesic effect of the drug with rapid onset and (2) a delayed hyperalgesic response resulting from cellular damage by hypertonic solutions.
Increase in analgesia by increasing dose could have potentially been counteracted by the increasing osmolarity, leading to the absence of a sigmoidal dose dependency.

Interaction between stimuli is not uncommon and is well documented in the literature. For example intraperitoneal injections of irritant agents can increase nociceptive thresholds in the tail and paws (Hitchens et al., 1967; Winter and Flatakaer, 1965). A burn on the back similarly raises tail flick threshold (Osgood et al., 1987). Nonetheless most interactions decrease rather than increase pain sensitivity, as we observed in the current study.

4.6.2. Hot Plate Test

Subcutaneous administration of ACBC generated a non-sigmoidal dose relationship in the hot plate assay - 60 mg/kg but not 30 or 120 mg/kg doses significantly prolonged paw licking latency. In this model, ACBC produced a more conventional pattern of responses over time. Peak response was obtained at 20 min and trailed off at an hour post-administration, which was consistent with duration of action in the formalin foot model. In contrast, high dose isovaline did not change hot plate response, suggesting a different mechanism of action. The non-significant results of high dose isovaline could also reflect the non-sigmoidal dose response relationship of these agents. Moreover, that no change was observed in the behaviour or breathing rate of isovaline and ACBC-treated mice, coupled with results from the MTD study and rotarod test, supports the safety of these agents for clinical use.

4.7. Mechanisms of Action of Isovaline & ACBC

Structural similarities between glycine and GABA with our amino acids of interest suggest these agents likely act by enhancing inhibition to produce antinociception. Isovaline appeared to
interact with the glycinergic system as it reversed strychnine-induced allodynia. Glycine also has a secondary excitatory action via the NMDA glycine$_B$ site. We attempted to distinguish between glycine$_A$ and glycine$_B$ receptor subtypes using the peripheral glutamate nociceptive models.

### 4.7.1. Glutamate-induced Paw Licking and Allodynia in Mice

Evidence in literature reported excitatory amino acids, including glutamate, are released in the thalamus, brainstem, spinal cord and in hind paws upon intraplantar administration of algogenic substances (formalin, capsaicin, substance P) or following inflammatory responses (Juranek and Lembeck, 1997; Omote et al., 1998; Sluka and Westlund, 1992; Teoh et al., 1996). Immunohistochemical staining indicated the presence of a large number of glutamate receptors (NMDA, AMPA, kainate) on unmyelinated axons in the glabrous skin of the hind paw (Carlton et al., 1995). Administration of glutamate locally within the paw also elicits dose-dependent nociceptive paw licking that lasts for 15 min (Beirith et al., 2002). Licking response can be inhibited by glutamate receptor blockers such as MK801 (NMDA receptor antagonist) and NBQX (AMPA and kainate receptor antagonist) (Beirith et al., 2002).

In the current study, ACBC suppressed glutamate-induced paw licking response, supporting *in vitro* findings. ACBC is a known selective partial agonist at the glycine$_B$ site, capable of inhibiting glycine binding in radioligand binding assays (Hood et al., 1989). Conversely, glycine produced a rightward shift in ACBC’s inhibitory effects in the assays. ACBC also does not interactive with strychnine-sensitive glycine$_A$ receptors or the NR2 subunit glutamate binding site on NMDA receptors (Hood et al., 1989). Moreover, we have demonstrated the lack of functional glycine$_A$ receptors in the glabrous skin of the mice, as concentration titration of strychnine did not produce any nociceptive responses.
Intraplantar administration of glutamate and glutamate agonists in rats also produce peripheral allodynia in the hind paw at lower concentrations than those required to induce licking behaviour (Carlton et al., 1995; Zhou et al., 1996). The current study established a glutamate-induced allodynia model in the mouse. Glutamate administration similarly decreased the PWT of the injected paw at concentrations lower than that required for the paw-licking assay in mice and was selected for a more sensitive test for detecting drug antagonism. Despite the structural similarities between isovaline and ACBC, isovaline, tested at a 4-fold range of doses, was not effective in reversing glutamate-induced allodynia. This suggests there are fundamental differences in the mechanisms of action between the two amino acids.

4.7.2. Evidence from Electrophysiology and Receptor Modelling

Electrophysiological research on thalamic slices of the rat identified distinct population of neurons sensitive to isovaline (Cooke et al., 2009). R-isovaline inhibited evoked action potentials with a long duration of action, none of which were reversed by strychnine. Much fewer neurons were sensitive to S-isovaline. Computer simulated structure modeling supports these findings; the two isoforms of isovaline appear to interact differently at the isoleucine binding complex. Favorable binding exists for the R-, but not for the S-enantiomer. Further investigations indicated R-isovaline likely inhibits action potential by a shunting mechanism mainly via potassium channels, which precludes the involvement of chloride-conducting glycine A and GABA A receptors. Activation of metabotropic GABA B receptors can produce potassium currents with a long duration of action. Indeed, the effect of R-isovaline was blocked by pre-application of CGP 35348, a selective GABA B receptor antagonist but not picrotoxin, a GABA A (and C) and glycine A
receptor antagonist. CGP 35348, however, was unable to reverse the effect of R-isovaline when R-isovaline was applied beforehand (unpublished results).

From X-ray crystallography, the GABA binding pocket of the receptor consists of two globular lobes separated by a hinge (Quirocho and Ledvina, 1996). Termed the “Venus flytrap”, these two lobes come together upon ligand-binding and stabilize the ligand-receptor complex (Bettler et al., 2004). At least two conformational states exist for the Venus flytrap - an open and a closed state, in which the closed state favours ligand binding (Galvez et al., 1999). Ligand interaction of the first lobe induces a conformational change to permit further stabilizing interaction with the second lobe. R-isovaline likely binds to the activator site and may stabilize the closed state as compared to CGP 35348. Presumably, the smaller molecular size of R-isovaline confers a tighter conformational change of the flytrap at the closed state which prevents subsequent displacement by the antagonist. However R-isovaline may not out-compete CGP when the receptor is in the open state. The slow (energetically unfavorable) off-rate can also explain its extended duration of action.

Pilot electrophysiology data also suggest activation of NMDA receptors for both R- and S-isomers under low but not high concentrations indicative of a partial agonist. Therefore, isovaline is likely an agent with mixed receptor activities. These interactions may explain the non-sigmoidal dose relationships obtained in the in vivo studies. Curiously the differences between enantiomers were not observed when they were administered independently into the lumbar intraspinal space (Wang, 2008). Both R- and S-isovaline were equally effective in reversing strychnine-induced allodynia, potentially due to in vivo enzyme directed isomerization.
Alternatively, isovaline may have different effects on neurons in the spinal cord as compared to those found in the thalamus.

4.7.3. Structure Activity Relationships

It is difficult to explain the difference between isovaline and ACBC in the glutamate-induced nociceptive models based on their chemical structures. Certainly ACBC, a ring-constrained analogue of isovaline, is expected to have similar receptor binding interactions as R-isovaline with comparable association constants. A dose-dependent partial agonist effect seems unlikely as a four-fold difference in concentration of R- and S- isovaline had been examined, none of which had any effect. Nonetheless, increase in side chain length would be expected to affect affinity at the glycine$_B$ site as well as a possible increase of antagonist activities. For example ACBC is a partial agonist at the glycine$_B$ site (Hood et al., 1989), while the cyclopropyl analog (1-amino-cyclopropane-carboxylic acid, ACPC) is an agonist (Nadler et al., 1988). Decreased steric hindrance of the non-ring-constrained isovaline may result in a greater agonist efficacy. Additionally, exogenous glutamate activates non-specific peripheral receptors, including NMDA, AMPA and kainate receptors, all of which are involved in generating nociceptive behaviour (Beirith et al., 2002; Zhou et al., 1996). We cannot rule out the possibility that isovaline has activities on other receptors that overshadow the effects of NMDA receptor antagonism.

4.8. Towards Better Analgesics Testing

As indicated before, demonstrable efficacy in animal models does not necessarily translate to suitability for clinical use. The measured endpoints are surrogate markers associated, but not necessarily linked to pain. Therefore, the use of more pain-specific behavioural markers may be
advantageous. For example, many of the traditional animal pain models focus on evaluation of pain-induced responses (e.g. licking, scratching and flinching). Drugs may decrease nocifensive responses by impairing the animal’s ability to respond to rather than to sense the noxious stimuli. One common strategy to control for motor effects has been to compare drug potency for antinociception to rotarod impairment. However, it is difficult to assess which potency differences are sufficient to identify true antinociceptive effects. Such ambiguities can be avoided by including measures of pain-related behavioral suppression.


While preclinical studies primarily identify pain-stimulated behaviours, chronic pain is equally associated with suppression of normally adaptive behaviours. This is utilized clinically in some pain inventories which assess suppression of daily activities such as walking, performance of household chores and social interactions (Ostelo and de Vet, 2005). Efficacy of intervention is measured in part by the restoration of these pain-suppressed behaviours. We can equally apply the same principles into animal pain models. For example the decreased and later drug-induced restoration of spontaneous locomotor activity, feeding and social interactions can be evaluated in trained animals given pain manipulations (Flecknell, 1994; Karas, 2002)(Fletcher, 2002). In the mouse model of MIA-induced osteoarthritis, we attempted to incorporate measurements of pain-suppressed behaviour. More specifically, impairment of hindpaw grip in OA mice is not subjective to false positive effects by CNS depressants. Further development of this assay may prove to be beneficial for the study of new analgesics.
4.8.2. Importance of Translational Studies

Off-target activities can lead to unwanted side effects. Most importantly, species differences in molecular biology of the target and tissue distribution can lead to false positives. For example, NK1 antagonists appeared to be promising agents – they have proven efficacious in animal pain models (Hill, 2000), demonstrated suitable systemic exposure and adequate CNS penetration in humans (Bergstrom et al., 2004). However, they had no demonstrable analgesic activities in clinical studies. In this case, the target is relevant to pain in rodents, but not in man. Therefore while animal models are useful in identifying potential drug candidates, one can only certify clinical relevance through clinical trials.

Indeed, the Certified Medical Representatives Institute (CMR) has reported that pharmaceutical lead candidates are subject to attrition rates of nearly 90% (reviewed in (Kola and Landis, 2004)). Analysis revealed that the majority of failures occurred in Phase II and Phase III clinical studies, which indicate a lack of therapeutic efficacy, safety or tolerance. Phase II studies address the efficacy and safety of the drug in limited patient populations, while Phase III studies are large scale “pivotal” trials, which compare the efficacy and safety of the agent to current therapies involving large numbers of patients, typically across multiple sites. Both are costly and time-consuming to conduct. As such, new perspectives on translational studies, which bridge the gap between preclinical and clinical studies, are highly beneficial for eliminating potential false positives prior to full-scale clinical trials.

Recent development of human pain models may provide hints on the effects of specific pain mechanisms. Koppert and colleagues developed an electrical hyperalgesia model to examine
central sensitivity and evaluated sensitivity of this model to selected analgesics (B. A. Chizh et al., 2004; Klede et al., 2003; Koppert et al., 2001; Koppert et al., 2004). In this procedure, a bipolar stimulation electrode is inserted intradermally, typically into the volar forearm of healthy volunteers. Stimulus parameters are titrated to achieve roughly equivalent pain intensities across subjects and the effects of drugs are evaluated against the background pain, or against secondary hyperalgesia (an indicator for central sensitization). This model detected efficacy of gabapentin and pregabalin (agents against neuropathic pain) but not the NK1 antagonist aprepitant (B. A. Chizh et al., 2007; Segerdahl, 2006). Such models will be useful in assessing efficacy of isovaline, ACBC and other glycine/GABA agonists that putatively act by increasing central inhibition.

4.9. Future Directions

Additional work is required to elucidate isovaline and ACBC’s mechanisms of antinociception. While whole animal models allow for integration of all response systems necessary to observe antinociception, they are crude methods in addressing drug responses from a finer mechanistic point of view. Similarly, electrophysiological slice recordings allow for precise analysis of responses from intact neural circuits, but results are difficult to interpret due to interactions of multiple receptor systems. The use of discrete receptor systems may be conducive to pinning down the specific receptor(s) responsible for our observed effects. In particular, electrophysiological studies of isolated receptor subtypes using expression systems such as Xenopus oocytes will be helpful in separating the mixed responses of these agents. Ideally, we should explore isovaline and ACBC’s effects on glycineA, glycineB, GABA_A, GABA_B and glutamatergic receptors in conjunction with slice and whole-animal models.
As demonstrated by our results, compounds which enhance inhibitory transmission and or suppress excitatory transmission appear to be a viable therapeutic strategy for the management of chronic pain. Characterization of additional structural analogues (e.g. 1-amino-cyclopropyl carboxylic acid and cycloleucine) to explore structure activity relationships will be helpful in designing more potent compounds and to further our understanding on their mechanisms of action.

The inverted wire mesh assay for the MIA-induced mouse osteoarthritis model appears to be a promising new animal model of nociception. The effects of other conventional analgesics (e.g. tramadol, acetaminophen) and agents known to induce false positives in other assays (e.g. neuromuscular blockers, sedatives, NK1 antagonists) can be explored in a dose-dependent fashion. Finally, our compounds of interest, isovaline and ACBC, can be studied in depth using this model.

Additional data on the pharmacokinetics of isovaline and ACBC are also desirable. Intravenous administration of ACBC in rodents demonstrated central penetration; however, it was rapidly cleared from the brain with a half-life of 4 min in mouse and 5 min in rat (Rao et al., 1990). Plasma and cerebrospinal fluid (CSF) concentrations of isovaline upon systemic administration have never been examined. Pharmacokinetic data of the amino acids in plasma and CSF by high performance liquid chromatography (HPLC) techniques will help establish the site of action of these agents. A correlation between efficacy and exposure at the putative target will increase our confidence in the drug’s mechanism of action. For example, since ACBC is rapidly cleared from
the brain, its hour-long analgesic effects are likely not due to direct actions on the cerebral
cortex. Examination of plasma drug levels will help in our exploration of its peripheral actions.
Chapter 5. Conclusion

In this thesis, we investigated the effects of isovaline and ACBC on several models of tonic and acute pain (formalin and glutamate-induced nociception in rats and mice, and heat-induced nociception, respectively). We have also established two new nociceptive assays (inverted wire mesh grip assessment and glutamate-induced allodynia) in mice. Attempts had been made to assess isovaline and ACBC’s efficacy in reversing pain-related behaviours in MIA-induced osteoarthritic rats. However, large variability between animals made it difficult to identify drug effects.

No toxicity was observed in rats and mice given high doses of isovaline - in the maximum tolerated dose (MTD) study, there were no significant changes to heart rate, blood pressure, breathing rate or ECG in rats at the maximum soluble dose. Isovaline and ACBC did not cause sedation or local irritation in rats. In contrast, morphine produced sedation and diclofenac produced local irritation. Again, neither isovaline nor ACBC altered behaviour or breathing rate in mice.

Subcutaneous administration of isovaline and ACBC significantly decreased formalin-induced paw licking behaviour. ACBC was approximately 10 times more potent than isovaline. Administration of MIA produced osteoarthritic-like changes to knee joints in mice, reflected by hind paw grip impairment similar to that seen in rats. The grip duration and duration the animals remained suspended on the inverted mesh were substantially decreased in MIA-treated animals. Morphine but not diclofenac reduced this grip impairment in the chronic phase (Day 14) of the
model. Two other assays were also examined (70° incline plane assay and the rotarod test); neither were effective in detecting the effects of morphine and diclofenac.

Non-sigmoidal dose-response relationships were obtained for both isovaline and ACBC in the hot water tail immersion and hot plate assays. Isovaline and ACBC increased tail flick latency time 5 min post injection. In the hot plate assay, ACBC delayed the onset to paw licking response, while high dose isovaline (1000 mg/kg) had no effect.

Glutamate administered intraplantarly into the rear hind paw elicited dose-dependent paw licking and allodynia in mice. Co-administration of ACBC suppressed the paw licking response, while isovaline was ineffective against glutamate-induced allodynia. Concentration titration of peripheral strychnine did not produce any nociceptive responses, suggesting an absence of functional glycine$_A$ receptors in glabrous skin of the mouse paw.

The observed effects are likely comprised of mixed activities in glycine and GABA receptor systems. Results from the glutamate-induced nociceptive models suggest isovaline and ACBC potentially have different mechanisms of action. An understanding of their agonist and partial agonist effects in isolated receptor system will be beneficial in elucidating their specific mechanisms of action. Our results confirmed the existence of pronounced antinociceptive effects of isovaline and ACBC in mural models without observable side effects. This supports our hypothesis that novel pharmacological interventions which elevate inhibitory transmission and/or suppress excitatory transmission are likely to be of therapeutic value.
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APPENDIX A

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0590

Investigator or Course Director: Bernard A. Macleod

Department: Anesthesiology, Pharmacology & Therapeutics

Animals:

Mice CD1-40

Start Date: November 1, 2007    Approval Date: December 14, 2007

Funding Sources:

Funding Agency: Various Sources
Funding Title: Jean Templeton Hugill Anesthesiology Research Fund

Funding Agency: Various Canadian Foundations (non-IHS)
Funding Title: Jean Templeton Hugill Anesthesiology Research Fund

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
# APPENDIX B

**THE UNIVERSITY OF BRITISH COLUMBIA**

## ANIMAL CARE CERTIFICATE

**Application Number:** A08-0360  
**Investigator or Course Director:** Bernard A. Marleau  
**Department:** Anesthesiology, Pharmacology & Therapeutics

**Animals:**
- Mice CD1 54

**Start Date:** May 15, 2008  
**Approval Date:** July 8, 2008

**Funding Sources:**

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<td>Various Canadian Foundations /non-HS/</td>
<td>Jean Templeton Hugill Anesthesiology Research Fund</td>
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**Unfunded title:** N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

*A copy of this certificate must be displayed in your animal facility.*

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APPENDIX C

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0502

Investigator or Course Director: Bernard A. Macleod

Department: Anesthesiology, Pharmacology & Therapeutics

Animals:

Rats Sprague-Dawley 49

Start Date: January 1, 2007

Approval Date: May 15, 2009

Funding Sources:

Funding Agency: Various Sources
Funding Title: Jean Templeton Hagill Anesthesiology Research Fund

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

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