

**EFFECTS OF ISOVALINE AND INVOLVEMENT OF METABOTROPIC GROUP II
GLUTAMATE RECEPTORS**

by

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Abstract

This thesis is comprised of manuscripts describing effects of isovaline, metabotropic GABA_B agonist, baclofen, and metabotropic glutamate (mGlu II) agonist, LY354740, on peripheral and central neurons. Our laboratory studies previously demonstrated analgesic properties of isovaline acting on peripheral GABA_B receptors while in brain slices, isovaline acted as atypical GABA_B agonist. Peripheral GABA_B and mGlu II receptors belong to same family of G-protein-coupled receptors. We examined if isovaline activated peripheral mGlu II receptors that inhibit nociception. We assessed post- and presynaptic effects of isovaline, with comparison to baclofen and LY354740 on neurons of somatosensory thalamus. We compared effects of isovaline, baclofen and LY354740 on electrically evoked contractions of guinea-pig ileum, a standard assay for GABA_B receptors.

In manuscript one, we hypothesized that mGlu II receptors mediate isovaline analgesia. We utilized a model of pain induced by prostaglandin-E₂ which sensitizes primary afferent fibers in hindpaw of mice. Isovaline produced analgesia which was blocked by mGlu II antagonist (LY341495). Therefore isovaline activated peripheral mGlu II receptors, in addition to GABA_B receptors.

In manuscript two, we hypothesized that isovaline activates presynaptic GABA_B and mGlu II receptors, thereby inhibiting GABA and glutamate release. We studied effects of isovaline, baclofen and LY354740 on inhibitory and excitatory postsynaptic currents (IPSCs and EPSCs) in thalamocortical neurons. We determined effects on electrically stimulated release of transmitters which we confirmed as producing GABAergic and glutamatergic currents. The agonists

decreased amplitudes of IPSCs and EPSCs during selective blockade of other postsynaptic currents, without affecting decay-time constants. GABA_B antagonist (CGP52432) and LY341495 blocked isovaline effects, implicating GABA_B and mGlu II receptors in transmitter release inhibition.

In manuscript three, we hypothesized that isovaline activates ileal GABA_B and mGlu II receptors. Isovaline decreased amplitude of muscle contractions and increased resting tension. CGP52432 antagonized baclofen-induced decreases in amplitude and tension, but not isovaline effects. LY354740 had no effect. These findings suggest novel targets for isovaline-like compounds in ileum.

The thesis demonstrates for the first time that isovaline produces analgesia in hindpaw and presynaptic inhibition in thalamus by activating mGlu II in addition to GABA_B receptors, while isovaline receptors are distinct from GABA_B receptors in ileum.

Lay Summary

In this thesis, we studied the effects of the analgesic amino acid isovaline in animal pain models. Isovaline relieves pain without causing side effects, usually observed with current analgesics. How isovaline induces its effect is unclear, we know that it acts partly by activating GABA_B receptors. Here, we supposed that isovaline also activates mGlu II receptors, which have important role in modulating pain. Our objective was to examine isovaline effects on mGlu II receptors in brain tissue and a pain model. We found that isovaline produces analgesia by activating mGlu II receptor, in addition to GABA_B receptors. These findings demonstrate that isovaline may serve as a prototypical member of a new class of analgesics that act on multiple receptor systems while having minimal toxicity.

Preface

The Animal Care Committee of University of British Columbia has approved the use of animals in the following experiments. The animal care certificate numbers were A11-0171, A13-0143 and A14-0269.

With the supervision of Drs. Ernest Puil and Bernard A MacLeod, my contributions to the first and second manuscripts were the design, execution, and analysis of the experiments and co-writing the manuscripts.

My contribution to the third manuscripts was in the design, execution of experiments, and analysis of results; the write-up was mainly the contribution of Mr. Tim Fung (as the first author) and Yahya Asiri, Richard Wall, Stephan Schwarz, Ernest Puil and Bernard MacLeod.

A version of the first manuscript was published in the journal of *Neuroscience* by Asseri KA, Puil E, Schwarz SKW, MacLeod BA, entitled “Group II metabotropic glutamate receptor antagonism prevents the antiallodynic effects of R-isovaline”. *Neuroscience* 2015; 293, 151-156.

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List of Abbreviations

aCSF	artificial cerebrospinal fluid
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
Baclofen	R(+)- β -(Aminomethyl)-4-chlorobenzenepropanoic acid hydrochloride
Bicuculline	(5S)-5-[(6R)-6,8-Dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl]-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-g]isoquinolinium iodide
cAMP	cyclic adenosine monophosphate
CGP35348	3-Aminopropyl-(diethoxymethyl)phosphinic acid
CGP52432	3-[[[(3,4 dichlorophenyl) methyl]- amino]propyl] diethoxymethyl) phosphinic acid
CGP7930	3,5-bis(1,1-Dimethylethyl)-4-hydroxy- β , β -dimethyl-benzenepropanol
CI	confidence interval
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
Cs ⁺	cesium ion
DL-AP5	DL-2-amino-5-phosphonopentanoic acid
DMSO	dimethyl sulfoxide
EPSCs	excitatory postsynaptic currents
GABA	γ -Aminobutyric acid
GDP	guanosine diphosphate
GI	Gastrointestinal
GIRK	G-protein coupled inwardly rectifying K ⁺ channel

GPCR	G-protein-coupled receptors
GTP	guanosine triphosphate
IASP	International Association for the study of pain
I-V	current-voltage relationships
IPSCs	inhibitory postsynaptic currents
IPSPs	inhibitory postsynaptic potentials
i.p.	intraperitoneal
Isovaline	2-Amino-2-methylbutyric acid
L-AP4	L-(+)-2-amino-4-phosphonobutyric acid
LTS	low-threshold Ca^{2+} spike
LY341495	(2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid
LY354740	(1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid
LY487379	2,2,2-trifluoro-N-[4-(2-methoxyphenoxy)phenyl]-N-(3-pyridinylmethyl) ethanesulfonamide hydrochloride
MAP-4	(S)-2-amino-2-methyl-4-phosphonobutanoic acid
mGluR II	group II metabotropic glutamate receptors
ML	medial lemniscus
NMDA	N-methyl-D-aspartate
nRt	nucleus reticularis thalami
PGE ₂	prostaglandin E ₂
PKA	protein kinase A
PLC	phospholipase C
PNS	peripheral nervous systems

R_i	input resistance
τ	decay time constants
TTX	Tetrodotoxin
VFT	venus fly trap domain
VPL	ventral posterior lateral
VPM	ventral posterior medial
V_h	holding potential
V_m	membrane potential

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Dedication

I dedicated this thesis to my wife, Amira, my parents and kids, for their love and encouragement, throughout my lifetime.

Chapter 1. General Introduction

1.1 Scope of the thesis

In this thesis, we investigated the effects of the non-proteinogenic α -amino acid isovaline on GABA_B and Group II metabotropic glutamate (mGlu II) receptors in peripheral and central nervous systems (PNS and CNS). We also investigated the effects of mGlu II agonist LY354740 and prototypical GABA_B agonist baclofen in peripheral and CNS neurons.

This thesis specifically addresses the following:

1. Does antagonism of mGlu II receptors prevent isovaline blockade of allodynia induced by prostaglandin E₂ (PGE₂)?
2. Does mGlu II agonist affect the firing and membrane properties of thalamic neurons?
3. Does mGlu II receptor antagonism prevent isovaline inhibition of thalamic neurons?
4. Does baclofen affect the release of the neurotransmitters, GABA and glutamate, from nerve terminals in thalamus?
5. Does isovaline affect muscle contractions of ileum, in the same way as baclofen?
6. Does a mGlu II agonist also affect muscle contractions of ileum?

This thesis describes the effects of isovaline on metabotropic glutamatergic and GABAergic receptors in the periphery and on CNS neurons. In the first manuscript on a rodent pain model, we examine the effects of isovaline on peripheral metabotropic glutamate receptors, known to inhibit nociception. The second manuscript examines the effects of mGlu II agonist (LY354740) on neurons of the ventrobasal nuclei in thalamus and compares the effects of isovaline as well as baclofen on inhibitory and excitatory neurotransmission. The third manuscript examines the

effects of isovaline and baclofen as well as LY354740, on electrically evoked, neuron-mediated contractions of smooth muscle in guinea pig ileum.

1.2 Background

1.2.1 Pain

Pain is defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Cortelli et al., 2013). Pain plays a critical role in the survival and well-being of animals. However, it is considered as one of the most common complaints in clinic, and can negatively affect the physical and psychological as well as the economic well-being. It has been estimated that about 10% of the world’s population suffer from chronic pain conditions (cf. Raffaelli and Arnaudo, 2017). Currently, the treatment of pain is inadequate and compromised by limited efficacy and side effects of therapeutic agents (cf. Park and Moon, 2010). Therefore, finding more effective analgesics with minimal adverse effects remains a primary goal of pharmacologists.

Pain signals are initiated in the periphery by nociceptors which are specialized primary afferent fibers that detect noxious stimuli ($A\delta$ and C nociceptors; Jankowski and Koerber, 2010). These signals or impulses are then transmitted along the axonal fibers of the dorsal root ganglion neurons to the dorsal horn of the spinal cord where secondary neurons convey the impulses to supraspinal structures including brainstem, thalamus and somatosensory cortex (Zhuo, 2007). Following peripheral tissue injury, pain mediators such as cytokines, hydrogen ions, substance P,

kinins, prostaglandin E₂ (PGE₂), and glutamate are released into the surrounding tissues (Woolf and Ma, 2007). The release of these mediators, also collectively known as the “inflammatory soup”, leads to peripheral sensitization by repeated activation of nociceptors (Woolf and Salter, 2000). Allodynia is a painful response to normally nonpainful stimuli, whereas hyperalgesia is an enhanced painful response to painful stimuli. Both allodynia and hyperalgesia are prominent characteristics of many pain conditions (Sandkühler, 2009). Several lines of evidence suggest that the glutamate release is enhanced in acute and chronic pain conditions in CNS and periphery (cf., Fundytus, 2001). A disinhibition mechanism, resulting from a reduction in the efficacy of GABAergic inhibitory pathways (e.g., in spinal cord, periphery and brain), has been also suggested to be involved in animal models for inflammatory, acute, and neuropathic pain (Enna and McCarron, 2006). Taken together, these studies suggest that GABA and glutamate receptors may promising targets for the development of new analgesics.

1.2.2 The family C G-protein-coupled receptors

Metabotropic or G-protein-coupled receptors (GPCR) superfamily is a large family of membrane receptors that are linked to effector proteins (e.g., ion channels) by signal transducers known as G- proteins. GABA_B and mGlu receptors are family C members of GPCRs. Family C is composed of a single polypeptide chain that spans the plasma membrane seven times with C- and N-termini. This family includes other receptors such as calcium-sensing and taste receptors. They regulate a wide range of physiological functions such as synaptic transmission, neurotoxicity and pain (Bettler et al., 1998; Brauner-Osborne et al., 2007).

In this thesis, we will deal only with mGlu II and GABA_B receptors. Both GABA_B and mGlu II receptors play pivotal roles in regulating synaptic transmission and neuron excitability (Rondard et al., 2011). Like other members of family C, they contain a seven-transmembrane domain, and large extracellular N-terminal and intracellular C-terminal domains (Figure 1.1; Rondard et al., 2011). While there are many structural similarities between the GABA_B and mGlu II receptors, there are some differences. For example, the extracellular domain of mGlu receptors contains a cysteine rich domain, which is absent in the extracellular domain of GABA_B receptors.

1.2.2.1 GABA_B receptors

GABA_B receptors are activated by the main inhibitory neurotransmitter, γ -aminobutyric acid (GABA). Unlike GABA_A and GABA_C subtypes of the GABA receptor family, GABA_B receptors inhibit adenylate cyclase and couple to G α_i and G α_o proteins (Greif et al., 2000; Morishita et al., 1990). They are GTP analog-sensitive and widely distributed throughout the CNS and periphery (Bowery et al., 1981; Hill et al., 1984). Heterodimerization between GABA_{B1} and GABA_{B2} subunits gives rise to a functional GABA_B receptor (Jones et al., 1998; Kaupmann et al., 1998). Each subunit is composed of seven transmembrane segments with intracellular C- and extracellular N-termini. The N-terminus of GABA_{B1} subunit contains the binding site of ligands on the bilobate proteins (Venus Flytrap or VFT). There are two isoforms of GABA_{B1} subunits, GABA_{B1a} and GABA_{B1b} (Hawrot et al., 1998). The C-terminus of GABA_{B2} subunits couples to G $\alpha_{i/o}$ proteins which inhibit adenylate cyclase (Robbins et al., 2001).

Binding of ligand to the VFT induces conformational changes which cause dissociation of $\beta\gamma$ subunits of G proteins and activation of inwardly rectifying K⁺ (GIRK) channels or inhibition of

voltage dependent Ca^{2+} channels (Bettler et al., 2004; Callaghan et al., 2008). The activation also may reduce the production of cyclic AMP as a result of $\text{G}\alpha$ -subunit-inhibition of adenylyl cyclase. Reduction in cyclic AMP decreases the level of protein kinase A (PKA) which affects the regulation of various cellular functions such as synaptic plasticity and GABA_B receptor desensitization (Mott and Lewis, 1994; Couve et al., 1998).

GABA_B receptors are distributed throughout the CNS and PNS (Desarmenien et al., 1984; Bowery et al., 1987; Enna and McCarson, 2006). GABA_B receptors exist on both the pre- and postsynaptic membranes of neurons. In the ventrobasal nuclei of thalamus, GABA_B receptors are expressed at presynaptic sites of axon terminals and postsynaptically on thalamocortical neurons (Emri et al., 1996; Connelly et al., 2013). Activation of presynaptic GABA_B receptors reduces release of GABA (Deisz and Prince, 1989; Nicoll et al., 1990) by attenuating voltage-dependent Ca^{2+} channels in the nerve terminal (Dittman and Regehr, 1996; Harayama et al., 1998). Presynaptic GABA_B receptors can serve as auto- or heteroreceptors that respectively modulate the release of inhibitory or excitatory transmitter from the nerve terminals (Bettler and Tiao, 2006). A primary function of postsynaptic GABA_B receptors is the inhibition of excitatory transmission (Luscher et al., 1997). Postsynaptic activation of GABA_B receptors by released GABA increases membrane conductance for K^+ and hyperpolarizes neurons. Unlike GABA_A and GABA_C receptors which mediate fast Cl^- -dependent inhibitory postsynaptic potentials (IPSPs), activated GABA_B receptors exhibit a delayed, slower increase in K^+ conductance which is characteristic of a slow IPSP component (Malouf et al., 1990; Nicoll et al., 1990).

In thalamus, activation of postsynaptic GABA_B receptors by GABA_B agonists produces an

increase in K^+ -conductance which inhibits firing (Crunelli and Leresche, 1991; Banerjee and Snead, 1995; Cooke et al., 2012). Activation of presynaptic GABA_B receptors reduces transmitter release by blocking high voltage-activated Ca^{2+} channels (Banerjee and Snead, 1995; Misgeld et al., 1995; Wu and Saggau, 1997). Several studies have implicated GABA_B receptors in the modulation of nociceptive information in thalamic pathways (Castro-Lopes et al., 1995; Lin et al., 1996). Application of baclofen, a prototypical agonist at GABA_B receptors modulates thalamocortical excitation by activating presynaptic GABA_B receptors (Zsuzsa Emri et al., 1996; Porter and Nieves, 2004).

Many studies have demonstrated that electrical or chemical stimulation of neurons of nucleus reticularis thalami (nRt) or perigeniculate equivalents in the visual system activates mostly GABA_A receptors in thalamocortical neurons (Huguenard and Prince, 1994; Shosaku et al., 1989). Glutamate stimulation of the perigeniculate nucleus evokes IPSPs and oscillations in thalamocortical neurons. These spindle-like oscillations are believed to underlie spindle oscillations in the EEG. At high intensity stimulation, both GABA_A and GABA_B receptors mediate the IPSPs. Only GABA_A receptors mediate the spindle-associated IPSPs evoked by low intensity stimulation. The slow IPSPs mediated by GABA_B receptors remains unaltered during GABA_A antagonism. Intense nRt stimulation or synchronous discharge evokes large GABA_B-, K^+ -mediated IPSPs, resulting in rebound low threshold spiking which is due to hyperpolarization-activated inward current (I_h) and a T-type Ca^{2+} current (Huguenard and Prince, 1994; Bal et al., 1995; Kim et al., 1997; Sanchez-Vives and McCormick, 1997). Physiological activation of GABA_B responses may require intense synaptic drive or repetitive stimulation that leads to enhanced transmitter release and diminished uptake of GABA (Otis and

Mody, 1992; Isaacson et al., 1993). These findings may have particular relevance to thalamic GABA_B receptor involvement in generalized spike-and-wave seizures and pain (Hosford et al., 1992; Enna and McCarson, 2006; Potes et al., 2006).

In the PNS, GABA_B receptors are located on primary afferent neurons and Schwann cells. Studies on the effects of baclofen on peripheral nerve have helped to demonstrate the existence of functional GABA_B receptors on afferent nociceptors in the rat (Desarmenien et al., 1984; Magnaghi et al., 2004). Previously, we showed that activation of peripheral GABA_B receptors produced an antinociceptive effect in the PGE₂ pain model in mice (Whitehead et al., 2012). GABA_B receptors also are found in the gastrointestinal (GI) tract of rodents (cf. Hyland and Cryan, 2010). Application of GABA_B agonists reduces the electrically evoked contractions of guinea pig ileum, suggesting the presence of functional GABA_B receptors (Kerr et al., 1990).

Pharmacologically, GABA_B receptors can be distinguished from GABA_A receptors by selectivity to baclofen-activation and insensitivity to the GABA_A antagonist, bicuculline (Bowery et al., 1981). There are several selective antagonists of the GABA_B receptor, including CGP52432 and CGP35348. In this study we used CGP52432, a potent competitive antagonist with an IC₅₀ of 85 nM (Lanza et al., 1993).

1.2.2.2 Inhibitory metabotropic glutamate receptors

Glutamate is a dicarboxylic amino acid which is the most common excitatory neurotransmitter in the CNS, acting on ionotropic and metabotropic types of receptors. The N-methyl-D-aspartate (NMDA), kainate and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)

receptors, ligand-gated ion channel receptors, mediate fast synaptic excitation (Dingledine et al., 1999). Group I, II and III metabotropic glutamate receptors which couple to G-proteins, mediate slow excitation or inhibition of neurons (Conn and Pin, 1997; Pin and Acher, 2002). According to their physiology, pharmacology and homology sequence, there are eight subtypes of receptors which can be classified into three groups. Group I mGlu (mGlu₁ and mGlu₅) receptors are coupled to phospholipase C (PLC) and Ca⁺² signalling, while group II (mGlu₂ and mGlu₃) and III mGlu (mGlu₄, mGlu₆ mGlu₇ and mGlu₈) receptors are negatively coupled to adenylyl cyclase (Schwartz and Alford, 2000; Goudet et al., 2009). Activation of group II or III mGlu receptors activate a slowly developing increase in K⁺ conductance and inhibit Ca⁺² currents (Dutar et al., 2000). These receptors are distributed throughout the CNS and PNS and may regulate nociceptive information processing. In this thesis we focus on group II mGlu receptors and potential involvement in nociception.

Like GABA_B receptors, mGlu II receptors are composed of a large N-terminal domain which contains the glutamate binding site - also known as VFT. The VFT is connected to the seven transmembrane domain by a cysteine-rich domain, which is responsible for the activation of G-protein (Niswender and Conn, 2010; Kniazeff et al., 2011). These receptors exist as a constitutive dimer by forming of a di-sulphide linkage between the N-terminal domains of two receptors (Doumazane et al., 2011; El Moustaine et al., 2012).

Ligand binding causes a closure of the VFT, which triggers conformational changes responsible for G-protein activation. These conformational changes are conveyed by cysteine-rich domain to the seven transmembrane domain and C-termini (Muto et al., 2007). C-termini activation causes

liberation of $\beta\gamma$ subunits of G proteins which inhibit adenylyl cyclase and reduce the formation of cyclic AMP. C-terminal activation also causes inhibition via activation of GIRK channels or inhibition of voltage-dependent Ca^{2+} channels (Conn and Pin, 1997). The mGlu II receptors may also couple to other signaling pathways such as protein kinase C which regulates synaptic transmission (Macek et al., 1998).

The mGlu II receptors are widely distributed through the CNS of mammals and are expressed in glial cells and neurons (Ferraguti and Shigemoto, 2006). Expression of mGlu II receptors in different peripheral tissues such as skin and myenteric plexus of the ileum has been demonstrated (Carlton et al., 2001; Chen and Kirchgeßner, 2002). After injury or inflammation glutamate is released into the subcutaneous tissue by keratinocytes which activates mGlu II receptors on primary afferent fibers (Genever et al., 1999; Miller et al., 2011). This endogenous activation of mGlu II receptors serves as a negative feedback mechanism and inhibits pain signal transmission (cf. Davidson et al., 2016). Although mGlu II receptors are found on both pre- and postsynaptic membranes, electrophysiological evidence suggest that they are mainly located presynaptically (Pin and Duvoisin, 1995). The main function of mGlu II is a modulatory role, regulating synaptic plasticity and neuronal excitability (Anwyl, 1999).

1.2.2.3 Crosstalk between G-protein receptors

Functional cross-talk between two different members of GPCRs including family C receptors has been demonstrated in many studies (Werry et al., 2003; Prezeau et al., 2010). Mammalian cells express several types of G-protein-coupled receptors, which upon activation, trigger multiple signaling pathways. Indeed, it has been suggested that these different signal transduction pathways can interact to integrate and fine-tune cellular responses (Flaherty et al., 2008). These

interactions imply important physiological roles, and in pathophysiological roles, may serve as a novel therapeutic drug targets to treat many neurological pathologies including pain (Agnati et al., 2003; Kaczor and Selent, 2011). In this thesis, cross-talk between GABA_B and mGlu II receptors has possible relevance to explanations of the atypical actions of isovaline.

Cross-talk may result from direct physical interactions between receptors (heterodimerization) or second messengers from different signaling pathways (Selbie and Hill, 1998; Milligan, 2006). Intramembrane receptor-receptor interaction between GPC-receptors can form heterodimers or even higher order oligomers (Milligan, 2009). These heterodimers can be formed by linking the two receptors with a disulfide bond (Breitwieser, 2004). They possess distinct functional profiles compared to their constituent homomers (Xu et al., 2014). The best example of such interaction is the mGlu II/5-HT_{2A} receptor heteromerization in native tissues (Gonzalez-Maeso et al., 2007).

Second messenger interaction is another form of crosstalk between GPC-receptors. This signaling crosstalk was observed in native and transfected cells without detecting physical interaction (Prezeau et al., 2010). An example of second messengers interaction is the interaction between GABA_B and mGlu_{1a} receptors (Rives et al., 2009). In a native and heterologous system expressing both receptor types, Rives et al., (2009) showed that the activation of GABA_B receptors potentiated mGlu_{1a}-mediated responses. This effect was observed without detection of cell-surface co-immunoprecipitation or energy transfer between GABA_B and mGlu_{1a} receptors. Thus, G-protein-mediated signaling can interact both temporally and spatially without the requirement of physical association. Novel drugs that target multiple receptor systems can be utilized as a pharmacological tool to explore the physiological functions of these GPCR

heteromers and also the treatment of several pathological disorders with high selectivity (Ferre et al., 2014). In this thesis, we examine possible involvement of crosstalk in isovaline action.

1.2.3 Ventrobasal nuclei of thalamus

In broad terms, the ventrobasal nuclei can be defined as a discrete group of neurons that relay somatosensory signals to structurally and functionally distinct areas of the cerebral cortex.

Throughout this thesis, we refer to the area studied as “ventrobasal” which includes the combined ventralposterolateral (VPL) and the ventral posteromedial (VPM) nuclei (Jones and Yang, 1985). Ventrobasal neurons are essential participants in the modulation of somatosensory information ascending the neuraxis (Sherman, 2005). Medial lemniscus provides the major somatosensory inputs to ventrobasal neurons including information on touch, proprioception and pressure (Tsumoto and Nakamura, 1974; Jones, 1991). Information about itch, nociception and temperature is transmitted to the ventrobasal neurons via the spinothalamic pathway (Welker, 1973). In rat ventrobasal thalamus, the medial lemniscal and trigeminothalamic and spinothalamic projections terminate in the same areas (Ma et al., 1986). Ventrobasal neurons relay somatosensory information to layer I to VI of primary somatosensory cortex (Herkenham, 1980). Ventrobasal neurons receive excitatory feedback projections from corticothalamic neurons of layer VI (Zhang and Deschenes, 1997; Alitto and Usrey, 2003). This reciprocal thalamo-corticothalamic interaction is thought to play an important role in processing somatosensory information including nociception and maintaining consciousness (Jung et al., 2004; Steriade, 2006). Abnormal activities or lesions of thalamocortical neurons may lead to pathological manifestations such as pain states (Yen and Lu, 2013).

In the ventrobasal nuclei, there are two types of neurons - thalamocortical neurons which comprise the majority and a small number of local interneurons (Yen and Jones, 1983; Harris and Hendrickson, 1987). Low-threshold Ca^{2+} spike (LTS) is an unique electrophysiological property of thalamocortical neurons. The LTS can be used as a criterion for identifying thalamocortical neurons, which can be evoked by depolarization from resting potentials more negative than -65 mV (Jahnsen and Llinas, 1964).

The main excitatory neurotransmitter released onto thalamocortical neurons is glutamate, released by stimulation of corticothalamic, medial lemniscal and trigemino-spinothalamic pathways (Mountcastle et al., 1963; Harris and Hendrickson, 1987; Salt and Eaton, 1996; Zhang and Deschenes, 1997; Sherman, 2016). GABA is the main inhibitory transmitter released onto thalamocortical neurons by stimulation of nRt and zona incerta (Bartho et al., 2002; Gentet and Ulrich, 2003) and possibly by local circuit neurons (Rainey and Jones, 1983; Harris and Hendrickson, 1987). Both GABA and glycine mediate inhibition of thalamocortical neurons evoked by stimulation of the medial lemniscal pathways, some of which may be disynaptic (Ghavanini et al., 2005; 2006). The heterogeneity of synaptic inputs is crucial to thalamocortical processing, gating and information transfer to the primary somatosensory cortex (Huguenard and Prince, 1994; Sherman and Guillery, 1996).

Stimulation of the medial lemniscus also results in activation of excitatory postsynaptic currents (EPSCs) in addition to inhibitory postsynaptic currents (IPSCs) in thalamocortical neurons of ventrobasal nuclei. These EPSCs are sensitive to blockade by antagonists of AMPA and NMDA receptors (Huntsman and Huguenard, 2000; Miyata and Imoto, 2006). The IPSCs or inhibitory

postsynaptic potentials (IPSPs) evoked by stimulation of the medial lemniscus or nRt are sensitive to blockade by GABA_A receptor antagonists and less often by glycine_A receptor antagonists (Browne et al., 2001; Turner and Salt, 2003; Ghavanini et al., 2005, 2006). Activation of ionotropic and/or metabotropic receptors for both GABA and glutamate at postsynaptic sites in the thalamus may affect somatosensory processing including pain signals (Turner and Salt, 2003; Chang et al., 2015). Metabotropic receptors for glutamate and GABA may modulate presynaptic release of amino acids in neurons of ventrobasal nuclei which receive nociceptive inputs (Potes et al., 2006; cf. Chiechio and Nicoletti, 2012). The latter possibility is considered in this thesis.

1.2.4 Isovaline

Isovaline (2-amino-2-methylbutyric acid) is a non-proteinogenic α -amino acid with close chemical similarity to endogenous amino acids, especially GABA and glycine (Figure 1.2). It was found originally in carbonaceous meteorites (Kvenvolden et al., 1970; Zhao and Bada, 1989). There is recent evidence that isovaline can be produced by several strains of microfungi (Bruckner et al., 2009). Isovaline is a chiral molecule and therefore has two stereoenantiomers, R- and S-isovaline. Both enantiomers can be actively transported across the small intestine via endogenous amino acid transporters (Evered et al., 1967).

In previous study, we have demonstrated that isovaline produces antinociceptive effects in acute and chronic pain models, including allodynia induced by the glycine antagonist, strychnine (MacLeod et al., 2010). Systemic application of isovaline blocks nociception in several rodent models without causing signs of sedation or respiratory depression. It is thought that the

antinociceptive effects result from its actions on peripheral receptors, partly a consequence of isovaline restricted distribution in peripheral tissues (Shiba et al., 1989; Whitehead et al., 2012). However, isovaline may reach brain concentrations that are sufficient to produce anxiolytic and antiepileptic effects in certain rodent models (Sase et al., 2013; Yu et al., 2014, 2015). In summary, isovaline can serve as a novel analgesic that may offer effective and safe pain relief.

Previous studies by Cooke et al. (2009) in brain slices showed that isovaline inhibits the activity and excitability of thalamocortical neurons by increasing K^+ conductance. This inhibition is long-lasting or poorly reversible but preventable by prior administration of the GABA_B antagonist CGP35348 or CGP52432. Pre-treatment of thalamocortical neurons with the positive allosteric modulator of GABA_B receptors, CGP7930, potentiates isovaline-induced inhibition or conductance increases (cf. Cooke et al., 2012). Application of picrotoxin, a chloride channel blocker of GABA_A and GABA_C receptors, had little or no effect on isovaline inhibition. The effect of isovaline is not susceptible to antagonism by d-tubocurarine, naloxone or strychnine. Cooke et al. (2012) suggested that the action of isovaline was mediated through G-protein coupled GABA_B receptors since intracellular application of a non-hydrolyzable substrate of GTP can block its inhibitory effect. In the studies of Cooke et al. 2009; 2012, the actions of isovaline were different from those of baclofen. Firstly, isovaline has a slow onset of action lasting for up to 2 h, and is not readily reversible. Secondly, co-application of isovaline with baclofen has no additive, sub-additive or synergistic effects. Thirdly, not all (<50%) baclofen-sensitive neurons respond to application of isovaline whereas most thalamocortical neurons respond to baclofen with an increase in K^+ conductance. For these reasons, the exact mechanism of isovaline remains unclear and requires further investigations.

1.2.5 LY354740

LY354740 (1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid) is a highly selective and very potent agonist of mGlu II receptors with an IC_{50} in the nanomolar range (Figure 1.1; Monn et al., 1997; Schaffhauser et al., 1997). It was developed in 1997 as an alternative to non-selective agonists such as (1SR,3RS)-ACPD, DGC-IV and L-CCG-IV (Schoepp et al., 1997). Like all mGlu II agonists, LY354740 has α -amino and carboxylic moieties and a conformationally restricted structure (Monn et al., 1999; Pin et al., 1999). It can cross the blood-brain barrier and has a limited oral bioavailability, presumably due to a poor ability to penetrate membranes (Johnson et al., 2002). For many research groups, LY354740 (also known as Eglumegad) has been utilized as a template for mGlu II receptor agonists (Brauner-Osborne et al., 2007).

In the formalin pain model, intraperitoneal injection of LY354740 produces a dose-dependent reduction in paw-licking time in phase II (Simmons et al., 2002). This analgesic effect occurs without affecting motor function. The antinociceptive effects of LY354740 have been also demonstrated in arthritis pain model in rat (Li and Neugebauer, 2006). In animal behavioral models, LY354740 has also been found to be effective in treating anxiety (Helton et al., 1998), psychosis (Moghaddam and Adams, 1998), drug addiction (Vandergriff and Rasmussen, 1999; Adewale et al., 2006), seizures (Kłodzinska et al., 1999) and neuronal injury (Allen et al., 1999). Currently, the therapeutic potential of LY354740 and related compounds is being investigated in several clinical trials. However, tolerance to the effects of LY354740 due to continuous activation of mGlu II receptors and poor oral bioavailability may limit its clinical use (Bergink and Westenberg, 2005; Dunayevich et al., 2008; Ahnaou et al., 2015). Thus, alternative

approaches are required to further exploit mGlu II receptors as potential therapeutic targets in various neurological disorders.

1.2.6. Baclofen

Baclofen ((4-amino-3-(4-chlorophenyl) butanoic acid) is an unnatural α -amino acid that is considered as the prototypical agonist of GABA_B receptors (Figure 1.1). Currently, baclofen serves as the only clinically available GABA_B receptor agonist, which has been approved for clinical use as a muscle relaxant for the treatment of spasticity (cf. Froestl, 2010). In 1962, baclofen was originally synthesized as a lipophilic derivative of GABA in order to improve blood-brain barrier penetration (Cates et al., 1984). Since it has a chiral center, baclofen exists as S- and R-enantiomers. In rat brain, the R-baclofen has higher binding affinity to GABA_B receptors with an IC₅₀ of 0.04 μ M, compare to the S-enantiomer which has an IC₅₀ of 33 μ M (Hill and Bowery, 1981; Froestl et al., 1995). Baclofen can be transported across the blood-brain barrier via large neutral amino acid transporter (Audus and Borchardt, 1986).

Besides its use as muscle relaxant, baclofen has also been shown to be effective in several pain conditions such as trigeminal neuralgia (Fromm et al., 1984), cluster headache (Hering-Hanit and Gadoth, 2001), and migraine (Hering-Hanit, 1999). There is a general belief that baclofen produces analgesia by reducing the release of excitatory neurotransmitters (e.g., glutamate and substance P) from primary afferent terminals (cf. Enna and McCarson, 2006). However, the beneficial clinical effects of baclofen are associated with side effects including hypothermia (cf. mice, Whitehead et al., 2012), sedation (Sawa and Paty, 1979), depression (Nakagawa et al., 1996), and muscular weakness (Ertzgaard et al., 2017). Chronic use of baclofen may result in the

development of tolerance and withdrawal signs which may progress rapidly after abrupt withdrawal (Garabedian-Ruffalo and Ruffalo, 1985; Nielsen et al., 2002). Therefore, further investigations for the development of a new analgesic/GABA_B agonist with tolerable side effects are highly warranted.

1.2.7 PGE₂-induced pain model

Prostaglandin E₂ (PGE₂) is one of the main chemical mediators that is released by leukocytes, endothelial cells and platelets in response to tissue injury or inflammatory insult. PGE₂ plays an important role in chronic pain conditions such as nociceptive, inflammatory and neuropathic pain (Dall'Acqua et al., 2014; St-Jacques and Ma, 2014; Natura et al., 2013). It is an arachidonic acid metabolite, synthesized by cyclooxygenases and prostaglandin E synthases (Nathoo and Barnett, 2004). The effects of PGE₂ are mediated by designated G protein-coupled receptor subtypes (EP₁₋₄) which are expressed by primary sensory neurons (Sugimoto et al., 1994; Oida et al., 1995). In addition to its diverse physiological functions, PGE₂ has been demonstrated to play an important role in pain processing and inflammation (Samad et al., 2002). The PGE₂ pain model is based on intraplantar administration of low-dose of PGE₂ which enhances pain sensation by sensitizing the peripheral terminals of sensory fibers (Dirig and Yaksh, 1999; Tilley et al., 2001). Such sensitization can lead to the development of mechanical allodynia which is pain caused by a tactile stimulus (such as light touch of the skin) that would not normally provoke pain (Merskey and Bogduk, 1994). Mechanical allodynia can be assessed by measuring the paw withdrawal threshold using the von Frey method (Bennett and Xie, 1988). The PGE₂ pain model has many advantages over the other models. For example, it is straightforward to perform and allows differentiation between the responses of both hind paws and provides stable level of pain

hypersensitivity over time which may last for up to one hour (Kassuya et al., 2007; Barrot, 2012). It has been used for screening potential analgesics and studying their mechanisms of actions (Kawabata, 2011; Barrot, 2012). Previous reports have demonstrated a good correlation between results obtained from the PGE₂ animal model and humans with pain (Ferreira, 1972; Kuhn and Willis, 1973). In this thesis, we used the PGE₂ model to investigate the effects of the new analgesic isovaline, as well as agonists and antagonists of mGlu II and GABA_B receptors.

1.3 Experimental rationale, objectives and hypothesis

Current strategies for discovery of analgesics are targeting excitatory and inhibitory receptors at synaptic and extrasynaptic sites in peripheral and central nervous systems. Analgesic drug research has moved towards reducing glutamatergic excitation and mimicking GABAergic and glycinergic inhibition.

Glutamate has a pivotal role in the processing of nociceptive information by activating two types of receptors: ionotropic and metabotropic receptors (cf., Conn et al., 2003). Modulation of glutamate excitation via blockade of ionotropic glutamate receptors and/or activation of certain metabotropic glutamate receptors have been proposed as novel therapeutic approaches to relieve pain (Urban et al., 1994; Simmons et al., 1998).

GABA acts on ionotropic and metabotropic types of receptors. The ionotropic GABA_A (Gage, 1992) and GABA_C receptors (Schlicker et al., 2004) which directly couple to chloride channels, mediate fast synaptic inhibition. Metabotropic GABA_B receptors (Bowery, 1999) located presynaptically, inhibit release of transmitter through inhibition of high voltage activated Ca²⁺

channels (Misgeld et al., 1995; Wu and Saggau, 1997). GABA_B receptors located postsynaptically, mediate slow inhibition through activation of K⁺ conductance (Bettler et al., 1998; Kaupmann et al., 1998). Many studies have implicated GABA_B receptors in the modulation of nociceptive information (Castro-Lopes et al., 1995; Lin et al., 1996).

The thalamus plays an important role in the transmission of painful stimuli from periphery to higher somatosensory centres (Guilbaud et al., 1994; Willis, 1995). Thalamic ventrobasal nuclei process and transmit nociceptive inputs from the spinothalamic tract terminations (Guilbaud et al., 1994; Price, 1995). The above studies implicate important roles of GABA_B and mGlu II receptors in these pain pathways.

In previous reports, we have shown that isovaline produces antinociceptive effects in pain models without producing sedation or respiratory depression (MacLeod et al., 2010). In the PGE₂-pain model, isovaline produces antalgic effects by activating GABA_B receptors. However, the isovaline analgesic effects are not completely blocked by application of GABA_B antagonist CGP52432, indicating other receptor involvement (Whitehead et al., 2012). Isovaline and the GABA_B agonist baclofen inhibit neurons, by activating metabotropic GABA_B receptors and increasing K⁺ conductance (Cooke et al., 2009, 2012). Since isovaline does not activate many baclofen-sensitive neurons, we considered the possibility that isovaline activated other inhibitory receptor types. As mentioned above, metabotropic glutamate receptors (mGluRs) and GABA_B receptors belong to the same family C of G-protein coupled receptors, and are distributed throughout the CNS and PNS. Activation of group II mGluRs inhibits nociceptive neurons also by increasing conductance for K⁺. Therefore, we hypothesize that isovaline can inhibit nociceptive neurons and produce analgesia by activating mGluRs.

In the first manuscript, our objective was to determine whether the analgesic effects of isovaline are mediated by mGlu II receptors in PGE₂-induced allodynia. In the second manuscript, our objective was to examine the presynaptic effects of isovaline, LY354740 and baclofen. In the third manuscript, our objective was to determine possible similarities or differences of isovaline's effects from those of baclofen and LY354740 in a standard preparation used for assaying GABA_B agonists.

This thesis will shed light on novel receptor actions of isovaline which serves as a prototypic analgesic, on GABA_B and metabotropic glutamate receptors.

1.4 Figures

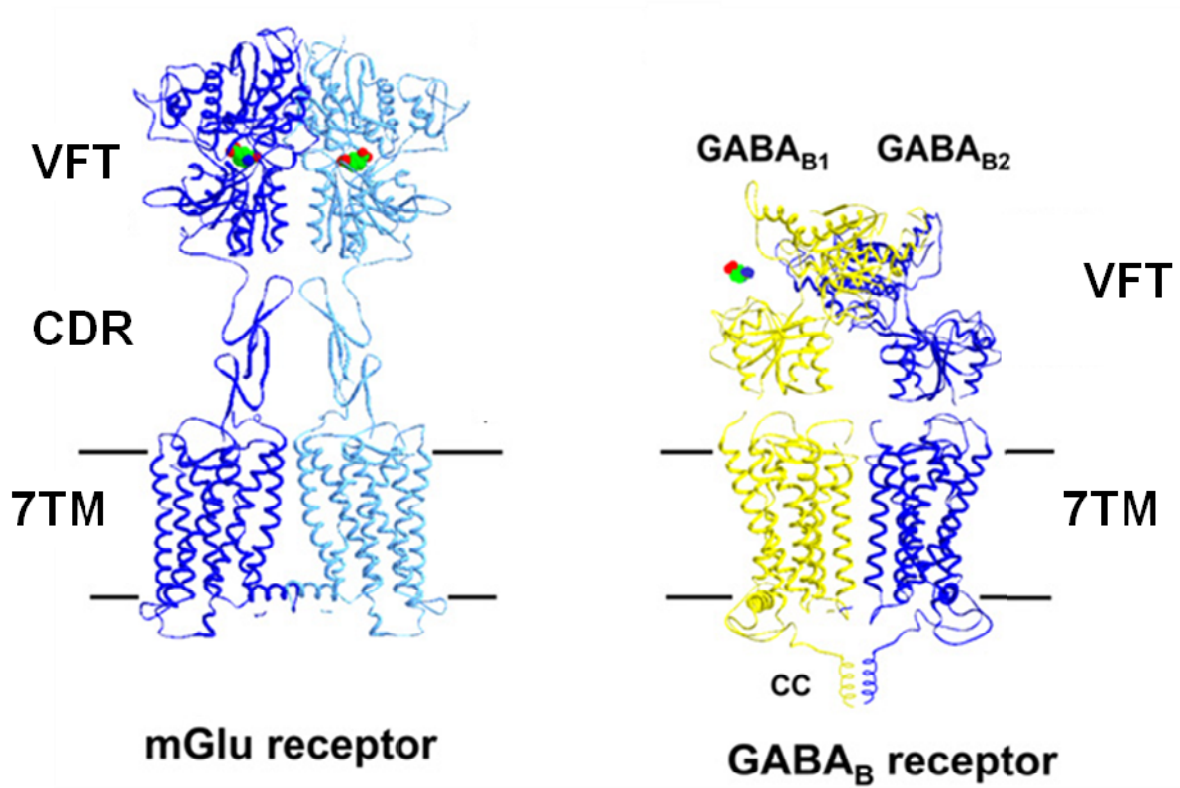
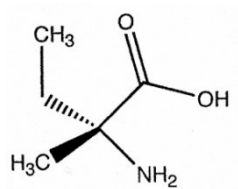
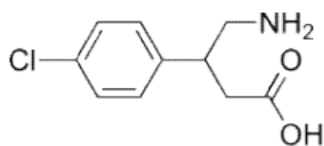


Figure 1.1 Schematic representation of structural organization of GABA_B and mGlu receptors.

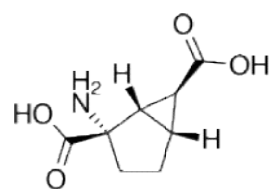
Figure adapted from (Rondard et al., 2011).



R-isovaline



Baclofen



LY354740

Figure 1.2 Chemical structures of R-isovaline, baclofen and LY354740.

Chapter 2. Antagonism of Group II Metabotropic Glutamate Receptors Prevents the Antiallodynic Effects of Isovaline

2.1 Introduction

Isovaline is an unusual amino acid that has antinociceptive properties in acute and chronic pain models. Consistent with its structural similarity to γ -aminobutyric acid (GABA), isovaline produces analgesia at least in part by activating GABA_B receptors in the periphery (Whitehead et al., 2012). While intrathecal application of isovaline may have antinociceptive effects, peripheral application results in analgesia without central nervous system (CNS) side effects presumably due to limited passage of isovaline across the blood-brain barrier (Shiba et al., 1989; MacLeod et al., 2010).

Studies on brain slices showed that bath application of isovaline increases membrane K⁺ conductance of neurons by activating GABA_B receptors (Cooke et al., 2009). The resulting inhibition of excitability by isovaline is attenuated by intracellular non-hydrolyzable GTP analogues, suggesting G-protein mediation. Although some *in vitro* actions did not mimic the effects of the prototypical GABA_B agonist (Cooke et al., 2012), we subsequently found the *in vivo* pharmacology of isovaline to be similar to GABA_B agonists, baclofen or GABA when applied intraplantarly (Whitehead et al., 2012). Co-application of isovaline and baclofen to thalamocortical neurons does not result in additive or synergistic effects and many baclofen-sensitive neurons do not respond to isovaline (Cooke et al., 2012). In *in vivo* studies, isovaline's analgesic actions are incompletely antagonized by a GABA_B antagonist, CGP52432, ((3-[[[3,4-dichlorophenyl)methyl]amino]propyl] diethoxymethyl) phosphinic acid) in contrast to baclofen- or GABA-mediated analgesia, suggesting that isovaline may interact with an additional receptor

mechanism (cf., Neale and Salt, 2006; Whitehead et al., 2012). Therefore, we considered the possibility that isovaline may interact with other G-protein coupled receptors (GPCRs) that mediate inhibitory effects.

Metabotropic glutamate receptors (mGlu receptors) belong to the same family C of GPCRs as GABA_B receptors. While group I mGlu receptors are excitatory, group II and III inhibit nociceptive neurons by increasing K⁺ conductance (Dutar et al., 2000; Yang and Gereau, 2003). Group II and III mGlu receptors are negatively coupled to adenylyl cyclase (Schwartz and Alford, 2000; Goudet et al., 2009) and may provide a novel therapeutic approach to relieve pain (Urban et al., 1994; Simmons et al., 1998). Our objective in the present studies was to test the hypothesis that isovaline's analgesic actions result from mGlu II or III receptor activation in a murine model of allodynia induced by intraplantar injection of prostaglandin E₂ (PGE₂).

2.2 Experimental procedures

2.2.1 Animals

The Animal Care Committee of The University of British Columbia approved these experiments. Adult female CD-1 mice (weight, 25-30 g) were maintained under conditions of a 12 h dark-light cycle and room temperature of 21 °C. Food and water were available ad libitum and each animal was used only once in the experiments which were conducted between 9:00 am and 17:00 pm.

2.2.2 Drugs

The group II mGlu agonist LY354740, antagonist LY341495, and positive allosteric modulator LY487379, as well as the GABA_B agonist RS-baclofen and the GABA_B antagonist CGP52432 were purchased from Tocris Cookson (Ellisville, MO, USA). Stock solutions were prepared by dissolving these drugs in equimolar amounts of NaOH and stored at 4 °C. R-isovaline was synthesized by Biofine International Inc. (Vancouver, B.C., Canada). MAP-4 was purchased from Precision Biochemicals Inc. (Vancouver, B.C., Canada). PGE₂ was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Solutions of CGP52432, R-isovaline, and PGE₂ for injection were freshly prepared from stock solutions just before an experiment. The drugs were diluted to their final concentration in normal saline with a pH of 7.4 and injected in a total volume of 20 µl subcutaneously through the plantar surface of the mouse hindpaw. Isovaline or LY354740 or baclofen, without or with an antagonist, was co-injected in the PGE₂-solution.

2.2.3 Mechanical allodynia induced by prostaglandin E₂

Experiments were conducted in a randomized and blinded manner in groups of 6-24 animals.

The methods used for determining drug effects on allodynia have been described in detail (Kassuya et al., 2007; Whitehead et al., 2012). For 10-15 min prior to an experiment, the animals were acclimatized on an elevated wire mesh platform. The paw withdrawal threshold was measured using von Frey filaments (Stoelting, Wood Dale, IL, USA). We defined paw withdrawal threshold as the minimum von Frey filament value required to elicit robust withdrawal in at least five out of six trials each separated by 5 s. Thresholds were measured at time 0 which was <1 min before the injection of PGE₂ (0.1 nM) through the plantar surface into the hindpaw to induce mechanical allodynia. A second measurement of mechanical threshold was performed at 15 min after injecting PGE₂. Von Frey filaments of increasing values (0.008-2.0 g) were applied to the plantar surface of the hindpaw until bending of the filament was observed (six trials per filament). Since the sensory response to von Frey filaments is linearly related to the logarithm of the stimulus, the log of the gram-response was used (Mills et al., 2012).

We determined the effects of LY354740 (10 pmol; Han et al., 2006), baclofen (0.3 µmol; Whitehead et al., 2012) and isovaline (14 µmol; Whitehead et al., 2012) on PGE₂ allodynia. The group II mGlu antagonist LY341495 (0.2 nmol; Yang and Gereau, 2003) and the GABA_B antagonist CGP52432 (20 nmol; Whitehead et al., 2012) or the group III mGlu antagonist MAP-4 (0.5 nmol; Hallock et al., 2009) were co-applied with each of the agonists. The percentage change in paw withdrawal threshold was measured at various doses of isovaline for constructing a dose-response relationship. Each data point is represented as an average of multiple measurements in different animals.

We determined the effects of isovaline at ED₁₀ (1 μmol) on PGE₂-induced allodynia in the absence and presence of the positive allosteric modulator LY487379 (0.3 nmol; Poisik et al., 2005). We also investigated potential interactions of baclofen and LY354740 by examining their effects before and after co-application with the antagonists, LY341495 and CGP52432. The agonists were injected into the ipsilateral paw and PGE₂ was injected into both paws. The contralateral paw injected with the vehicle served as a control.

2.2.4 Statistical analysis

Data are presented as mean ± 95 % confidence interval (CI), with n as the number of animals. Prism software (GraphPad, San Diego, CA, USA) was used for the statistical analyses. We used ANOVA followed by Bonferroni's Multiple Comparison Test to compare treatment effects on PGE₂-induced allodynia. For the dose response relationships, we used ANOVA followed by Bonferroni's Multiple Comparison Test to compare the effects of isovaline relative to baseline values. Estimation of ED₅₀s was done by fitting the data using a Hill equation ($Y = \text{bottom} + [\text{top} - \text{bottom}] / [1 + 10^{([\log \text{ED}_{50} - X] * \text{Hill slope})}]$) where the top and bottom are plateaus in the units of the y-axis. A *p* value of < 0.05 was used for statistical significance.

Mills and co-workers showed that a more rigorous analysis in determining changes in pain thresholds from von Frey data was obtained by the use of a logarithmic scale, in accord with Weber's Law. This was found to be necessary if parametric analysis is used (Mills et al., 2012).

2.3 Results

2.3.1 Analgesic effects of isovaline

We first determined a dose-response relationship for the effects of isovaline on allodynia, obtaining an estimate of an ED₅₀. Application of R-isovaline prevented PGE₂-induced allodynia in dose-dependent manner (Figure 1). Applying isovaline in single s.c. doses of 1, 3.5, 7, 14, and 28 µmol yielded an ED₅₀ of 6 µmol (95% CI 3–12). Figure 2 shows that isovaline (7 µmol) co-injected with PGE₂ prevented the allodynic response. As in a previous report using RS-isovaline (Whitehead et al., 2012), the analgesic effect of R-isovaline was localized to the ipsilateral hindpaw (n = 6; Kruskal-Wallis test, See Appendix A).

2.3.2 Group II mGlu antagonism blocked the analgesic effects of isovaline

When co-applied with isovaline at its ED₅₀, the mGlu II antagonist, LY341495, (0.2 nmol) the analgesic effect of isovaline was absent (Figure 2). The antagonist by itself had no effect on the baseline withdrawal threshold (cf. Figure 4). To determine if activation of group III mGlu receptors contributed to analgesic effects of isovaline, we co-applied isovaline with the selective group III antagonist, MAP-4 (0.5 nmol). MAP-4 did not alter the analgesia produced by isovaline (Figure 2). LY341495 at 0.2 nmol was selective for group II receptors because it did not antagonize the reduction in PGE₂-allodynia produced by a group III agonist (L-AP4; 2 nmol; n = 4, data not shown). These results indicated that while group III receptors did not contribute significantly, group II receptors participated in the analgesic effects of isovaline.

2.3.3 Time course of analgesia effects of isovaline

Figure 3 shows the time course of the analgesic effect of isovaline (single injection at its ED₅₀). Saline, isovaline, and isovaline in combination with LY341495 were injected into the hindpaws of five groups of animals. The paw withdrawal threshold was measured using von Frey filaments at 10-min intervals. An injection of PGE₂ induced allodynia that lasted for ≥ 60 min in the control group. Isovaline at its ED₅₀ blocked the allodynia for 60 min and this effect was absent on co-injection of isovaline with LY341495 (0.2 nmol).

2.3.4 Group II mGlu agonist produced an analgesia effect similar to isovaline

Figure 4 shows that an injection of the mGlu II agonist, LY354740 (10 pmol), produced analgesia similar to isovaline. As in the case of isovaline (Whitehead et al., 2012), the analgesic effect of LY354740 was localized to the injected side and was not observed on the contralateral hindpaw (n = 5; data not shown). The effect of LY354740 was antagonized by co-injected LY341495 (0.2 nmol).

2.3.5 Positive allosteric modulation of group II mGlu receptors potentiated isovaline's analgesic effect

Figure 5 shows that LY487379, a positive allosteric modulator of group II mGlu receptors, potentiated the action of isovaline (ED₁₀). Alone, LY487379 had no effect on PGE₂-induced allodynia, suggesting no endogenous activation of mGlu II receptors. This suggests that the analgesic effect of isovaline had occurred through activation of mGlu II receptors which were susceptible to modulation by LY487379.

2.3.6 Absence of crosstalk between mGlu II and GABA_B receptors

Since group II mGlu receptors and GABA_B receptors are both C family members of GPCRs, we determined responses to potential crosstalk between these receptor types. As shown in Figure 6, application of the mGlu II antagonist, LY341495, did not affect the actions of baclofen. While the GABA_B antagonism reduces the peripheral analgesic effect of isovaline (Whitehead et al., 2012), it does not alter the analgesic effect of the mGlu II agonist, LY354740 (Figure 7). This indicates an absence of crosstalk between mGlu II and GABA_B receptors.

2.4 Discussion

Our objective was to determine if isovaline's actions against PGE₂-allodynia involve mGlu receptor activation, which would complement the known mediation by peripheral GABA_B receptors (Whitehead et al., 2012). In the PGE₂-model, we demonstrated that isovaline prevented allodynia through mGlu activation. The effect was not present in the contralateral paw and was confined to the site of injection, indicating a peripheral action. The specific activation of the mGlu II receptors was independent of GABA_B receptors. However, activation of GABA_B receptors also produced analgesia in the mouse preparation. Our data indicate that isovaline acts peripherally on both GABA_B and mGlu II receptors to produce analgesia.

Involvement of mGlu receptors in PGE₂-induced allodynia was inferred from the effects of mGlu agonist applications. Activation by the selective group II agonist, LY354740, prevented the reduction in paw withdrawal threshold in the PGE₂ mouse model. Peripheral activation occurred since the analgesic effect of locally injected LY354740 was restricted to the ipsilateral limb. In the paw pad, nociceptive primary afferent neurons express mGlu II receptors (Carlton et al., 2001). High concentrations of LY354740 activate group I mGlu receptors (cf. Schoepp et al., 1999). We used low concentrations of LY354740 and did not observe an increase in mechanical hypersensitivity as would be expected from group I activation (Walker et al., 2001). The results are in agreement with previous reports (Yang and Gereau, 2003; Yamamoto et al., 2007) that group II activation by LY354740 produces analgesia in rodent pain models.

We demonstrated for the first time that group II receptors participate in isovaline-induced analgesia. An antagonist of mGlu II receptors (LY341495) prevented subcutaneous isovaline

from producing an analgesic effect. *In vitro* studies show that LY341495 is highly selective for group II receptors with an $IC_{50} = 21$ nM (Kingston et al., 1998; Odagaki et al., 2011). Much higher concentrations of LY341495 are required for group III antagonism (cf. Schoepp et al., 1999). We observed that a group III antagonist (MAP-4) failed to alter the analgesic effect of isovaline. The group II antagonist LY341495 at a low dose did not block the analgesic effect induced by the group III agonist (L-AP4). A positive allosteric modulator (LY487379) of group II receptors ($EC_{50} = 0.3$ μ M, Johnson et al., 2003; Schaffhauser et al., 2003) increased the analgesic effects of isovaline. These observations demonstrate the selective participation of mGlu II receptors in the analgesia due to isovaline.

Combined with our previous findings, the present results demonstrate that isovaline activation of either mGlu II or GABA_B receptors can produce an analgesic effect. We did not observe evidence for crosstalk/activation of mGlu or GABA_B receptors in the responses to isovaline. The GABA_B antagonist, CGP52432, did not alter the mGlu II agonist effects of LY354740 whereas an antagonist of mGlu II receptors did not affect the analgesia produced by baclofen. Isovaline apparently acts independently on the inhibitory receptor systems of glutamate and GABA. The independent actions of isovaline may involve an additional complex network. For example, LY341495 blockade of group II receptors may increase extracellular glutamate levels (cf. Schwann cells, Pinard et al., 2003; Wu et al., 2005). However it is not clear if resulting excitatory effects would mask the analgesic effect produced by GABA_B receptor activation.

In conclusion, the results of the present studies demonstrate that in addition to GABA_B receptors, peripheral mGlu II receptors mediate the analgesic actions of isovaline. Acting at both

peripheral metabotropic glutamate and GABA_B receptor targets and apparently devoid of CNS side effects, isovaline is a prototype of a new class of analgesics (Singh et al., 2013; cf. Fowler et al., 2014).

2.5 Figures

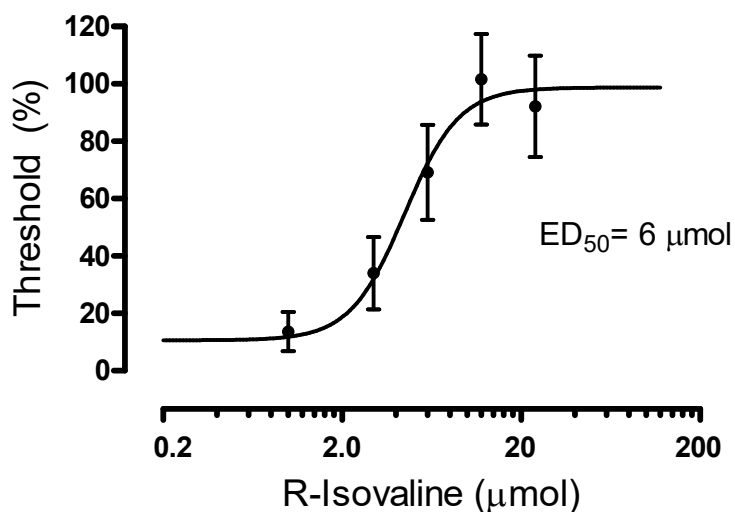


Figure 2.1. Dose-response relationship for isovaline's analgesic effects. The y-axis shows the percent of baseline paw withdrawal threshold to the application of von Frey filaments. The mechanical threshold was measured at 15 min after the injection of PGE₂. Each data point represents the response (mean \pm 95% CI) of intra-plantar injection of isovaline in naïve mice (n = 6-7). The estimated ED₅₀ was 6 μ mol.

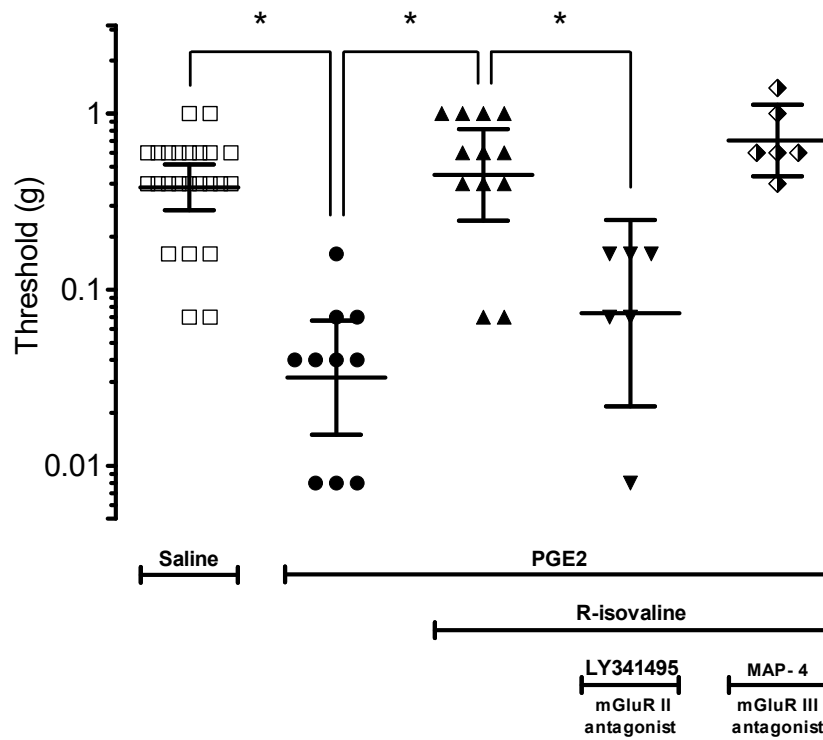


Figure 2. 2. Selective antagonism of isovaline's analgesic effects by LY341495 in naïve mice.

Each treatment cluster of data represents threshold force (g) exerted by von Frey filaments (means \pm 95% CI). Isovaline (14 μ mol) produced a significant reduction in allodynia induced by PGE₂ (0.002 pmol). This analgesia was blocked by mGlu II antagonist (LY341495, 0.2 nmol) but not by a mGlu III antagonist, MAP-4 (0.5 nmol). Each cluster shows responses at 15 min after PGE₂ intraplantar co-injection with drugs. * $P < 0.05$; one-way ANOVA followed by Bonferroni's Multiple Comparison Test (n = 6-24).

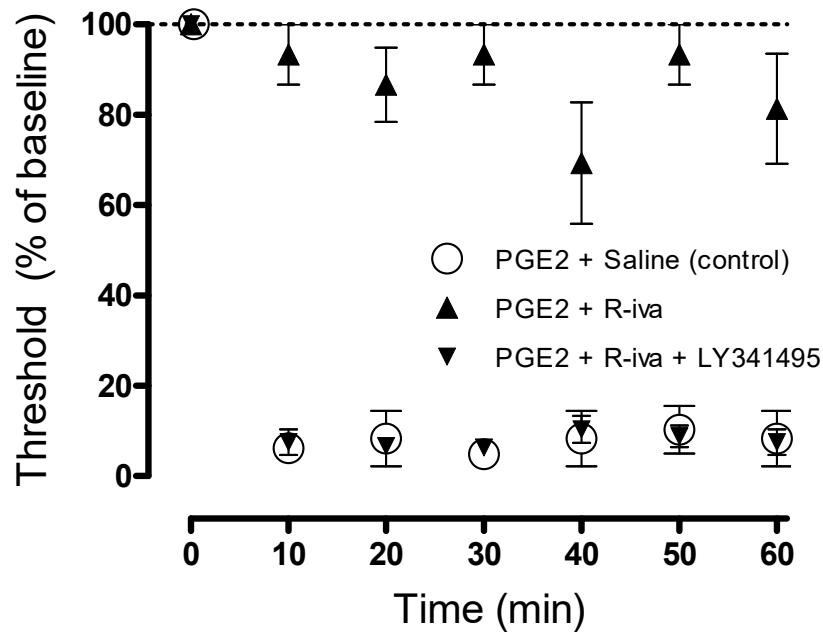


Figure 2.3. Time course of the analgesic effects produced by single intraplantar injection of isovaline at the ED₅₀ (6 μ mol). The y-axis shows the percent of baseline paw withdrawal threshold to the application of von Frey filaments. Data represent responses of 5 mice in each treatment group recorded at 10 min intervals after the injection of PGE₂ at time zero (means \pm 95% CI). At its ED₅₀, isovaline prevented the allodynia for 60 min. The analgesia was absent on co-injection of isovaline with LY341495 (0.2 nmol).

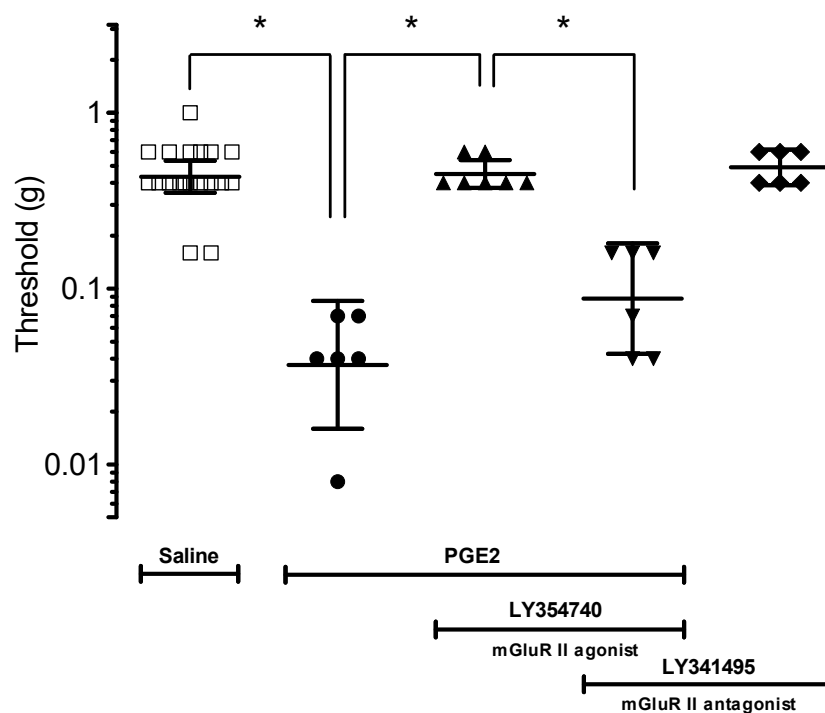


Figure 2.4. Analgesic effects of the mGlu II agonist, LY354740 in naïve mice. Each treatment cluster of points represents threshold force (g) exerted by von Frey filaments (means \pm 95% CI). LY354740 (10 pmol) produced significant blockade of allodynia induced by PGE₂ (0.002 pmol). The analgesia was antagonized by mGlu II antagonist LY341495 (0.2 nmol), which itself had no effect. Each cluster shows responses at 15 min after PGE₂ intraplantar co-injection with drugs.

* $P < 0.05$; one-way ANOVA followed by Bonferroni's Multiple Comparison Test ($n = 6-19$).

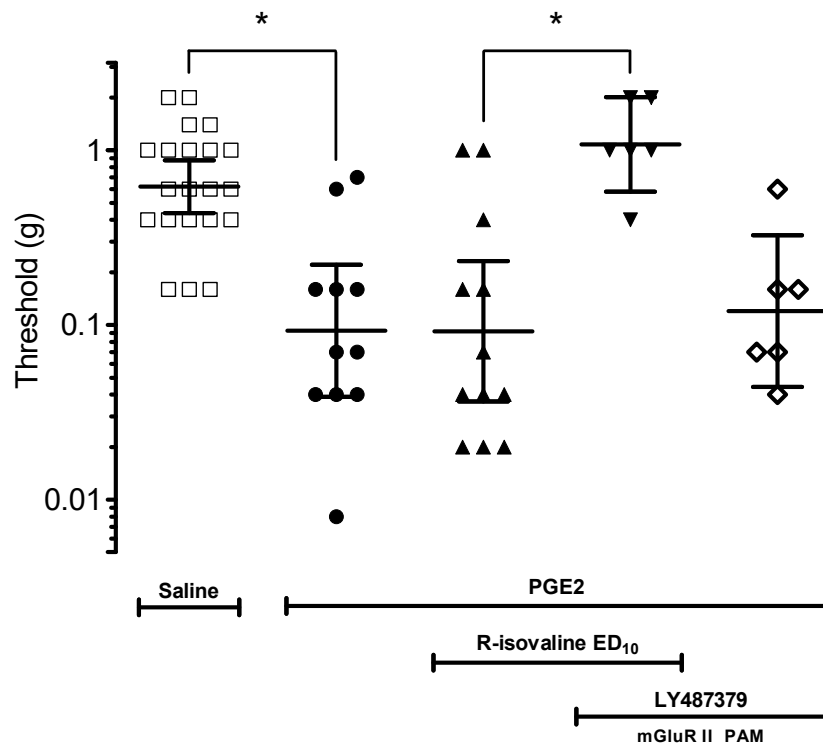


Figure 2.5. Isovaline's analgesic effect was potentiated by mGlu II positive allosteric modulator (LY487379) in naïve mice. Each treatment cluster of points represents threshold force (g) exerted by von Frey filaments (means \pm 95% CI). The ED₁₀ (cf. Fig 1) of isovaline did significantly reduce the allodynia induced by PGE₂. LY487379 (0.3 nmol) co-applied with ED₁₀ (1 μ mol) of isovaline prevented the allodynia produced by PGE₂. Each cluster shows responses at 15 minutes after PGE₂ intraplantar co-injection with drugs. * $P < 0.05$; one-way ANOVA followed by Bonferroni's Multiple Comparison Test (n = 6-21).

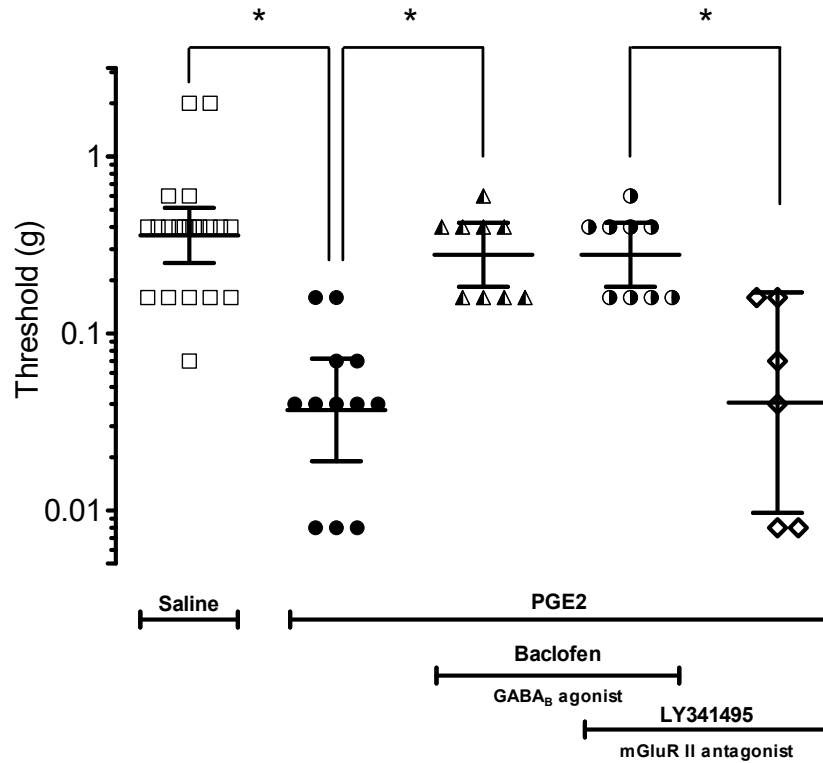


Figure 2.6. Group II mGlu antagonist LY341495 did not affect the analgesic effect of the GABA_B agonist baclofen in naïve mice. Each treatment cluster of points represents threshold force (g) exerted by von Frey filaments (means \pm 95% CI). LY341495 (0.2 nmol) had no effect on the analgesic effect produced by baclofen (0.3 μ mol). Each cluster shows responses at 15 min after PGE₂ intraplantar co-injection with drugs. * $P < 0.05$; one-way ANOVA followed by Bonferroni's Multiple Comparison Test ($n = 6-21$).

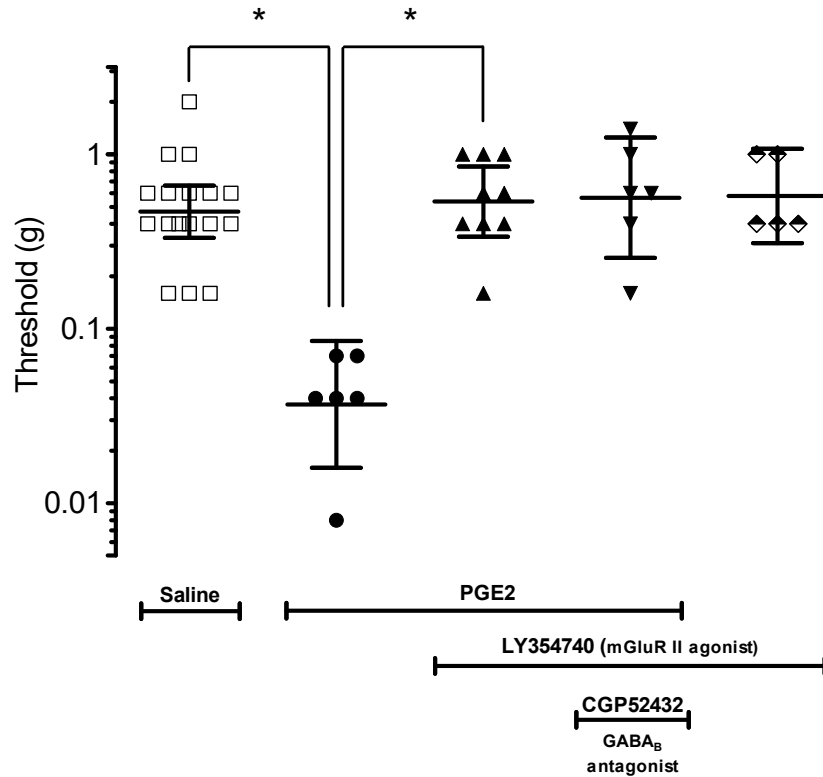


Figure 2.7. GABA_B antagonist CGP52432 did not alter analgesic effect of the mGlu II agonist LY354740 in naïve mice. Each treatment cluster of points represents threshold force (g) exerted by von Frey filaments (means \pm 95% CI). CGP52432 (20 nmol) had no effect on analgesia produced by LY354740 (10 pmol). Each cluster shows responses 15 min after PGE₂ intraplantar co-injection with drugs. * $P < 0.05$; one-way ANOVA followed by Bonferroni's Multiple Comparison Test ($n = 6-17$).

Chapter 3. Isovaline Activates Metabotropic Group II Receptors which Modulates GABA and Glutamate Release in Thalamocortical Neurons

3.1 Introduction

Isovaline is an unusual non-proteinogenic amino acid that exhibits antinociceptive properties in animal models. On systemic administration, isovaline distributes mostly to the periphery (cf. Shiba et al., 1989) and produces analgesia by acting on metabotropic GABA_B receptors for γ -aminobutyric acid (GABA) and group II (mGlu II) receptors for glutamate (Whitehead et al., 2012; Asseri et al., 2015). Antagonists of GABA_B- or mGlu II-receptors only partly block isovaline's antinociceptive actions in pain models (Whitehead et al., 2012; Asseri et al., 2015). Metabotropic group II and GABA_B receptors belong to family C of G-protein coupled receptors and have a similar functional profile. Immunohistochemical and in situ hybridization studies show mGlu II and GABA_B expression in thalamic nuclei (Ohishi et al., 1998; Liang et al., 2000; Gu et al., 2008). In neurons of ventrobasal nuclei, pre- or postsynaptic activation of these receptor subtypes affects somatosensory inputs and processing of pain-related signals (Chiechio & Nicoletti, 2012; Chang et al., 2015).

In some *in vivo* models, systemically administered isovaline may reach concentrations in the brain that are sufficient to produce anxiolytic and antiepileptic effects (Sase et al., 2013; Yu et al., 2014, 2015). In brain slice preparations which avoid the issue of blood-brain barrier penetration, isovaline acts partly like the prototypical GABA_B agonist, baclofen. Both amino acids inhibit action potential firing of thalamic neurons by increasing K⁺-conductance but their actions are not additive on co-administration (Cooke et al., 2012). Many baclofen-sensitive

neurons are unaffected by isovaline. These findings suggest that isovaline acts on multiple receptor systems. While GABA_B-antagonists prevent its inhibitory effects on firing (Cooke et al., 2012), it is not known whether mGlu II-antagonists block the inhibitory actions of isovaline on thalamic neurons.

Metabotropic group II receptors do not have a clear role in ventrobasal thalamus but participate in presynaptic modulation of GABAergic transmission in hippocampus and spinal cord (Gerber et al., 2000; cf. Sherman, 2014; Davidson et al., 2016). In the thalamus, high concentrations of the mGlu II agonist, LY354740, and less selective mGlu II agonists reduce inhibitory postsynaptic potentials (IPSPs) and inhibitory postsynaptic currents (IPSCs) evoked by electrical stimulation or physiological activation of thalamic reticular nucleus (nRt) (Salt and Eaton, 1995; Turner and Salt, 2003; Liu et al., 2015). Changes in the postsynaptic conductance property do not accompany this reduction, consistent with activation of mGlu II receptors on nerve terminals that release GABA onto thalamocortical neurons (Turner and Salt, 2003; Pin and Duvoisin, 1995). Presynaptic activations of mGlu II or GABA_B subtypes reduce Ca²⁺-dependent transmitter release while their postsynaptic activations cause inhibition through an increased K⁺-conductance (Misgeld et al., 1995; Stefani et al., 1996; Conn and Pin, 1997; Dutar et al., 2000).

In the present studies, we sought to determine whether isovaline acts on mGlu II and GABA_B receptors at nerve terminations in ventrobasal thalamus. We compared the effects of isovaline, LY354740, and baclofen on membrane properties, IPSCs and excitatory postsynaptic currents (EPSCs), hypothesizing that isovaline inhibits transmitter release by activating mGlu II in addition to GABA_B receptors. Our specific hypothesis was that isovaline inhibits GABA and

glutamate release by activating GABA_B and mGlu II receptors.

3.2 Experimental procedures

3.2.1 Animals

All experiments were carried out with the approval of the Animal Care Committee of The University of British Columbia.

3.2.2 Slice preparation

Thalamic slices were prepared from Sprague-Dawley (P11-13) rats of either sex as previously described (Cooke et al., 2009). Briefly, rats were decapitated while under isoflurane anesthesia; the brain was removed from the cranium and submerged in artificial cerebrospinal fluid (aCSF) at 4 °C. The composition of the aCSF was (in mM): 124 NaCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 2 MgCl₂, 2 CaCl₂, 10 dextrose, with pH = 7.3-7.4. Parasagittal slices were cut in an ice-cold slurry of aCSF to a 250 µm thickness using a Vibroslicer (Campden Instruments Ltd., London, England). The slices were warmed and incubated in aCSF, saturated with 95% O₂ and 5% CO₂ at 23-25 °C. The aCSF had an osmolarity of 297-313 mOsmoles. After incubation for ≥1 h, a slice was transferred to the recording chamber (2 ml volume) and perfused with oxygenated aCSF (95% O₂: 5% CO₂) at a rate of ~2 ml/min. Thalamocortical neurons of ventrobasal nuclei and the medial lemniscus site for stimulation were visually identified with the aid of a differential interference contrast microscope (Axioscope II, Zeiss, Germany).

3.2.3 Electrophysiological recording

For recording and data analysis, we used pClamp (Clampfit, Axon Instruments, Sunnyvale, CA, USA). Recording micropipettes were made from thin-wall borosilicate glass tubing (World

Precision Instruments, Sarasota, USA), with tip resistances ranging from 4 to 8 M Ω . Whole-cell patch clamp recordings were performed at 23 C° using a List EPC-7 (HEKA, Lambrecht, Germany) in current- and voltage-clamp modes. Signals were filtered at 3 kHz, digitized at 10 kHz (Digidata 1322A, Axon Instruments), and analyzed using pClamp 8.0 software (Axon Instruments, Sunnyvale CA). During the experiment, series resistance was monitored frequently and data were discarded if series resistance increased by >20%. Only one neuron in each slice was recorded for analysis.

For studying the effects on membrane properties, the micropipettes were filled with a “normal solution” containing (in mM): 133 K-gluconate, 12 KCl, 4 NaCl, 1 EGTA, 10 HEPES, 0.5 CaCl and Na₂-phosphocreatine with an osmolarity of 293 mOsm. Prior to recording, Na₂GTP (0.3 mM) and MgATP (3 mM) were added to the internal pipette solution. The pH was adjusted to 7.3-7.4 using gluconic acid (25%) or CsOH (8 M) solution. Current-voltage (I-V) relationships were performed from a holding potential (V_h) of -70 mV, stepping to -40 mV and then in -5 mV steps, to -110 mV.

For studying the presynaptic effects, kynurenic acid (1 mM) was added to bath solution to block ionotropic glutamate receptors and was used to isolate IPSCs from EPSCs. In order to record miniature IPSCs, tetrodotoxin (TTX 1 μ M) was added to bathing solution to block Na⁺-dependent action potentials. Cs⁺ was applied intracellularly in all voltage-clamp experiments to block postsynaptic K⁺-conductances. Micropipettes were filled with a solution containing (in mM): 16.5 CsOH, 128.5 CsCl, 10 EGTA, 4 NaCl, 10 HEPES, 0.5 CaCl₂, 3 MgATP, 2.7 Na₂-

phosphocreatine and 0.3 Na₂GTP, with an osmolarity of 307 mOsm. E_{Cl} as calculated using the Nernst equation was -70 mV.

We used electrical stimulation of the main somatosensory input to evoke EPSCs and IPSCs in thalamocortical neurons. Square pulse stimuli were applied (0.1 Hz, duration 0.05-1 ms) to the medial lemniscus using a bipolar tungsten electrode (World Precision Instruments) in conjunction with a stimulus isolator unit (Digitimer, Hertfordshire, UK). The stimulus was adjusted for evoking a maximal response with no failures (1.1 x threshold). For these experiments, micropipettes were filled with solution containing (in mM): 110 Cs₂SO₄, 5 EGTA, 4 NaCl, 5 HEPES, 0.5 CaCl₂, 5 MgATP, 2 MgCl₂, 2.7 Na₂-phosphocreatine and 0.3 Na₂GTP, with an osmolarity of 289 mOsm. E_{Cl} as calculated using the Nernst equation was -70 mV. The holding potentials for recording IPSCs were 0 mV and -70 mV for recording EPSCs (Pan et al., 2002). We verified that there was no contribution of a GABAergic component to EPSCs by applying bicuculline (20 μM) which produced little or no change in several recorded EPSCs.

3.2.4 Drugs

All drugs were applied in the bathing solution at a rate of ~2 ml per minute. Bicuculline methiodide (20 μM) was applied in the bathing solution for identifying GABA_Aergic IPSCs. Bicuculline and kynurenic acid were purchased from Sigma-Aldrich Inc. (St. Louis, USA). CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; Tocris Cookson, Ellisville MO, USA) and DL-AP5 (DL-2-amino-5-phosphonopentanoic acid; Precision Biochemicals Inc. (Vancouver, B.C., Canada) were applied to identify the glutamatergic EPSCs. R-baclofen and the mGlu II antagonist, LY341495, were purchased from Tocris Cookson. Stock solutions were prepared by

dissolving these drugs in equimolar amounts of NaOH which were stored at 4 °C. R-isovaline was synthesized by Biofine International Inc. (Vancouver, B.C., Canada) and Nagase & Co. Ltd. (Osaka, Japan). Tetrodotoxin citrate was purchased from Alomone Labs (Jerusalem, Israel). Solutions were prepared from stock solutions just before an experiment.

3.2.5 Statistical analysis

In this study, we examined a total of 172 neurons. Data are presented as mean \pm 95% confidence interval (CI) and number of neurons (n). Prism 5 software (GraphPad, San Diego, CA, USA) was used for the statistical analyses. We used repeated measure ANOVA followed by Bonferroni's Multiple Comparison Test to compare treatment effects. Estimation of EC₅₀ was obtained through fitting the following equation to the data:

$$Y = \text{bottom} + [\text{top-bottom}] / [1 + 10^{([\log \text{EC}_{50} - X] * \text{Hill slope})}]$$

where the top and bottom are plateaus in units of the y-axis. A *P* value of < 0.05 was used for statistical significance.

Ten IPSCs and 10 EPSCs evoked at 0.1 Hz were averaged and analyzed off-line using pClamp software. The decaying phase of EPSCs at $V_h = -70$ mV was fitted with a single exponential function to determine decay time constant of predominantly AMPA-mediated components of lemniscal EPSCs (Miyata and Imoto, 2006). In order to determine the decay time of IPSCs at $V_h = 0$ mV, the falling phase was fitted with one of the following functions:

$$y = Ae^{-t/\tau} \quad \text{or} \quad y = A_1e^{-t/\tau_1} + A_2e^{-t/\tau_2}$$

Where A is the peak amplitude and τ is the decay time constant for the monoexponential IPSCs.

A_1 and A_2 are the peak amplitudes of the fast and slow phases of the biexponential IPSCs, having time constants τ_1 and τ_2 , respectively.

For recording miniature IPSCs, TTX was applied to block action potential dependent release of synaptic transmitter. Changes in frequency of miniature IPSCs are attributable to changes in frequency of presynaptic transmitter release whereas changes in amplitude of miniature IPSCs are attributable to changes in postsynaptic sensitivity to transmitter (De Koninck and Mody, 1994; Isaacson and Walmsley, 1995). Spontaneous and miniature IPSCs were detected and analyzed using Mini Analysis software (Synaptosoft, Decatur, GA, USA). The threshold for amplitude detection was set at 3 times RMS of the noise. A minimum of 250 events for each neuron was required for inclusion in the analysis. We used Kolmogorov-Smirnov test to compare the changes in peak amplitude or inter-event interval (i.e., frequency).

The reduction in IPSC amplitude or frequency at a given concentration of agonist was calculated as percentage of baseline and used to construct a concentration-response curve. Different concentrations of agonist were applied in a step-wise manner and cumulatively. Mean values of the IC_{50} were obtained by averaging multiple measurements of different neurons and fitted by the Hill-equation (see above).

3.3 Results

Before studying isovaline effects, we examined the effects of the mGlu II agonist, LY354740, on the postsynaptic properties and spontaneous or miniature IPSCs.

3.3.1 Postsynaptic effects of LY354740

As shown Figure 1, bath application of LY354740 (1 μ M) did not alter passive and active properties of thalamocortical neurons recorded with normal pipette solution. In voltage-clamp, LY354740 (1 μ M) did not significantly affect the currents evoked in 5 neurons or their I-V relationships (Fig. 1C,D). The lack of effects of LY354740 on these parameters observed over a wide concentration range (0.01–5 μ M) in 5 neurons, suggests that functional mGlu II receptors are absent on the postsynaptic membrane.

3.3.2 Effects of LY354740 on spontaneous IPSCs

We investigated possible presynaptic effects of LY354740 on spontaneous IPSCs using Cs⁺ containing microelectrodes to block postsynaptic K⁺ conductances, and additional application of kynurenate in the bath solution to block ionotropic glutamate receptors. As shown in Fig. 2A, LY354740 (0.1 μ M) reversibly decreased the number of spontaneous currents that occurred over sequential 6.3 min epochs. This decrease was evident from the rightward shift in the cumulative distribution of inter-event intervals, without affecting the peak amplitude distribution (Kolmogorov-Smirnov test; Fig. 2B). In a summary of n=8 neurons with spontaneous IPSCs, LY354740 decreased the mean frequency by 64%, from 0.8 Hz (95% CI, 0.5–1.0) to 0.29 Hz (95% CI, 0.12–0.46; paired t-test, $P < 0.05$), without altering their amplitude (Fig. 2C). The mean

peak amplitude was 55 pA (95% CI, 35–74) before, and 49 pA (95% CI, 35–62) after LY354740 (paired t test). The depressant effect on frequency was concentration-dependent, with an IC_{50} of 0.026 μ M (Fig. 2D). The mean decay constant of IPSCs in the 8 neurons was unaffected by LY354740 application (Fig. 2E). The decrease in frequency without corresponding changes in peak amplitude of spontaneous IPSCs suggests that LY354740 acts presynaptically to reduce transmitter release.

3.3.3 Effects of LY354740 on miniature IPSCs

Figure 3 shows the effects of LY354740 on miniature IPSCs during applications of TTX to block action potential-dependent transmitter release, as well as during Cs^{+} - and kynurenate-blockade of potentially interfering postsynaptic receptor activations. As shown for the neuron in Figure 3A and 3B, LY354740 (0.1 μ M) reduced the frequency of miniature IPSCs while having little or no effect on peak amplitude. In a summary of n=8 neurons, LY354740 (25 nM) reduced the mean frequency of miniature IPSCs by 66%, from 0.39 Hz (95% CI, 0.22–0.56) to 0.13 Hz (95% CI, 0.06–0.19; $P < 0.05$; Fig. 3C). In the same neurons, LY354740 did not significantly affect mean amplitude (before LY354740, 43 pA [95% CI, 34–52]; after, 44 pA [95% CI, 32–56]; one-way ANOVA followed by Bonferroni's Multiple Comparison Test). LY354740 produced a concentration-dependent reduction in mean frequency with an IC_{50} of 20 nM (Fig. 3D). LY354740 had little or no effect on the decay time constant (Fig. 3E). These data suggest that LY354740 acts at presynaptic sites, decreasing transmitter release.

3.3.4 Antagonism of the effects of LY341495 on miniature IPSCs

Using the selective mGluR II antagonist, LY341495 (Schoepp et al., 1997), we sought to confirm that the depressant effect of LY354740 on frequency is mediated by mGlu II receptors on nerve terminations. LY354740 reduced the mean frequency of miniature IPSCs from 0.39 Hz (95% CI, 0.22–0.56) to 0.13 Hz (95% CI, 0.06–0.19; $P < 0.05$; Fig. 3C). LY341495 (1 μ M) co-applied with LY354740 largely prevented the reduction in miniature IPSC frequency and the rightward shift in inter-event intervals, without affecting peak amplitude and decay time constant (Fig. 3). In $n=8$ neurons, LY341495 reversed the LY354740-induced reduction in mean frequency from 0.13 Hz (95% CI, 0.06–0.19) to 0.35 Hz (95% CI, 0.24–0.46; one-way ANOVA followed by Bonferroni's Multiple Comparison Test; $P < 0.05$; Fig. 3C). The above data indicate that LY354740 reduces the frequency of miniature IPSCs by activating mGlu II receptors at presynaptic sites, independent of action potentials.

3.3.5 Effects of LY354740 on evoked synaptic currents

LY354740 (0.1 μ M) reduced the peak amplitude of the IPSCs evoked by medial lemniscal stimulation in neurons voltage-clamped at 0 mV (Fig. 4A). In $n=10$ neurons, LY354740 decreased the mean peak amplitude of evoked IPSCs by 50% from 306 pA (95% CI, 143–468) to 154 pA (95% CI, 81–228; Fig. 4B). This reversible reduction was concentration-dependent, with an IC_{50} of 0.021 μ M (Fig. 4C). Co-application with LY341495 (1 μ M) almost eliminated the LY354740 reduction in peak amplitude (297 pA, 95% CI, 140–455; one-way ANOVA followed by Bonferroni's Multiple Comparison Test; $P < 0.05$; Fig. 4A,B). The data suggest that LY354740 reduces peak amplitude of IPSCs by activating presynaptic mGlu II receptors.

LY354740 (0.1 μ M) reduced the peak amplitude of EPSCs evoked in neurons voltage-clamped at -70 mV. The reversible reduction was prevented by co-application of 1 μ M LY341495 (Fig. 4D). In 7 neurons, LY354740 decreased the mean peak amplitude of EPSCs by 49% from 178 pA (95% CI, 74–283) to 90 pA (95% CI, 20-160; Fig. 4E). Co-application with LY341495 eliminated the reduction (176 pA; 95% CI, 78–273; one-way ANOVA followed by Bonferroni's Multiple Comparison Test; $P < 0.05$; Fig. 4E). The findings that LY354740 reduces peak amplitude of EPSCs implicate the activation of presynaptic mGlu II receptors.

3.3.6 Nature of transmitter of synaptic currents

Previous studies demonstrated that GABA and glycine are co-transmitters in ventrobasal thalamus (Ghavanini et al. 2005). Using a GABA_A antagonist, bicuculline, and glycine_A antagonist, strychnine, we determined the nature of the transmitter mediating the IPSCs. Bicuculline (20 μ M) eliminated all spontaneous and miniature IPSCs in 2 separate groups of 8 neurons (cf. Figs. 2A-3A), demonstrating the GABA_Aergic nature of the neuronal inputs that may originate from nRt (Cope et al., 2005).

Before applying bicuculline, we applied strychnine, given the lower percentage of purely glycinergic transmission in ventrobasal thalamus (cf. Ghavanini et al., 2005; Mathers et al., 2009). Strychnine (2 μ M), reduced the peak amplitude of evoked IPSCs by a mean of 38% in 6 of 25 tested neurons, but did not eliminate IPSCs evoked by medial lemniscal stimulation. Co-application of bicuculline (20 μ M) with strychnine abolished the IPSCs (n=25, data not shown). These data confirm that in most cases, the transmitter of IPSCs was GABA which often evoked responses at GABA_A receptors but in a few other cases, glycine which activated glycine

receptors (Zhang et al., 1997; Ghavanini et al., 2005). The latter is consistent with the suggestion that GABA and glycine are co-transmitted in some ventrobasal neurons (cf. Mathers et al., 2009).

We determined the nature of the excitatory transmitter by applying CNQX, an antagonist of AMPA receptors and DL-AP5, an antagonist of NMDA receptors. CNQX (50 μ M) alone reduced the amplitude of EPSCs by 93% in 3 of 4 neurons. Co-application with DL-AP5 (100 μ M) eliminated the residual EPSCs in 7 out of 7 neurons (data not shown). These results confirmed the purely glutamatergic nature of lemniscal transmission (Hsu et al., 2010) and are consistent with those of Miyata and Imoto (2006) indicating that AMPA receptors for glutamate predominantly mediate medial lemniscal EPSCs.

3.3.7 Effects of baclofen on evoked synaptic currents

The GABA_B agonist, baclofen (0.3 μ M) reversibly reduced the peak amplitude of medial lemniscal IPSCs (Fig. 5A-B). The mean peak amplitude in 12 neurons was 359 pA (95% CI, 244–475). Baclofen reduced mean peak amplitude by 61% to 137 pA (95% CI, 44–229). Co-application with the GABA_B receptor antagonist, CGP52432 (10 μ M) blocked the reduction by 82% (293 pA, 95% CI, 171–415; one-way ANOVA followed by Bonferroni's Multiple Comparison Test; $P < 0.05$; Fig. 5B). This reduction was concentration-dependent, with an IC₅₀ of 0.1 μ M and reversible in all neurons (Fig. 5C).

Baclofen (0.3 μ M) also depressed the amplitude of EPSCs (Fig. 5D,E). The control peak amplitude in 6 neurons had a mean of 220 pA (95% CI, 70–371). Baclofen reversibly reduced

the peak amplitude by 80 % to 43 pA (95% CI, 29–116). Co-application with CGP52432 eliminated the depressant effect (210 pA, 95% CI, 73–348; one-way ANOVA followed by Bonferroni's Multiple Comparison Test; $P < 0.05$; Fig. 5D,E). These data suggest that baclofen reduces the peak amplitude of EPSCs by activating presynaptic GABA_B receptors.

3.3.8 Effects of isovaline on evoked synaptic currents

Isovaline (400 μ M) reduced the peak amplitude of medial lemniscal IPSCs and EPSCs (Figs. 6). In a summary of $n=8$ neurons, the mean peak amplitude of IPSCs before application was 373 pA (95% CI, 276–469). Isovaline application reduced the peak amplitude by 30 % to a mean of 262 pA (95% CI, 170–354; one-way ANOVA followed by Bonferroni's Multiple Comparison Test; $P < 0.05$; Fig. 6B). Isovaline produced a concentration-dependent reduction in mean IPSC amplitude, with an IC_{50} of 284 μ M (Fig. 6C). In a summary of $n=5$ neurons, isovaline (400 μ M) depressed the mean peak amplitude of EPSCs by 57% from 280 pA (95% CI, -39–601) to 119 pA (95% CI, 103–343; one-way ANOVA followed by Bonferroni's Multiple Comparison Test; $P < 0.05$; Fig. 6E). Unlike baclofen and LY354740, the depressant effects of isovaline on IPSCs and EPSCs were not reversible after 40 min of washing with normal solution. The above data show that isovaline reduces the peak amplitude of IPSCs and EPSCs.

3.3.9 GABA_B antagonism of isovaline effects on IPSCs and EPSCs

We examined possible involvement of GABA_B receptors in the effects of isovaline on IPSCs and EPSCs. Previously, Cooke et al. showed that GABA_B receptors mediated isovaline's postsynaptic effects (Cooke et al., 2012). During conditions that isolated presynaptic from postsynaptic effects (cf. Methods), co-application of CGP52432 (10 μ M) blocked the inhibitory

effects of isovaline on IPSCs and EPSCs (Fig. 7). In a summary of $n=5$ neurons, the IPSCs had mean peak amplitude of 302 pA (95% CI, 124–481). Isovaline (400 μ M) depressed the mean peak amplitude of IPSCs by 46% to 162 pA (95% CI, 24–300; Fig. 7B). Co-application with CGP52432 (10 μ M) reduced the depressant effect by 78% to 236 pA (95% CI, 71–400; one-way ANOVA followed by Bonferroni's Multiple Comparison Test; $P < 0.05$; Fig. 7B). In a summary of $n=5$ neurons, the control peak amplitude of EPSCs was 188 pA (95% CI, 74–301). Isovaline (400 μ M) depressed the mean peak amplitude of EPSCs by 57% to 81 pA (95% CI, -27–190; Fig. 7D). Co-application of CGP52432 partly reversed the inhibitory effect of isovaline by 79% back to 148 pA (95% CI, 86–209; one-way ANOVA followed by Bonferroni's Multiple Comparison Test; $P < 0.05$; Fig. 7D). These data provide evidence that GABA_B receptors are involved in the effects of isovaline on IPSCs and EPSCs.

3.3.10 mGlu II antagonism of effects of isovaline on IPSCs and EPSCs

In view of possible involvement of mGlu II receptors in the effects of isovaline (cf. Introduction), we sought to determine if mGlu II antagonism blocked its effects on IPSCs and EPSCs. Co-application of LY341495 (1 μ M) with isovaline (400 μ M) blocked its depressant effects on both IPSCs and EPSCs (Fig. 8A,C). In a summary of $n=9$ neurons, isovaline depressed the peak amplitude of IPSCs by 37% from 295 pA (95% CI, 202–387) to 186 pA (95% CI, 121–251; Fig. 8B). LY341495 partly blocked the depressant effect on IPSCs by 84% (247 pA, 95% CI, 178–316; one-way ANOVA followed by Bonferroni's Multiple Comparison Test; $P < 0.05$; Fig. 8B). In summary of $n=5$ neurons, isovaline depressed the mean peak amplitude of EPSCs by 52% from 212 pA (95% CI, 82–342) to 101 pA (95% CI, 4–196; Fig. 8D). LY341495 reversed the depressant effect on EPSCs by 97% (206 pA; 95% CI, 35–377; one-way ANOVA

followed by Bonferroni's Multiple Comparison Test; $P < 0.05$; Fig. 8D). These data demonstrate that mGlu II receptors mediate the depressant effects of isovaline on IPSCs and EPSCs.

These effects of isovaline on IPSCs and EPSCs were likely due to isovaline acting on presynaptic receptors since 1) internal Cs^+ application precluded postsynaptic effects of isovaline or endogenous GABA_B activation; 2) a mGlu II agonist had no postsynaptic effects; and, 3) activation of mGlu II receptors by a mGlu II agonist had a purely presynaptic effect on miniature synaptic currents. This raised the possibility of the relative contributions of GABA_B and mGlu II receptors to the presynaptic effects of isovaline. Co-application of both mGlu II and GABA_B antagonists with isovaline did not eliminate the depressant effects of isovaline on IPSCs and EPSCs in 13 neurons (data not shown).

3.4 Discussion

The present studies demonstrate that the novel experimental analgesic, isovaline, suppressed synaptic inhibition and excitation in thalamus. The actions of isovaline mimicked selective agonist actions at GABA_B and mGlu II receptors of nerve terminations on thalamocortical neurons. Like these agonists, isovaline suppressed IPSCs by decreasing the release of GABA, and EPSCs by decreasing the release of glutamate. These effects of isovaline were mediated by GABA_B and mGlu II receptors as discussed below.

3.4.1 Presynaptic effects of isovaline

Isovaline and mGlu II agonists acted on nerve terminals, depressing the amplitude of both IPSCs and EPSCs evoked by medial lemniscal stimulation. Previous studies demonstrated the presynaptic effects of baclofen which reduced transmitter release from lemniscal terminations in ventrobasal neurons (Bessah et al. 2006). We also showed that isovaline has postsynaptic GABA_B actions similar to baclofen on thalamocortical neurons, increasing K⁺ conductance (Cooke et al., 2012). In the present experiments, we used intracellular Cs⁺ to block postsynaptic G-protein-mediated K⁺ conductances, including possible, endogenous GABA_B-mediated conductance, thereby uncovering the presynaptic effects of isovaline. The presynaptic effects were evident from the decreased amplitudes of medial lemniscal IPSCs and EPSCs without changes in their decay-time constants. Isovaline acted on IPSCs in a concentration-dependent manner with an IC₅₀ of 284 μM. The GABA_B antagonist, CGP52432, or the mGlu II antagonist LY341495, partly reversed the depressant effects of isovaline on IPSCs and EPSCs. A combination of both antagonists did not eliminate the depressant effects of isovaline. It is

unclear why we could not obtain a full blockade using both antagonists. However, this could be due to one of the following: 1) involvement of new receptor types; 2) activation of potential mGlu II/GABA_B heterodimer which has distinct pharmacological profile and less sensitivity to antagonists; or, 3) activation of anatomically distinct terminals that only activate one type of receptors (i.e., mGlu II or GABA_B receptors). The susceptibilities to these antagonists are consistent with GABA_B and mGlu II receptor mediation of antiallodynic effects of isovaline (Whitehead et al. 2012; Asseri et al., 2015).

3.4.2 Presynaptic effects of LY354740

LY354740 suppressed the frequency but not the amplitude of spontaneous and miniature IPSCs in thalamocortical neurons in ventrobasal nuclei (cf. Copeland et al., 2017). Frequency suppression was not due to activation of group I or III receptors since their activation would require higher concentrations than used here (cf. Schoepp et al., 1999). In our experiments, a selective mGlu II receptor antagonist, LY341495, suppressed the depressant effects of LY354740 on IPSCs, consistent with the previous findings (Copeland et al., 2017). On medial lemniscal stimulation, thalamocortical neurons of ventrobasal nuclei receive disynaptic inhibitory inputs mainly through nRt (Ma et al. 1987; Gentet and Ulrich, 2003) and possibly local circuit neurons (Harris and Hendrickson, 1987). GABA and glycine are known co-transmitters of the inhibition evoked by stimulation of medial lemniscal pathways (Ghavanini et al., 2005; 2006). The heterogeneity of synaptic inputs is crucial for processing by thalamocortical neurons and transferring information to the primary somatosensory cortex (Huguenard and Prince, 1994; Sherman and Guillery, 1996). While the IPSCs originated from nRt, EPSCs were

due to glutamate released directly from medial lemniscal axon terminations (Salt and Eaton, 1996).

LY354740 decreased the amplitude of the evoked IPSCs without altering postsynaptic membrane properties. This action had an IC_{50} of 20 nM and within the range reported for LY354740's actions at mGlu II receptors in a recombinant expression system (Schoepp et al., 1997). Copeland et al., (2017) showed that mGlu II receptors activation in ventrobasal neurons reduces astrocyte-dependent GABA release. It is unlikely that the inhibitory effects of LY354740 was due to activation of astrocytic mGlu II receptors since the application of LY354740 had no apparent effect on the holding current which would reflect the actions by the released GABA (Jia et al., 2005). However the possibility that GABA released by astrocytes activated GABA_A receptors at both pre- and postsynaptic sites cannot be ruled out. In summary, LY354740-induced depression of IPSCs evoked by medial lemniscal stimulation is attributable to actions on transmitter release by nerve terminals.

The effects of LY354740 application on EPSCs in ventrobasal nuclei have received little attention. In this study, LY354740 reversibly suppressed medial lemniscal EPSCs in a concentration-dependent manner. Co-application of LY341495 and LY354740 produced no such effect, implicating group II mGlu receptors on nerve terminals which may mediate glutamate release by medial lemniscal terminations. We demonstrated that mGlu II receptor activation reduces the peak amplitude of medial lemniscal EPSCs. Hence, LY354740 not only suppresses excitation but also inhibition in thalamocortical neurons due to medial lemniscal stimulation (Ngomba et al., 2011). These findings allude to the complex circuitry and mechanism of action

of mGlu II receptor agonists when dealing with therapeutic targets in animal models (e.g., pain, epilepsy).

3.4.3 Functional GABA_B heteroreceptors on thalamocortical neurons

This study demonstrates that functional GABA_B receptors are present on axonal terminations activated by medial lemniscal stimulation. During Cs⁺-blockade of postsynaptic GABA_B-actions, baclofen reversibly reduced the amplitudes of IPSCs and EPSCs without affecting their decay-time constants. GABA_B receptors mediated these presynaptic effects, as shown by complete blockade with CGP52432. The effects of R-baclofen on the IPSCs were concentration-dependent with an IC₅₀ of 100 nM, similar to the IC₅₀ (800 nM) observed for the effects of racemic baclofen on miniature IPSCs (Bessah et al., 2006). The presynaptic effects of baclofen on the EPSCs observed in this study are consistent with the reduction in medial lemniscal EPSP amplitude in ventrobasal neurons (Emri et al., 1996; cf. also corticothalamic EPSCs, Porter and Nieves, 2004).

Crosstalk between GABA_B and mGlu II receptors was not evident in our experiments. GABA_B antagonism did not affect the action of LY354740 on mGlu II receptors on IPSCs and EPSCs in 5 neurons (cf. Kantamneni, 2015). Antagonism of mGlu II receptors had no effects on baclofen activation of GABA_B receptors in 7 neurons. While baclofen reduced transmitter release by activating presynaptic GABA_B receptors (see above), no crosstalk was observed between GABA_B and mGlu II receptor systems.

3.4.4 Possible nature of released transmitter producing IPSCs

We examined the nature of transmitters released by the medial lemniscus, by applying antagonists of GABA_A and glycine receptors during the electrical stimulation. The GABA_A antagonist bicuculline, eliminated spontaneous, miniature and medial lemniscal IPSCs in 24 of 42 neurons tested, indicating a GABAergic nature of the activated pathways. Strychnine did not affect spontaneous or miniature IPSCs in all 16 tested neurons. The above data and GABA_B receptor expression on GABAergic terminals Bessah et al., 2006 are consistent with our findings that activation of presynaptic GABA_B receptors by baclofen modulates GABA release on ventrobasal neurons.

3.4.5 Possible nature of released transmitter producing EPSCs

We attempted to elucidate the nature of released transmitters by determining if glutamate antagonists affected the changes in EPSCs induced by the released transmitter during stimulation of the medial lemniscus. An AMPA receptor antagonist (CNQX) but not a NMDA receptor antagonist (DL-AP5) eliminated medial lemniscal EPSCs, indicating glutamate acted as the transmitter at AMPA receptors. These observations are consistent with findings of Miyata and Imoto (2006) that a majority of EPSCs evoked by medial lemniscal stimulation result mainly from activation of AMPA receptors on thalamocortical neurons in the ventrobasal nuclei.

3.4.6 Presynaptic role of mGlu II and GABA_B receptors

Our studies demonstrate for the first time that isovaline acts on GABAergic terminals as well as glutamatergic terminals on thalamocortical neurons. GABA acting on GABA_B receptors of nerve terminals modulates glutamate release in the medial geniculate body (Luo et al., 2011).

Presynaptic GABAergic inhibition modulates ascending somatosensory inputs including pain signals onto thalamocortical neurons (cf. Sherman and Guillery, 1996). Presynaptic mGlu II receptors regulate GABA release in thalamic reticular nucleus (Govindaiah and Cox, 2006), hippocampus (Fitzsimonds and Dichter, 1996), and cerebellum (Mitchell and Silver, 2000). As heteroreceptors, presynaptic group II mGlu receptors may regulate the release of GABA from terminations on thalamocortical neurons, providing potential targets for antinociceptive drugs. We speculate that isovaline reduces synaptic transmission associated with painful inputs by activating both presynaptic mGlu II and GABA_B receptors on terminations of the medial lemniscal pathway.

3.4.7 Conclusion

The present study demonstrates that presynaptic activation of GABA_B or mGlu II receptors decreases the release of GABA and glutamate in the thalamus. The novel experimental analgesic, isovaline, reduces the release of GABA and glutamate by activating mGlu II and GABA_B receptors on nerve terminal inputs to thalamocortical neurons. Our findings support the notion that mGlu II receptors represent promising targets for novel therapeutic approaches to the relief of pain (Simmons et al., 2002; Chiechio et al., 2004).

3.5 Figures

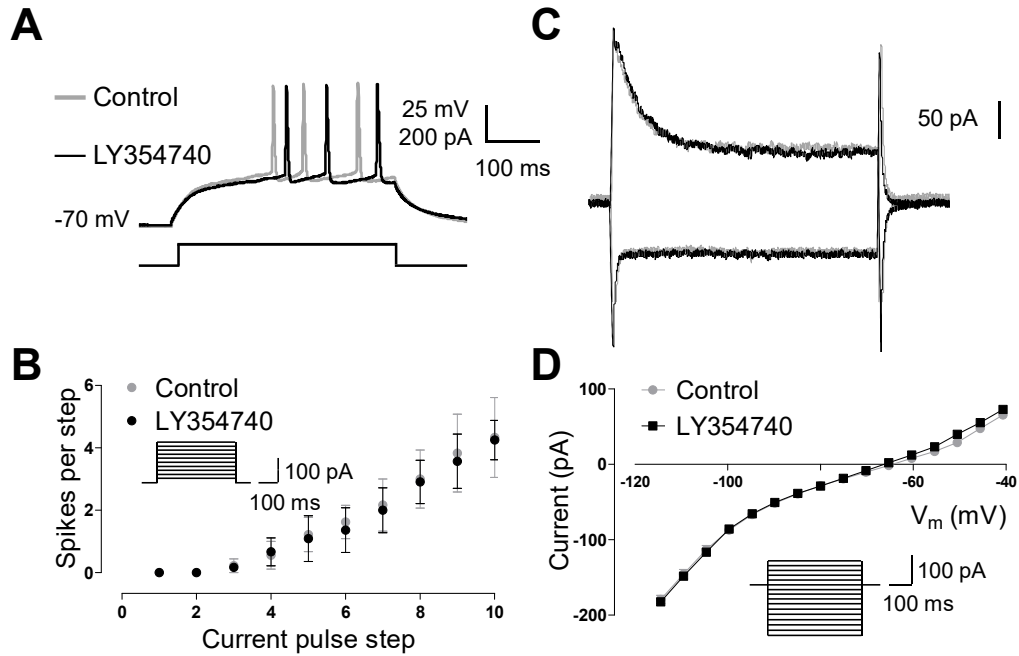
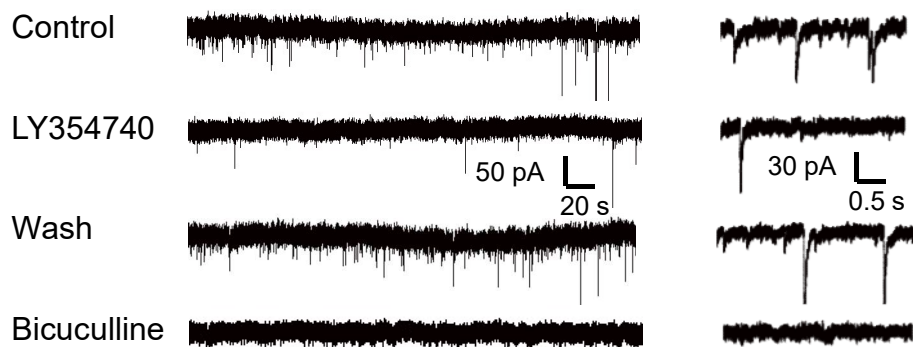
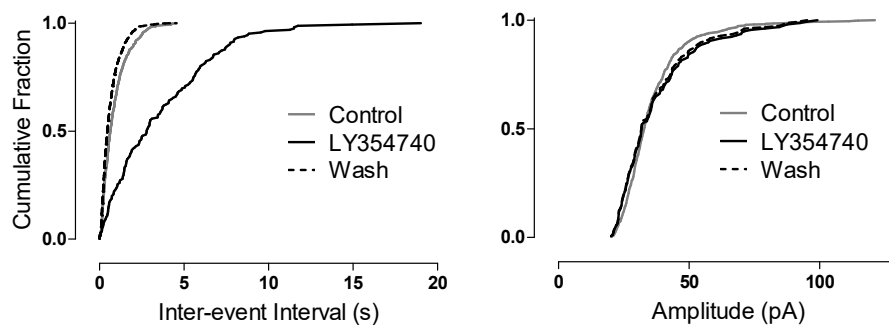


Figure 3.1. mGlu II agonist LY354740 had no effects on membrane properties in 5 neurons. Application of LY354740 (1 μ M) did not affect action potential firing in neuron (A) or total number of spikes/400 ms-pulse in 5 neurons (B). (C) LY354740 (1 μ M) had no effects on currents evoked by voltage step to -110 mV from holding potential of -70 mV. (D) LY354740 (1 μ M) did not alter the current-voltage relationship in 5 neurons (error bars omitted for clarity). Control (grey) and LY354740 (black) traces were overlapped.

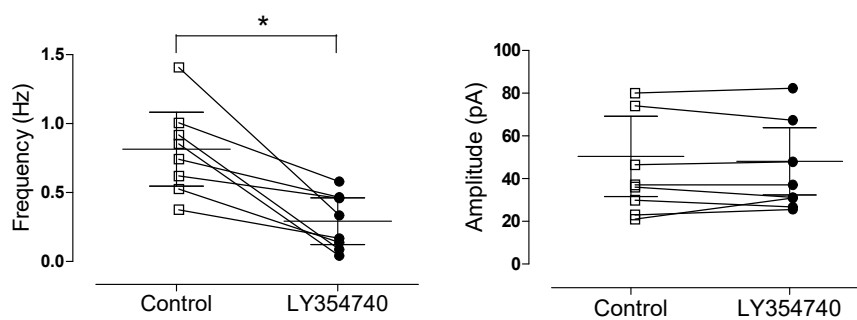
A Spontaneous IPSCs



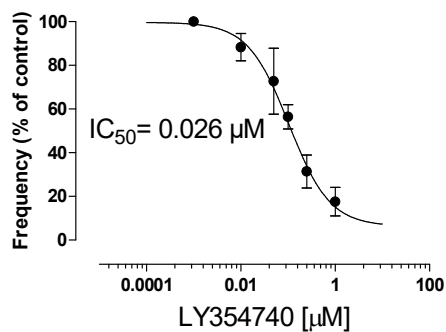
B



C



D



E

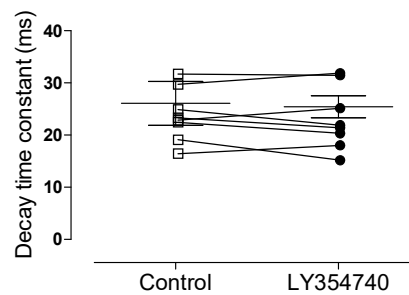


Figure 3.2. LY354740 reduces frequency of spontaneous IPSCs without affecting peak amplitude or decay time constant. (A) Currents of representative neuron recorded over 6.3 min epochs show that application of LY354740 (100 nM) decreased spontaneous IPSC frequency with recovery after 40 min wash. (B) Normalized cumulative probability histograms of inter-event interval and amplitude before, during and after application of LY354740 (same neuron as in A). LY354740 produced a rightward shift of cumulative probability curve representing a decrease in frequency (Kolmogorov-Smirnov test), but no corresponding shift in peak amplitude. (C) In 8 neurons LY354740 decreased spontaneous IPSC frequency without affecting the peak amplitudes (average of 10, paired *t*-test). (D) LY354740 decreased spontaneous IPSC frequency in a concentration-dependent manner with IC_{50} of 0.026 μ M. (E) In 8 neurons, LY354740 did not affect the decay time constants of spontaneous IPSCs (average of 10, paired *t*-test). Data were presented as mean \pm 95% CI. * $P < 0.05$. $V_h = -70$ mV.

A Miniature IPSCs

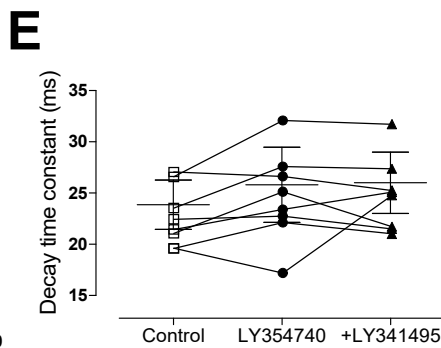
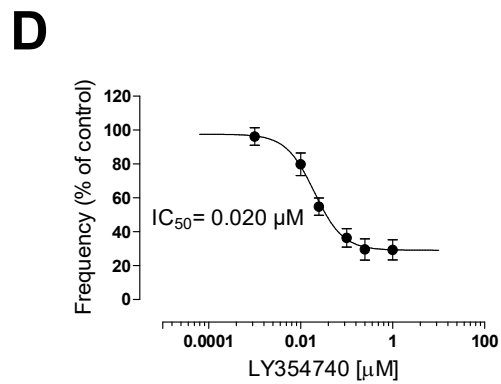
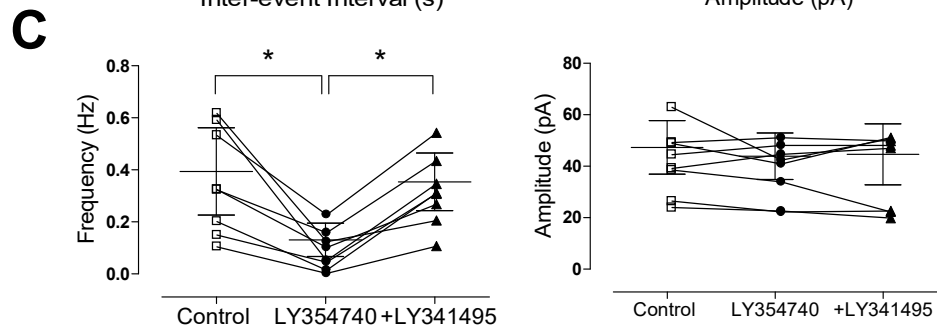
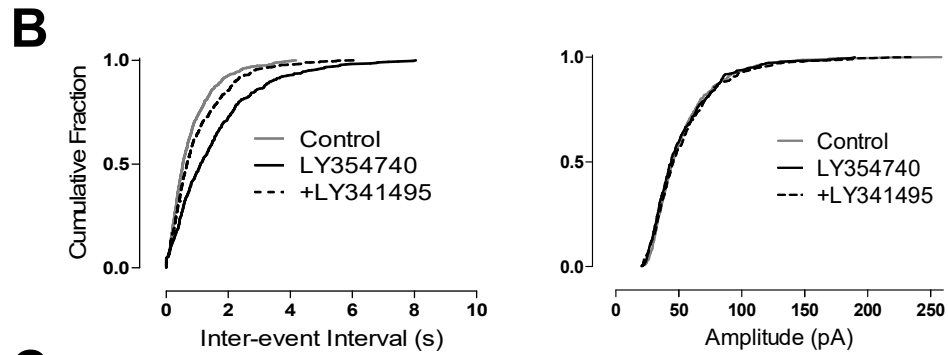
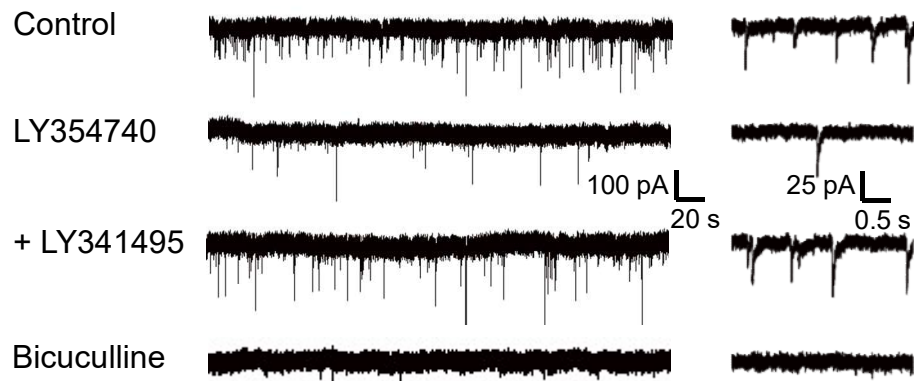


Figure 3.3. mGluR II antagonism of the decrease in miniature IPSC frequency induced by LY354740 (A-E). (A) Co-applied LY341495 (1 μ M) blocks the decrease in miniature IPSC frequency in neuron induced by LY354740 (100 nM). (B) Co-application of LY341495 (1 μ M) with LY354740 antagonizes rightward shift of cumulative inter-event intervals representing frequency (Kolmogorov-Smirnov test), without affecting amplitude. (C) In 8 neurons, co-application of LY341495 (1 μ M) with LY354740 antagonizes the decrease in frequency without affecting amplitude (average of 10, one-way ANOVA followed by Bonferroni's Multiple Comparison Test). (D) LY354740 decreased the frequency in a concentration-dependent manner with an IC_{50} of 0.020 μ M. (E) In 8 neurons, co-application of LY341495 with LY354740 had no effect on decay time constants of miniature IPSCs (average of 10, one-way ANOVA followed by Bonferroni's Multiple Comparison Test). Data were presented as mean \pm 95% CI. * $P < 0.05$. TTX was used to block action potentials. $V_h = -70$ mV.

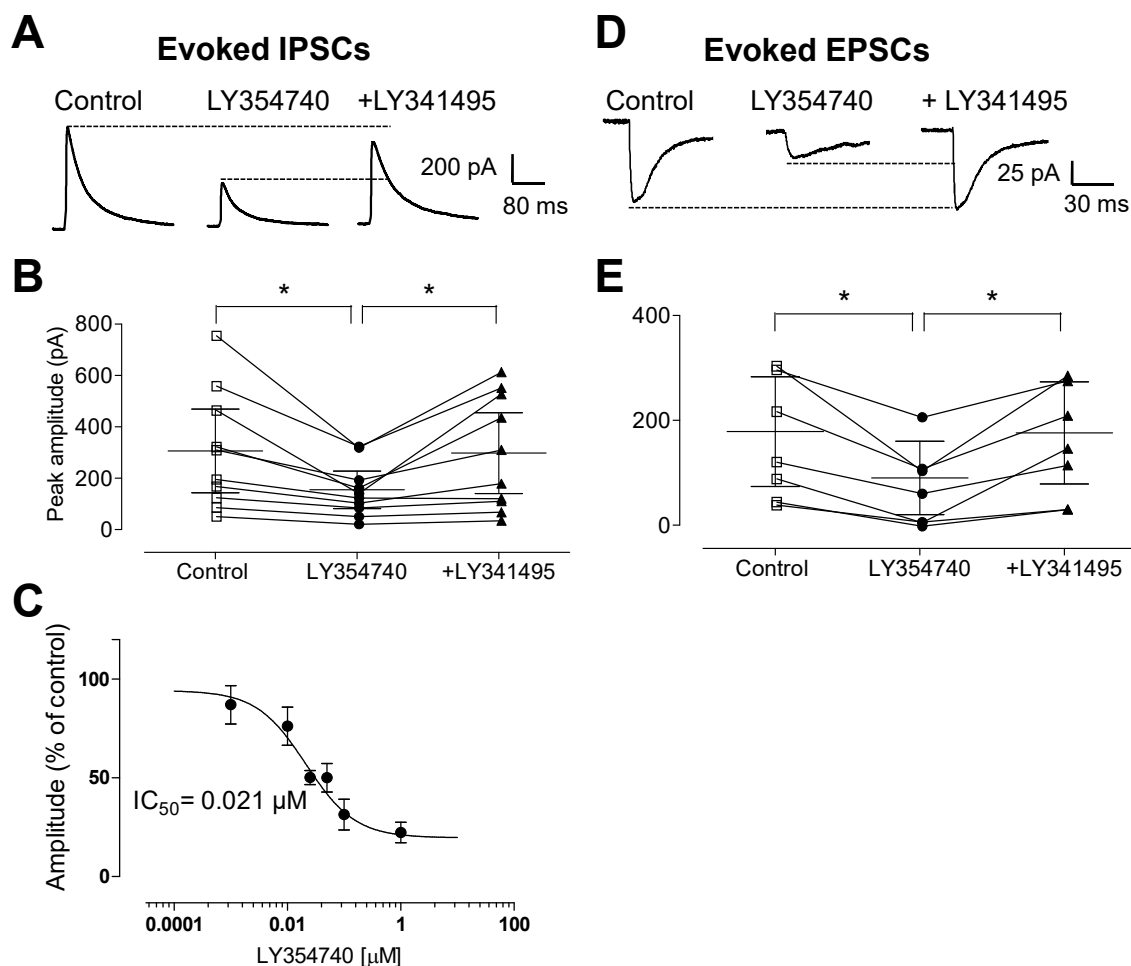


Figure 3.4. Activation of mGlu II receptors decreases amplitude of IPSCs and EPSCs evoked by medial lemniscal stimulation. (A) Application of LY354740 (0.1 μ M) in neuron reduced IPSCs (average of 10) which was antagonized by LY341495 (1 μ M). (B) In 10 neurons, co-application of LY341495 (1 μ M) with LY354740 antagonizes the decrease in peak amplitude of IPSCs (average of 10). (C) LY354740 decreased the peak amplitude IPSCs in a concentration-dependent manner with an IC₅₀ of 0.021 μ M. (D) Application of LY354740 (0.1 μ M) in neuron reduced evoked EPSCs (average of 10) which was antagonized by LY341495 (1 μ M). (E) In 10 neurons, co-application of LY341495 (1 μ M) with LY354740 antagonizes the decrease in peak amplitude of EPSCs (average of 10). Data were presented as mean \pm 95% CI. * $P < 0.05$; one-

way ANOVA followed by Bonferroni's Multiple Comparison Test. $V_h = 0$ mV for IPSCs and $V_h = -70$ mV for EPSCs.

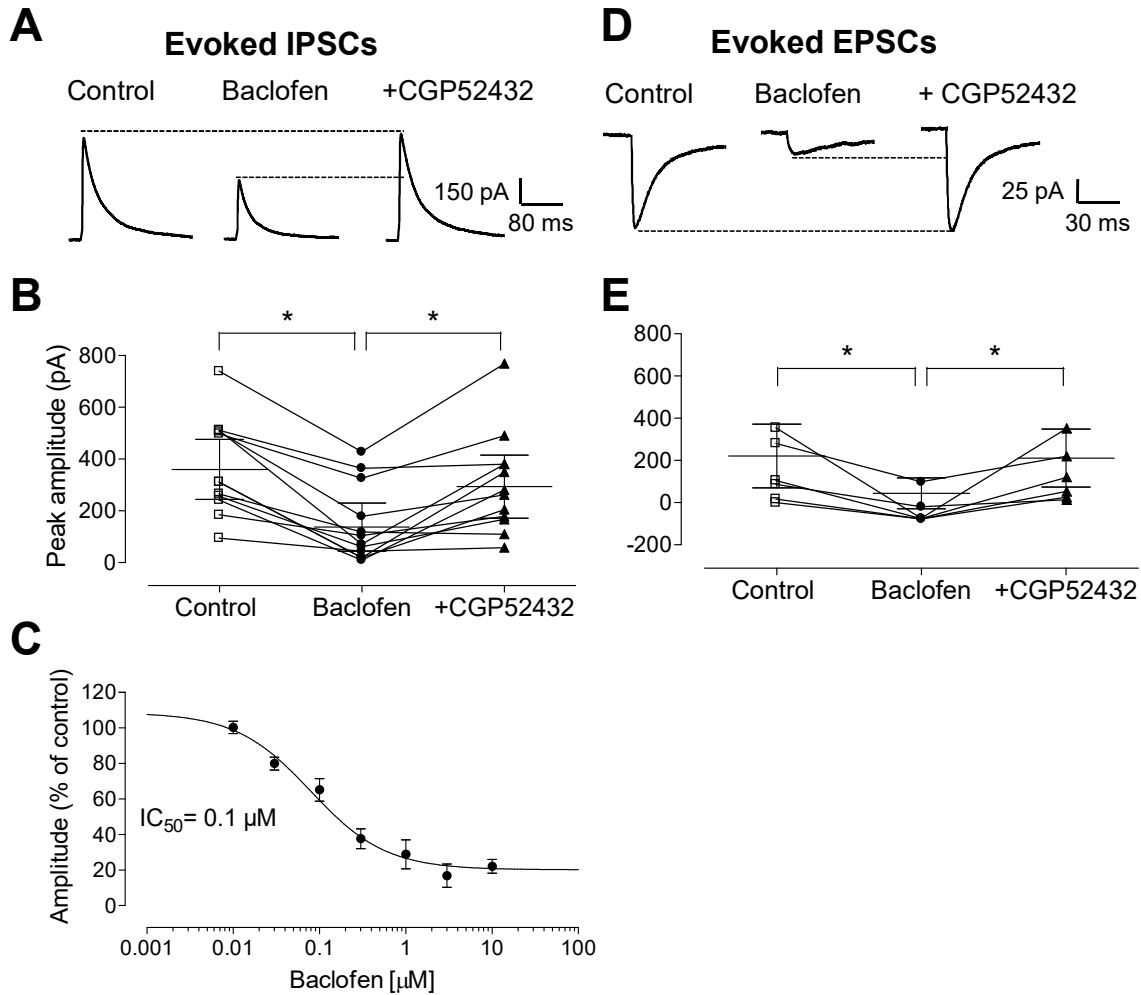


Figure 3.5. Activation of GABA_B receptors decreases amplitude of evoked IPSCs and EPSCs.

(A) Application of baclofen (0.3 μM) to neuron reduced IPSC amplitude (average of 10) which was blocked by GABA_B antagonist, CGP52432 (10 μM). (B) In 10 neurons, co-application of CGP52432 (10 μM) with baclofen antagonizes the decrease in peak amplitude of IPSCs (average of 10). (C) Baclofen decreased the peak amplitude of IPSCs in a concentration-dependent manner with an IC_{50} of 0.1 μM . (D) Application of baclofen (0.3 μM) to neuron reduced EPSCs (average of 10) which was antagonized by CGP52432 (10 μM). (E) In 10 neurons, co-application of CGP52432 (10 μM) with baclofen antagonizes the decrease in peak amplitude of EPSCs (average of 10). Data were presented as mean \pm 95% CI. * $P < 0.05$; one-way ANOVA

followed by Bonferroni's Multiple Comparison Test. $V_h = 0$ mV for IPSCs and $V_h = -70$ mV for EPSCs.

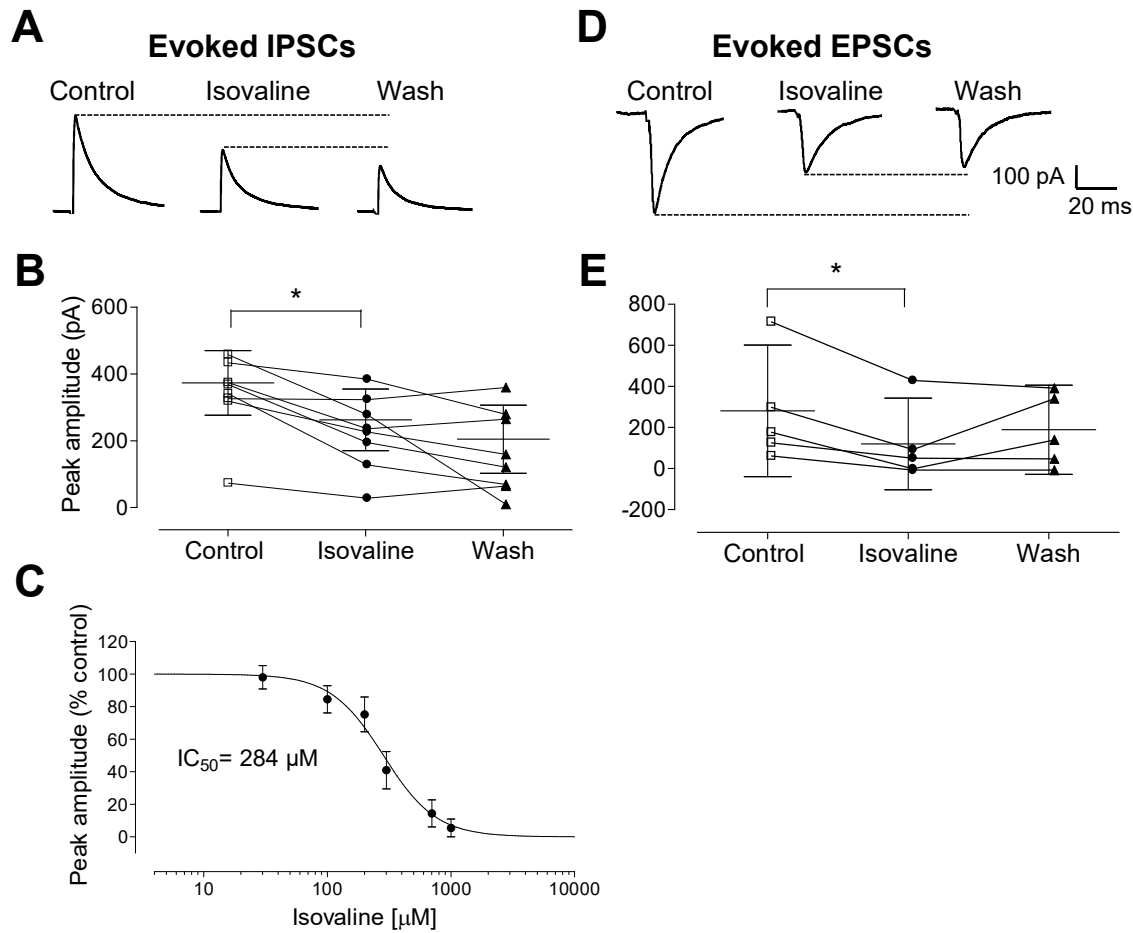


Figure 3.6. Isovaline decreases amplitudes of evoked IPSCs and EPSCs. (A) Application of isovaline ($400 \mu\text{M}$) to neuron reduced IPSC amplitude (average of 10). (B) In 8 neurons, isovaline decreased IPSC peak amplitude (average of 10). (C) Isovaline decreased the peak amplitude IPSCs in a concentration-dependent manner with an IC_{50} of $284 \mu\text{M}$. (D) Application of isovaline ($400 \mu\text{M}$) to neuron reduced EPSCs (average of 10). (E) In 5 neurons, isovaline decreased EPSC peak amplitude (average of 10). Washing for 40 min in (B-E) did not reverse the inhibitory effects of isovaline. Data were presented as mean \pm 95% CI. * $P < 0.05$; one-way ANOVA followed by Bonferroni's Multiple Comparison Test. $V_h = 0 \text{ mV}$ for IPSCs and $V_h = -70 \text{ mV}$ for EPSCs.

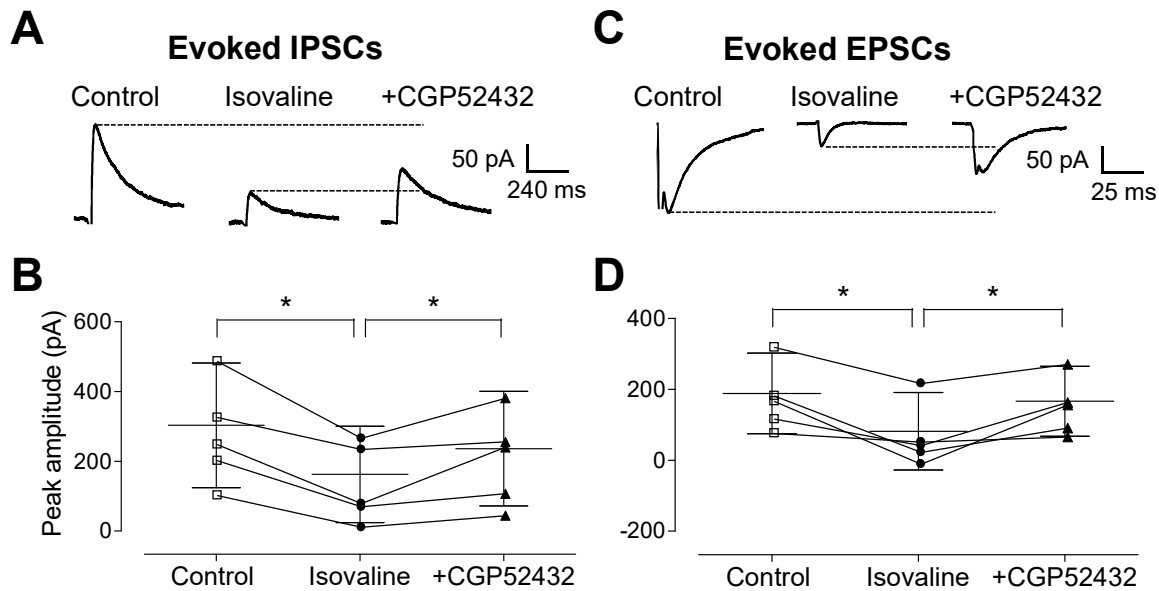


Figure 3.7. GABA_B receptor antagonism with CGP52432 reversed the inhibitory effects of isovaline on evoked IPSCs and EPSCs. (A) Application of isovaline (400 μ M) to neuron decreased peak amplitude of IPSCs (average of 10). Co-application of CGP52432 (10 μ M) with isovaline blocked the decrease. (B) In 5 neurons, co-application of CGP52432 (10 μ M) with isovaline (400 μ M) antagonized the decrease in peak amplitude of IPSCs (average of 10). (C,D) Application of isovaline (400 μ M) to neuron decreased peak amplitude of EPSCs (average of 10). Co-application of CGP52432 (10 μ M) with isovaline blocked the decrease in 5 neurons. Data were presented as mean \pm 95% CI. * $P < 0.05$; one-way ANOVA followed by Bonferroni's Multiple Comparison Test. $V_h = 0$ mV for IPSCs and $V_h = -70$ mV for EPSCs.

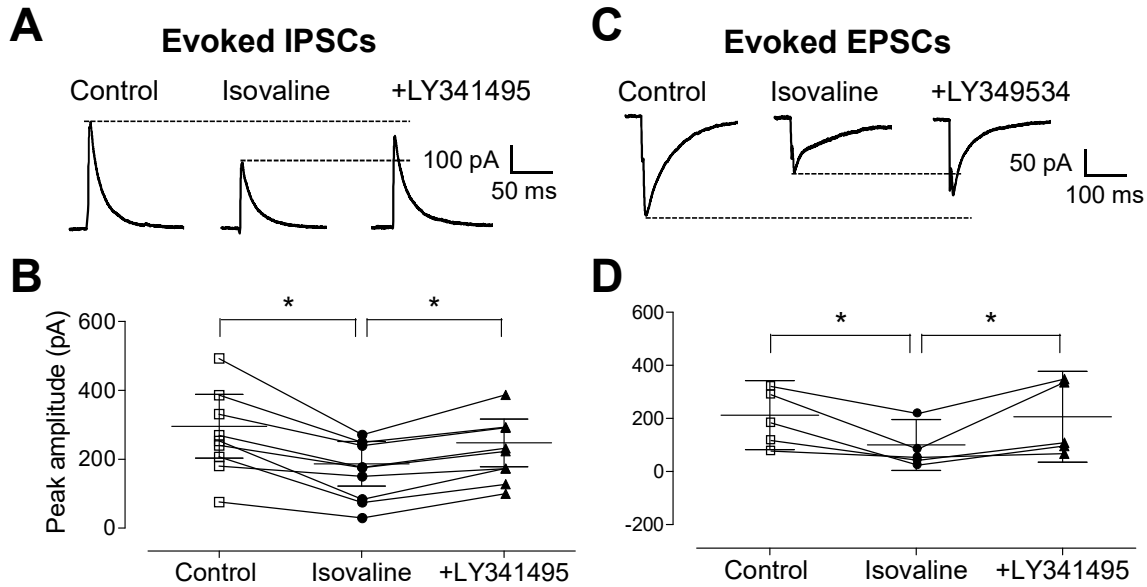


Figure 3.8. mGlu II receptor antagonism with LY341495 reversed the inhibitory effects of isovaline on evoked IPSCs and EPSCs. (A) Application of isovaline (400 μ M) to neuron decreased peak amplitude of evoked IPSCs (average of 10). (B) In 9 neurons, co-application of mGlu II receptor antagonist, LY341495 (1 μ M) blocked the inhibitory effect of isovaline (average of 10). (C,D) Application of isovaline (400 μ M) to neuron decreased peak amplitude of EPSCs (average of 10). In 5 neurons, co-application of LY341495 (1 μ M) with isovaline partially blocked the inhibitory effect of isovaline. Data were presented as mean \pm 95% CI. * P < 0.05; one-way ANOVA followed by Bonferroni's Multiple Comparison Test. V_h = 0 mV for IPSCs and V_h = -70 mV for EPSCs.

Chapter 4. Differential Effects of Isovaline and the GABA_B Agonist, Baclofen, in the Guinea Pig Ileum

4.1 Introduction

Isovaline (2-Amino-2-methylbutanoic acid) is a non-proteinogenic amino acid with a chemical structure similar to the major inhibitory neurotransmitter, γ -aminobutyric acid (GABA). The initial pharmacological studies showed that isovaline produced peripheral antinociception in rodent assays including the formalin foot assay and allodynia induced by prostaglandin E₂ or strychnine (MacLeod et al., 2010; Whitehead et al., 2012; Asseri et al., 2015). These studies on the analgesic effects were extended by our recent demonstration that isovaline co-administered with the hypnotic, propofol, produces general anesthesia (Whitehead et al., 2015). Isovaline also prevents seizures in 4-aminopyridine and pilocarpine models (Shin et al., 2011; Yu et al., 2014, 2015). While the specific targets mediating the anesthetic and anticonvulsant effects are not known, GABA_B receptors participate in the antiallodynic effects of isovaline as indicated by their partial reversibility by the GABA_B receptor antagonist, CGP35348 (Whitehead et al. 2012). Both GABA_{B1} and GABA_{B2} subunits have been detected in keratinocytes and nerve endings, consistent with GABA_B mediation of isovaline effects in the periphery (Whitehead et al., 2012; Corell et al., 2015).

Subsequent investigations have demonstrated an additional involvement of mGlu II receptors in isovaline analgesia *in vivo*. LY341495 nearly abolished the antiallodynic effect of isovaline which was potentiated by LY487379, a positive allosteric mGlu II receptor modulator (Asseri et al., 2015). On the basis of these findings, we undertook the present experiments to shed further light on the receptor mechanisms of isovaline's effects in the guinea pig ileum which expresses

functional GABA_B and mGlu II receptors (Larzabal *et al.* 1999; Kirchgessner 2001; Chen & Kirchgessner 2002; Hyland & Cryan 2010).

Originally developed by Paton and Zar (1968), this gut assay involves isolation of a small length of ileal tissue and applying transmural electric stimulation, increased luminal pressure, or receptor agonists to evoke muscle contraction. In the present experiments, we used transmural electrical stimulation which elicits muscle contraction neurogenically, by activating the myenteric plexuses of Auerbach and Meissner (cf. Paton and Zar, 1968). GABA_B agonists attenuate spontaneous and stimulated contractions by actions that are dose-dependent and susceptible to blockade by GABA_B antagonists (Giotti *et al.*, 1983; Ong and Kerr, 1983; Kerr *et al.*, 1990). In contrast, the effects of mGlu II agonists have received less study. Chen and Kirchgessner (2002) showed that group II/III glutamate receptor agonists inhibited N-type calcium channels in neurons of the myenteric plexus of guinea pig ileum. They also demonstrated immunoreactive staining of group II/III receptors, implicating glutamatergic inputs. Hence, we hypothesized that isovaline would decrease contractions through an intrinsic activation of both GABA_B and mGlu II receptors. We sought to compare isovaline's effects to those of the prototypical GABA_B agonist, baclofen, as well as the specific mGlu II receptor agonist, LY354740.

4.2 Experimental procedures

4.2.1 Animals

Hartley guinea pigs of either sex weighing 300–400 g were housed at 21°C and 55% relative humidity on a 12 h light dark-cycle with lights on at 07:00 AM. Food and water were available *ad libitum*. All procedures were carried out with approval from the Animal Care Committee at The University of British Columbia and complied with the Canadian Council on Animal Care. This study is reported according to ARRIVE guidelines (Kilkenny et al., 2010). A total of 28 tissues from 14 animals were used in these studies. These numbers were based on previous studies investigating the effects of baclofen in the guinea pig ileum (Kerr *et al.* 1990).

4.2.2 Experimental methods

On the day of testing, animals were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital (65 mg · kg⁻¹). The abdomen of the animal was opened via a V-shaped incision and a separate incision was made into the chest cavity. The ileum was then located and a 20–30 cm segment was excised 2–3 cm distal to the ileocecal valve. The ileal segment was divided into 2–3 cm segments and quickly transferred into a organ bath containing 20 ml of continuously aerated Tyrode's solution composed of (mM): NaCl 137, KCl 2.68, glucose 5.55, NaH₂PO₄ 0.42, NaHCO₃ 11.9, MgCl₂ 1.05, and CaCl₂ 1.8 (pH 7.4). The segments were mounted with 5-0 silk sutures threaded through one wall at each end of the segment with a preload tension of 1 g and allowed to equilibrate with the aerated solution for 1 h prior to testing. Tissues were primed for testing after the equilibration period via stimulation with parallel platinum electrodes delivering pulses (pulse width 10 ms, 0.1 Hz, sub-maximal voltage) generated by a Grass S9 stimulator

(Natus Neurology Inc., Warwick, RI, USA). The tissue was stimulated for 3 min followed by a 7-min recovery period, and this protocol was repeated for a total of 10 cycles. Contractions were measured isometrically with a Grass FT03 force transducer (Natus Neurology Inc., Warwick RI, USA), recorded with Powerlab/8sp software (ADInstruments, Colorado Springs, CO, USA), and displayed using Chart 5 software (ADInstruments). Following priming, drugs were added 1 min after the start of electrical stimulation and remained in the organ bath for 2 min before washing. In the antagonist studies, CGP52432 was added to the bath for a final concentration of 3 μ M at 5 min before application of baclofen or isovaline. Effects of drugs on contractions were recorded as the measured change in tension in relation to the preload tension.

4.2.3 Statistical analysis

Muscle contraction traces were analyzed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) by measuring peak amplitude of contraction force. Spike amplitudes during drug application were compared to spike amplitudes obtained prior to application and are expressed as a percentage of pre-drug amplitudes. Interpulse tension was determined by calculating the average of cyclic minima of contractions for the 1 min and 2 min periods before and after drug application. The data are expressed as a percentage change from pre-application values. In cases of normalization, data were normalized to pre-drug application values to account for differences in contractility and resting tension between tissues. Concentration-response curves were produced using a least squares fit of a log[inhibitor] versus response model according to the equation:

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

Where Top and Bottom are plateaus of the curve and the IC_{50} referring to the concentration yielding a response halfway between Top and Bottom. Grubb's test was used for the detection of outliers which were excluded without replacement. A two-tailed, parametric t test was used to compare isovaline and baclofen data. IC_{50} s for isovaline and baclofen were taken to be different if there was no overlap of 95% confidence intervals (CIs). Data are presented as individual points with each point and summary bars presented as mean with 95% CI. A P value < 0.05 was considered to be statistically significant.

4.2.4 Drugs and chemicals

Baclofen, LY354740, and CGP52432 were purchased from Tocris Bioscience (Ellisville, Mo, USA). Tetrodotoxin (TTX) was purchased from Alomone Labs (Jerusalem, Israel). R-isovaline was a generous gift from Nagase & Co. Ltd (Osaka, Japan). Baclofen and LY354740 was dissolved in equimolar sodium hydroxide solution, TTX was dissolved in water, and isovaline was dissolved in Tyrode's solution.

4.3 Results

Application of TTX (300 nM) abolished the electrically evoked ileal contractions (Fig. 1A). This blockade, which was reversible, demonstrated that the contractions resulted from action potential-dependent stimulation of neural tissue, and not likely the smooth muscle.

4.3.1 Isovaline's effects on guinea pig ileum differ from those of baclofen

Isovaline reversibly reduced the amplitude of evoked contractions and increased the interstimulus resting tension over the tested range of concentrations (1–100 mM) in $n = 8$ tissues sampled from 4 animals (Fig. 1). The maximum estimated inhibition was 60% (95% CI, 37– 82) (Fig. 1C). A non-linear least squares fit of the reductions in contraction amplitude by isovaline indicated an IC_{50} of 17.8 mM (95% CI, 3.5–91.1). Isovaline also produced an increase in the area under the curve over the range of 10–100 mM in the same tissues with a maximum increase of 200% (95% CI, 112–288) and an EC_{50} of 6.4 mM (95% CI, 0.9–46.1) (Fig. 1D). To ensure these effects were not due to changes in osmolarity from the addition of high concentrations of isovaline, a 370 mOsm sucrose solution (iso-osmotic to 100 mM isovaline) dissolved in Tyrode's solution was used as a control. Addition of the sucrose solution produced a decrease in interstimulus resting tension which is in contrast to isovaline, suggesting the observed effects of isovaline were not due to changes in osmolarity (data not shown). Lower doses starting at 10 μ M were initially tested, but no effect was detected (data not shown).

Baclofen reversibly reduced the amplitude and resting tension of the contractions over a concentration range (1–100 μ M) lower than isovaline in $n = 6$ tissue samples from 3 animals (Fig. 2). A non-linear least squares fit of the reductions in contraction amplitude indicated an

IC₅₀ of 3 μ M (95% CI, 1–9). The maximum inhibition was 32% (95% CI, 17– 46). Unlike isovaline, baclofen decreased the resting tension. The maximum decrease in resting tension was 143% (95% CI, –329– –42) and was approached at a dose of 100 μ M (Fig. 3B). A logistic curve fit of the reductions in resting tension indicated an IC₅₀ of 65 μ M (95% CI, 3–1496) in $n = 10$ tissues from 5 animals (Fig. 3C).

4.3.2 Isovaline decreases contractions despite GABA_B receptor antagonism

Representative traces of baclofen and isovaline in the presence and absence of CGP52432 are shown in Figure 4. Applied alone, CGP52432 did not consistently affect the responses to electrical stimulation (data not shown). Pre-treatment and co-application with 3 μ M CGP52432 did not alter the mean amplitude of the responses to 10 mM isovaline in $n = 6$ tissues from 3 animals (control, 80%, [95% CI, 68– 93]; CGP52432 76%, [95% CI, 60–92]; difference between means, 5%, [95% CI, –16–25]) (Fig. 4E). CGP52432 pretreatment and co-application prevented the decrease in spike amplitude induced by baclofen. CGP52432 blocked the decrease in the mean amplitude of the responses to 100 μ M baclofen in $n = 5$ tissues from 3 animals (control, 94% [95% CI, 84–103]; CGP52432, 78%, [95% CI, 64– 92]; difference between means, –16%, [95% CI, –26– –6]) (Fig. 4F).

4.3.3 Metabotropic group II glutamate agonist LY354740 does not affect contractility of guinea pig ileum

Figure 5A illustrates the effects of LY354740 on evoked contractions in a representative record. LY354740 tested at 0.05, 0.1, 0.3, 1, 3, and 10 μ M did not inhibit or enhance electrically evoked

contractions and did not affect resting tension in 4 tissue samples of guinea pig ileum (Fig. 5B). The effects of isovaline were not antagonized by (1 μ M) of the mGlu II antagonist, LY341495 (data not shown).

4.4 Discussion

In this study, we found that isovaline reversibly inhibited electrically evoked contractions of the guinea pig ileum. This inhibition was baclofen-like but much to our surprise, we found no evidence of mediation of isovaline's effects by neuronal GABA_B receptors. Moreover, isovaline increased resting tension whereas baclofen decreased resting tension in addition to the peak amplitude by interacting with GABA_B receptors. It was unlikely that the effects of isovaline involved group II receptors since LY354740 itself did not have an effect on the ileum. The non-involvement of GABA_B and mGlu II receptor subtypes in the mediation of isovaline responses in the ileum compared to their involvement in cutaneous and CNS tissues (cf. Introduction) raises the possibility of differences in metabotropic receptor compositions.

Isovaline reversibly decreased electrically evoked contractions with an IC₅₀ of 18 mM and caused a maximum inhibition of nearly 60%. The concentration-response curves of isovaline and baclofen for reducing contraction amplitude had similar trajectories although baclofen had a several thousand-fold lower IC₅₀. Baclofen decreased contractions with an IC₅₀ of 3 μM and caused a maximum inhibition of 35% which is consistent with values in the literature (Ong and Kerr, 1983; Kerr et al., 1990). However, prior administration of CGP52432, a GABA_B antagonist, blocked the effects of baclofen, but not the effects of isovaline on contraction amplitude.

The differential effects on resting tension further highlight the differences between isovaline and baclofen. While decreasing the peak amplitude, isovaline increased the area under the contraction curve. The greater area under the curve was likely due to an increase in resting

tension. To ascertain if the greater area did not result from changes in osmolarity on addition of high concentrations of isovaline to the bath, we applied a 370 mOsm sucrose solution which was iso-osmotic to 100 mM isovaline. The sucrose control and isovaline solutions had different effects, suggesting that the observed increase in resting tension by isovaline was not due to changes in osmolarity. It is possible that the effects of isovaline on resting tension may have been a consequence of isovaline actions on transport proteins present in the ileum (Christensen, 1962). A high concentration of isovaline could potentially overwhelm this transport mechanism leading to its sequestration by cells thereby preventing relaxation of the ileum.

We investigated the possibility that the effects of isovaline were mediated by mGlu II receptors but could not demonstrate the functional presence of these receptors. LY354740, in concentrations up to 10 μ M, did not produce detectable effects in the ileum. LY354740 has been found in nanomolar concentrations (e.g., 10 nM) to activate mGlu II receptors in rat CNS (Schoepp *et al.* 1999). While mGlu II receptors have been identified by immunocytochemistry in the myenteric plexus of guinea pig ileum (Larzabal *et al.*, 1999; Chen and Kirchgessner, 2001; Kirchgessner, 2002), they may play a role in other aspects of enteric nervous system function. The present findings suggest that mGlu II receptors in the ileum do not modulate resting tension or contractile responses.

Our studies showed that application of TTX abolished electrically evoked contractions, demonstrating their neurogenic nature. In contrast to the effects of isovaline on guinea pig ileum previous studies established that isovaline acts at GABA_B receptors to produce analgesia and inhibition of CNS neurons (Cooke *et al.*, 2012; Whitehead *et al.*, 2012). In the studies by Cooke

et al. (2012), isovaline increased membrane conductance in <50% of thalamic neurons in contrast to the reliable inhibition produced by baclofen. Similarly, AtT-20 cells transfected with GABA_B receptor subunits showed an insensitivity to isovaline while consistently responding to baclofen or GABA application (Pitman et al., 2015). Molecular studies show that populations of GABA_B receptor subtypes are not homogeneous at different anatomical locations (Calver et al., 2000; Marcoli et al., 2000; Enna, 2001). GABA_B agonists are up to three orders of magnitude more potent when applied to central than in the case of peripheral tissues, implicating tissue-dependent receptor types (Lehmann et al., 2010). The different rank order of potency for GABA_B antagonists in blocking baclofen inhibition in rat spinal cord compared to guinea pig ileum also suggests distinct sets of receptors (Sawynok, 1986). Heterogeneity of the GABA_B receptor subtype appears likely in these tissues since several GABA analogues exhibit differential agonist activity at GABA_B receptors in the CNS and periphery (Kerr et al., 1993, 1994, Ong et al., 1994, 1992, 1990). The present findings suggest that isovaline has actions different from baclofen's, possibly by acting on a subset of GABA_B receptors.

In summary, we found that isovaline affects electrically evoked contractions in guinea pig ileum in a manner that is distinct from baclofen. Future studies on receptor compositions in guinea pig ileum may reveal differences from the known compositions of metabotropic GABA receptors and may lead to the discovery of therapeutic targets with selective effector mechanisms.

4.5 Figures

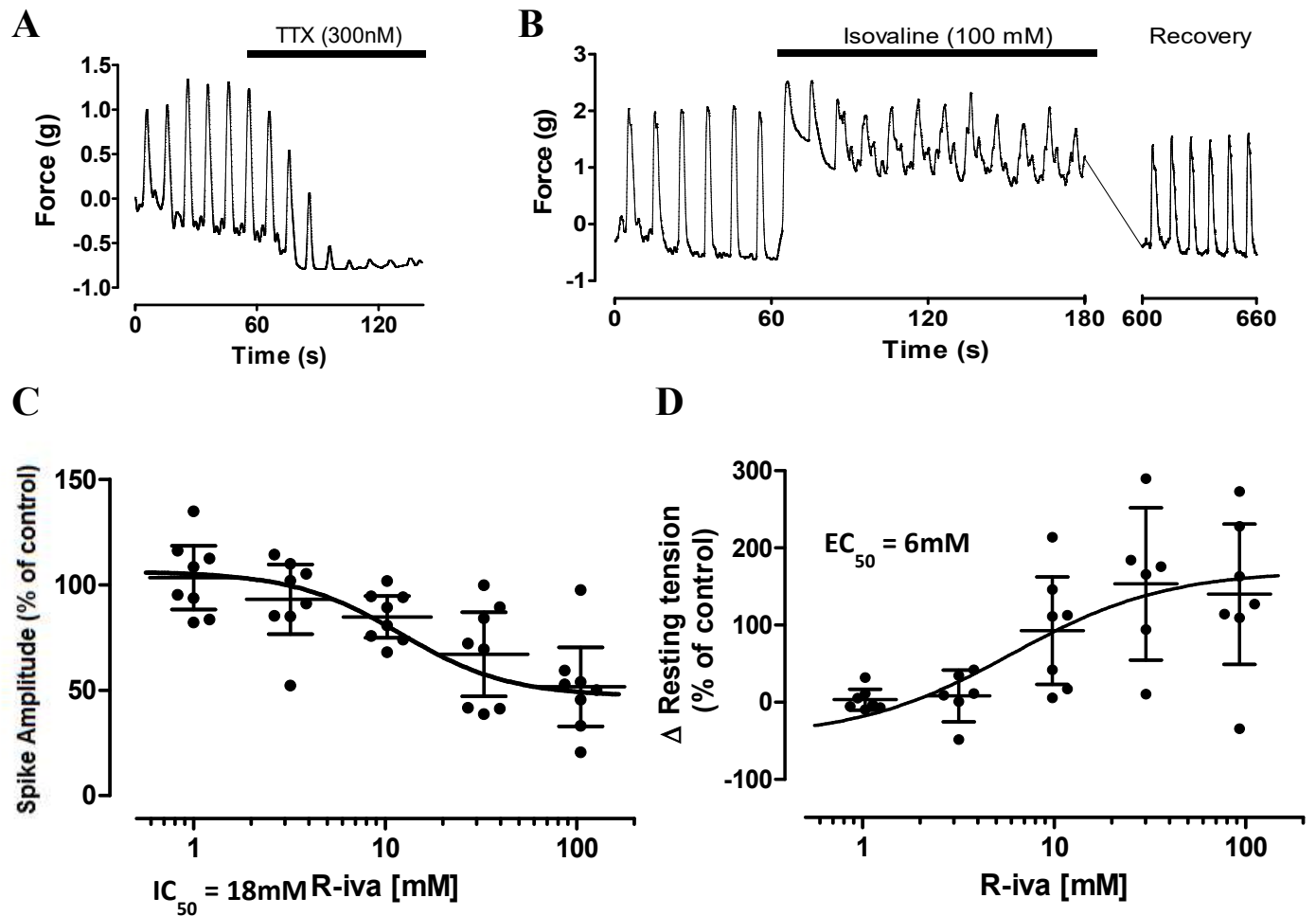


Figure 4.1. Isovaline decreased the amplitude of electrically evoked contractions, but increased the area under the curve. (A) Representative trace of contractions before and during application of 300 nM tetrodotoxin (TTX). (B) Representative trace of contractions at baseline and in the presence of 100 mM isovaline (R-iva) and tissue recovery following wash. (C) Isovaline decreased the amplitude of evoked contractions in a concentration-dependent manner with an IC_{50} of 18 mM ($n = 8$ tissues from 4 animals). (D) Isovaline increases the baseline tension in a concentration dependent manner with an IC_{50} of 6 mM ($n = 7$ tissues from 4 animals with

outliers excluded). Data are responses measured as a percentage reduction in contraction amplitudes prior to drug addition. Each point represents an individual recording with the summary bars presented as mean \pm 95% CI.

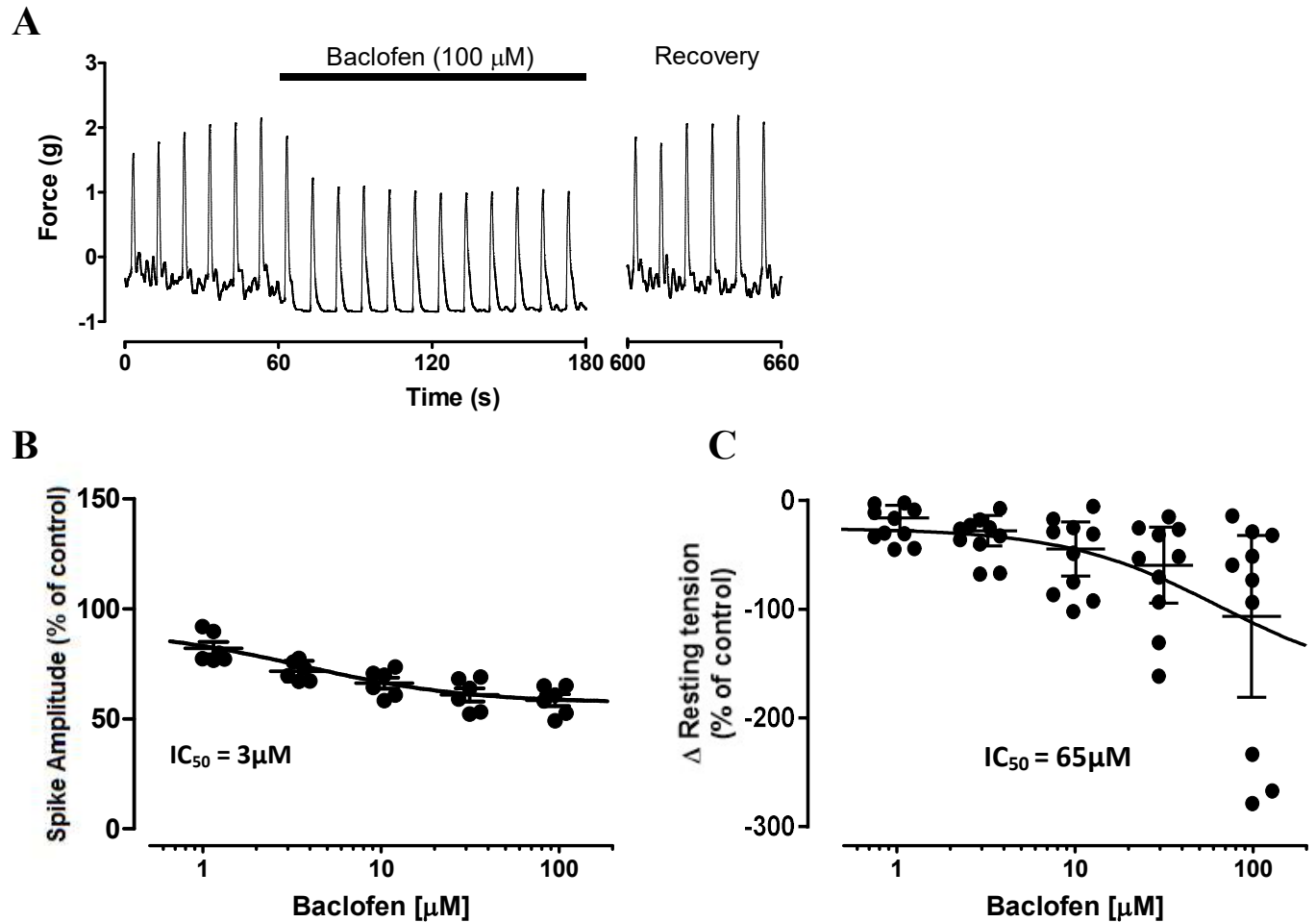


Figure 4.2 Baclofen partially inhibited electrically evoked contractions in a concentration-dependent manner. (A) Representative trace of contractions at baseline and in the presence of 100 μ M baclofen (left panel) and tissue recovery following wash. (B) Baclofen decreases spike amplitudes of electrically evoked contractions in a concentration-dependent manner with an IC_{50} of 3 μ M ($n = 6$ tissues from 3 animals). (C) Baclofen decreases resting tension in a concentration dependent manner with an IC_{50} of 65 μ M ($n = 10$ tissues from 5 animals). Data are responses measured as a percentage reduction in contraction heights prior to drug addition. Each point represents an individual recording with the summary bars presented as mean \pm 95% CI.

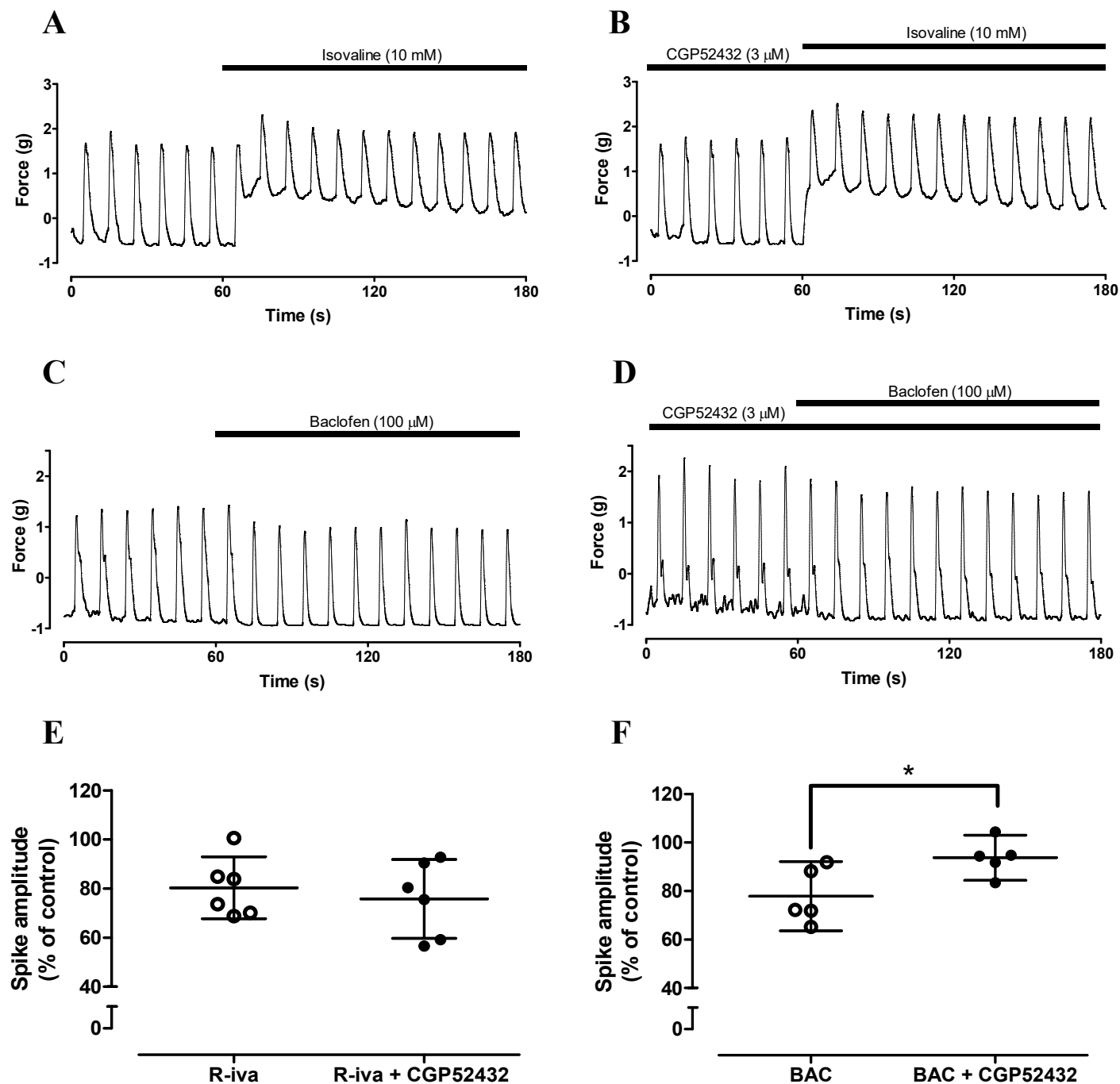


Figure 4.3 CGP52432 antagonizes the effects of baclofen but not isovaline. Representative traces of isovaline's (R-iva) effects on contractions in the absence (A) and presence (B) of CGP52432. Representative traces of baclofen's effects on ileal contractions in the absence (C)

and presence (D) of CGP52432. (E) Pre-treatment with 3 μ M CGP52432 did antagonize the effects of 10 mM isovaline (R-iva). $n = 6$ tissues from 3 animals. (F) 100 μ M baclofen (BAC) reduced contractions while pretreatment with 3 μ M CGP52432 antagonized the effect of 100 μ M baclofen on spike amplitude of contractions. * indicates a statistically significant difference, $n = 5$ tissues from 3 animals. Data are responses measured as a percentage reduction in contraction amplitudes prior to drug addition. Each point represents an individual recording with the summary bars presented as mean \pm 95% CI.

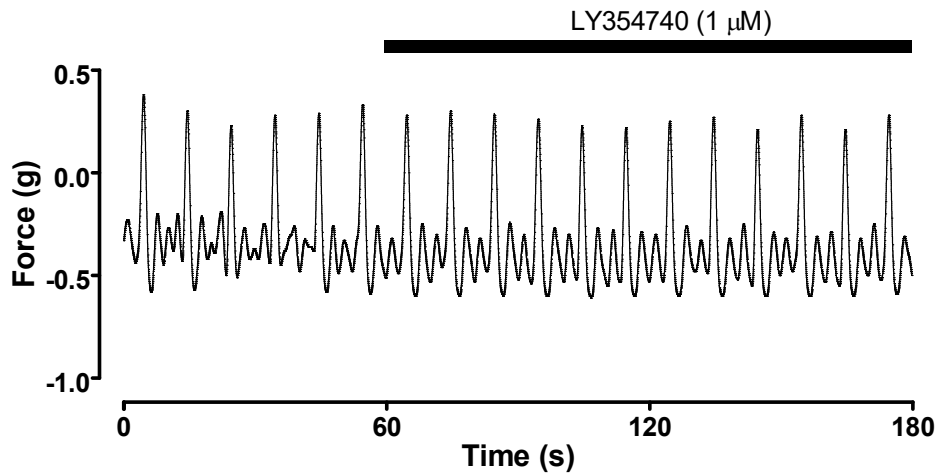
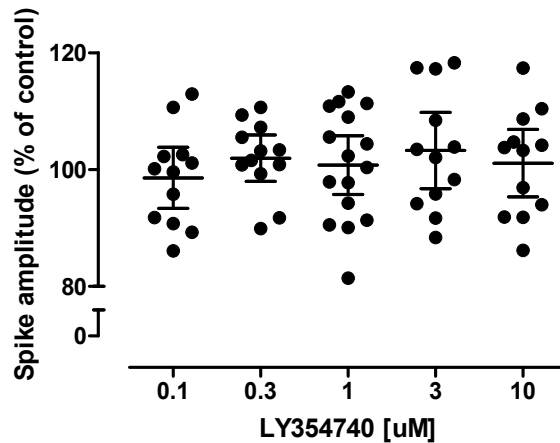
A**B**

Figure 4.4 LY354740 does not affect electrically evoked contractions . (A) Representative trace of contractions at baseline and in the presence of 1 μM LY354740. (B) Peak amplitudes of contraction in the presence of LY354740 expressed as a percentage of baseline. Data are responses measured as a percentage reduction in contraction amplitudes prior to drug addition. Each point represents an individual recording with the summary bars presented as mean \pm 95% CI ($n = 12$ tissues from 6 animals).

Chapter 5. General Discussion

5.1 Summary of results

In this thesis, we studied the inhibitory effects of isovaline both *in vivo* and *in vitro* in three body tissues. An objective was to determine whether isovaline activated mGlu II receptors in central and peripheral neurons, in addition to the previously shown interactions with GABA_B receptors. We used a pain model (*in vivo*), showing that isovaline reversed the PGE₂-induced allodynia. This reversal was blocked by mGlu II receptor antagonist, LY341495, suggesting a contribution of mGlu II receptors to isovaline-induced analgesia. Therefore, the analgesic effects of isovaline were attributable to interactions with peripheral GABA_B and mGlu II receptors.

We used patch clamp techniques in thalamic slice preparations, determining that isovaline decreased transmitter release by activating presynaptic mGlu II and GABA_B receptors. In an absence of functional postsynaptic mGlu II receptors, LY354740 decreased frequency of spontaneous and miniature GABAergic IPSCs, suggesting that mGlu II receptors have a presynaptic modulatory role on GABA-containing terminals. Isovaline, LY354740 or baclofen decreased lemniscal IPSCs and EPSCs, supporting hypothesized presynaptic actions of these agonists.

The effects of isovaline at GABA_B receptors in brain slices were atypical and mostly irreversible (Chapter 3) as in previous studies (cf. Cooke et al. 2012; see also Pittman et al. 2015). Therefore we considered the possibility that GABA_B receptors in some peripheral tissues may be distinct from GABA_B receptors in the CNS. For practicality, we chose the guinea pig ileum preparation as a standard assay for GABA_B responsiveness (Bowery et al., 1981; Ong et al., 1992). In the

guinea-pig ileum (*in vitro*), we showed that isovaline and baclofen decreased the amplitude of muscle contractions whereas a mGlu II agonist had no effects. A GABA_B antagonist did not block the effects of isovaline while blocking baclofen effects. Therefore, nociceptors or receptor mechanisms for isovaline in skin and thalamic tissues may differ from the gastrointestinal tract.

In summary, the main results indicate that isovaline can activate peripheral (skin) and central (thalamic) mGlu II receptors in addition to GABA_B receptors. The following sections highlight the above and discuss their implications and future directions for experiments and therapeutics.

5.2 Analgesic effects of isovaline mediated by peripheral mGlu II receptors

The first manuscript addressing the peripheral interaction of isovaline with inhibitory metabotropic glutamate receptors (mGluRs), examined the effects on mechanical allodynia produced by PGE₂. Subcutaneous isovaline produced a dose-dependent analgesic effect only in the injected hindlimb. Group II but not group III mGluRs mediated this effect which was potentiated by a mGlu II receptor positive allosteric modulator. These findings help to explain previous observations that the GABA_B antagonist, CGP52432, did not eliminate the peripheral analgesia due to isovaline (Whitehead et al., 2012). Thus, the mechanism of action of the experimental analgesic, isovaline, involves at least two metabotropic receptor targets, and may serve as a potential prototype of a new class of analgesics.

The agonists for mGlu II and GABA_B receptor subtypes - LY354740 and baclofen - readily penetrate into CNS and are selective with IC₅₀s of ~20 nM and ~85 nM (reviewed by Audus and

Borchardt, 1986; Nikiforuk et al., 2010; Schoepp et al., 1999). There was no crosstalk between these receptor subtypes when activated by baclofen and LY354740. Systemic administration of LY354740 and baclofen produces in addition to analgesia, central side effects such as hypothermia, motor impairment and reduction in cognitive performance (Jones et al., 2005; Spinelli et al., 2005; Wieronska et al., 2012; Ertzgaard et al., 2017). We previously reported that systemic isovaline produces analgesia without CNS effects due to its inability to cross the blood-brain barrier (Shiba et al., 1989; MacLeod et al., 2010; Whitehead et al., 2012). Thus, analgesic isovaline has an advantage over mGlu II and GABA_B agonists.

There are limitations in this study, such as the method used for measuring mechanical allodynia induced by intraplantar injection of PGE₂ and the difficulty in estimating the drug concentrations at the injection sites. While our data agree with previous reports (Yang and Gereau, 2003; Kassuya et al., 2007), the assessment of tactile allodynia in pain models using the von Frey filament test is not always precise (cf. Kawabata, 2011). Mechanical thresholds obtained with von Frey filaments can only approximate the exact threshold value. The force generated by the filaments is represented by discrete log values (0.02, 0.07, 0.16, 0.4 g etc.). Although these force applications are discontinuous, the test is intended to assess a sensory continuum (Bove, 2006). This may limit accurate estimation of paw withdrawal thresholds (Chaplan et al., 1994). Another limitation is the unknown concentrations of agonists and antagonists at the receptor sites and distribution following subcutaneous injection. Therefore, it would be difficult to compare the effects of the agonists in peripheral and brain tissues.

Future investigations should be directed toward determining peripheral functions of mGlu II and GABA_B receptors, given their wide distribution especially in sensory systems (Ohishi et al., 1993; Carlton et al., 2001). While these receptor subtypes have received extensive study on modulation of transmitter release in the CNS, participation in peripheral sensory physiology is unclear. The present study demonstrated the presence of functional mGlu II and GABA_B receptors in nociception of peripheral tissues (Reis and Duarte, 2006; Zammataro et al., 2011; Whitehead et al., 2012). These receptor subtypes have been implicated in neuropathic pain (Bridges et al., 2001; Patel et al., 2001; Fisher et al., 2002). GABA_B agonists produce a dose-dependent antinociceptive effect in neuropathic pain model involving chronic constriction injury (CCI) in rodents (Smith et al., 1994; Franek et al., 2004). In the CCI model, activation of mGlu II receptors by selective agonists produces antinociceptive effects (Osikowicz et al., 2008). These findings suggest potential targets in peripheral tissues for the drug treatment of chronic pain.

A drawback of clinical use of mGlu II agonists in treatment of pain and nicotine dependence is the development of tolerance after repeated dosing (Jones et al., 2005; Liechti et al., 2007). Repeated doses of baclofen administered to patients with muscle spasms results in tolerance (Nielsen et al., 2002). Tolerance after chronic use of isovaline has not been investigated; it would be interesting to examine this possibility after multiple administrations of isovaline.

5.3 Presynaptic effects of isovaline in ventrobasal neurons

The second manuscript addressed isovaline's inhibitory actions on neurotransmission and possible receptor mechanisms in ventrobasal thalamus. Previous studies in thalamic slices examined the postsynaptic effects of isovaline and the basis for inhibition of neuronal firing. The actions of isovaline were mediated by activation of GABA_B receptors, with some differences from the prototypical GABA_B agonist, baclofen (cf. Introduction). Subsequent *in vivo* studies by Whitehead et al. (2012) showed that isovaline produced analgesia mediated in part by GABA_B receptors. In the first manuscript, we confirmed the GABA_B mediation of the analgesic effects and additionally demonstrated an involvement of peripheral mGlu II receptors (Asseri et al., 2015). We will now discuss the main findings of our studies in thalamic slices, demonstrating that both GABA_B and mGlu II receptors mediate the presynaptic effects of isovaline in thalamocortical neurons.

We determined the effects of isovaline on presynaptic metabotropic receptors by using ionotropic receptor blockade and Cs⁺ in the recording electrode to block postsynaptic K⁺-mediated effects, thereby isolating the presynaptic effects. We found that isovaline decreased the peak amplitude of IPSCs and EPSCs evoked by medial lemniscal stimulation without affecting their decay time constants. The inhibitory effects of isovaline on types of synaptic currents were partly blocked by application of either a mGlu II antagonist, LY341495 or a GABA_B antagonist, CGP52432. The data suggest that isovaline reduces release of neurotransmitters GABA and glutamate by activating mGlu II and GABA_B receptors at nerve terminals. These effects of isovaline are more likely attributable to action resulting in autoinhibition rather than to presynaptic inhibition. While the term “presynaptic inhibition” describes inhibition of

presynaptic neuron by a transmitter released by an interneuron, autoinhibition is a negative feedback caused by activation of receptors located on the nerve terminal from which the transmitter is released. There is little or no anatomical basis for the synaptic arrangement that provides presynaptic inhibition. The organization of the ventrobasal nuclei in the rat is relatively simple (De Biasi et al. 1988).

The thalamic results describing activation of mGlu II receptors by isovaline are consistent with LY354740-like effects in behavioral including pain models (cf. Section 5.2). Activation of mGlu II receptors by LY354740 induces anxiolytic behavior in mice. In an elevated plus maze model of anxiety, systemic administration of LY354740 increases the time spent in the open-arm, suggesting an anxiolytic effect (Schoepp et al., 2003; Linden et al., 2004). Although isovaline distributes mostly to the periphery (cf. Shiba et al., 1989; MacLeod et al., 2010; Whitehead et al., 2012), in some *in vivo* models, systemically administered isovaline may reach concentrations in the brain that are sufficient to produce anxiolytic and antiepileptic effects (Sase et al., 2013; Yu et al., 2014). Thus, under certain disorders or pathophysiological conditions such as epilepsy, anxiety and nerve injury (Kovacs et al., 2012; Skultetyova et al., 1998; Reinhold and Rittner, 2017) the integrity of the blood-brain barrier may alter to allow entry of previously non-penetrating compounds. Sase and colleagues (2013) reported that systemic administration of isovaline induced robust anxiolytic effects on the elevated plus maze model. Our findings that isovaline acts as a mGlu II agonist in CNS suggest further investigations on the receptor basis of the anxiolytic effect.

The metabotropic receptor effects of isovaline mimic presynaptic inhibition which has an important role in regulating excitatory synaptic transmission (Thompson et al., 1992). Excessive glutamatergic synaptic activity has been implicated in several neurological and psychiatric disorders such as anxiety, epilepsy and depression (Javitt, 2004; Dudek, 2009). Anxiety, epileptic and depressive disorders may result from neuronal dysfunctions that involve mGlu II or GABA_B receptor subtypes (Helton et al., 1998; Alexander and Godwin, 2006; Kumar et al., 2013; Park et al., 2015; Alexander, 2017; Chen et al., 2017). However, the adverse effects of GABA_B and mGlu II receptor agonists may limit their clinical use (cf. Introduction). Unlike the conventional GABA_B and mGlu II agonists, isovaline is expected to be efficacious, and may have few side effects. Isovaline represents a first-in-class, safe and effective, drug candidate for the treatment of these disorders. Future directions for the current study should be aimed at investigating the efficacy of isovaline in animal models of anxiety, epilepsy and depression.

Drug combination therapy targeting different receptor systems can improve the treatment of various pain conditions (Ortiz et al., 2012; Eisenberg and Suzan, 2014). The main objective of such therapy is to attain synergistic interaction between two dissimilar analgesics (Tallarida, 2001). The interaction would be beneficial since lower doses are required to produce sufficient antinociceptive effects thereby reducing the side effects observed with monotherapy. By targeting both mGlu II and GABA_B receptors (Whitehead et al., 2012; Asseri et al., 2015), isovaline is expected to improve the treatment outcomes in chronic conditions such as neuropathic pain that are refractory to current pharmacotherapies. In light of current findings, further investigation is warranted to determine whether a combination of mGlu II and GABA_B agonists could produce synergistic effects in other disorders involving both receptors.

5.4 Presynaptic effects of LY354740 in ventrobasal neurons

In the second manuscript, we also examined the inhibitory actions of LY354740 in ventrobasal thalamus. Previous investigations used conventional electrode solutions, extracellular AMPA and NMDA antagonists and non-hydrolyzable substrates of GTP to isolate and study the effects of LY354740 or APDC on GABAergic IPSPs evoked by nRt stimulation (Turner and Salt, 2003; Liu et al., 2015). The studies suggested a presynaptic location of mGlu II receptors on nRt terminals in ventrobasal thalamus, based on observed reductions of IPSP or IPSC amplitude by the mGlu II agonists which were partly blocked by a mGlu II antagonist, LY341495. These studies were unclear because of the use of an unselective group II mGluR agonist (APDC; Liu et al. 2015) or high concentrations of agonist (1-10 μ M of LY354740; Turner and Salt, 2003).

The current objective was to determine the pre- and postsynaptic effects of LY354740 on thalamic ventrobasal neurons. LY354740 had no postsynaptic actions on the active or passive membrane properties but had significant presynaptic actions. LY354740 reduced the peak amplitude of evoked IPSCs in a concentration-dependent manner, without affecting the decay time constants. In an absence or presence of action potential-evoked release, LY354740 respectively reduced the frequency of spontaneous and miniature IPSCs. LY341495, a selective mGlu II receptor antagonist, blocked the reductions. The GABA_A antagonist bicuculline eliminated both types of IPSCs, revealing their GABAergic nature. The studies demonstrated that activation of mGlu II receptors decreases the release of GABA. LY354740 also reduced the peak amplitude of evoked EPSCs without affecting the decay time constants. LY341495 blocked the reductions, demonstrating the specificity for mGlu II receptors. The data suggested that LY354740 decreases the release of glutamate by activation of presynaptic mGlu II receptors. As

hetero- or autoreceptors, presynaptic mGlu II receptors may regulate the release of GABA or glutamate, suggesting a thalamic target for antinociceptive drugs.

Future studies should include assessment of the role of mGlu II receptors in regulating local neural network in thalamus which controls the output of thalamocortical neurons during various states of consciousness (Steriade, 2004). This may provide insight about the role of mGlu II receptors in disorders of consciousness such as absence seizures (cf. Alexander and Godwin, 2006).

5.5 Presynaptic effects of baclofen in ventrobasal neurons

The presynaptic effects of baclofen on lemniscal IPSCs and EPSCs bring to mind the complexity of GABAergic interactions in somatosensory thalamus. Selective GABA_B activation by baclofen reduced the peak amplitudes of IPSCs and EPSCs without affecting their decay time constants. These data showed that transmitter release was reduced from nerve terminals in GABAergic and glutamatergic neurons. The reduction in GABA release would result in a reduced postsynaptic response to GABA, reducing mostly GABA_A activation. Also activation of presynaptic GABA_A receptors reduces the presynaptic release of GABA (Park et al., 2017). We cannot exclude the possibility that GABA_A receptors may mediate in part the presynaptic effects of isovaline (cf. Zheng-Xiong et al., 1996). Therefore, future experiments should address this possibility that isovaline's presynaptic actions are mediated in part by GABA_A receptors.

5.6 Isovaline effects on ileal muscle contractions

The third manuscript describes the effects of isovaline on peripheral metabotropic receptors in guinea-pig ileum. In this project, we utilized the guinea-pig ileum *in vitro* preparation to demonstrate the effects of isovaline, finding that they were not attributable to actions on GABA_B and group II metabotropic glutamate receptors. Both metabotropic subtypes are expressed in guinea pig ileum (cf. Introduction). Isovaline in millimolar concentrations reversibly decreased the amplitude of muscle contractions and increased muscle tension. Baclofen reversibly reduced contraction amplitude as reported previously (Ong and Kerr, 1983; Kerr et al., 1990), but decreased tension. The GABA_B antagonist, CGP52432 antagonized these effects but did not prevent the effects of isovaline on contraction amplitude or tension. Surprisingly, the group II metabotropic glutamate receptor agonist, LY354740, had no effects on ileal muscle contractions. Hence, the effects of isovaline in the ileum are unlike the other peripheral and thalamic receptor actions (Whitehead et al., 2012; Cooke et al., 2012; Asseri et al., 2015). The data suggest that mechanism of isovaline action in ileal muscle is independent of GABA_B receptors and involves a new receptor.

Future directions: Since the actions of isovaline were inconsistent with activation of GABA_B receptors, it would be interesting to examine the compositions of metabotropic GABA receptors in guinea pig ileum. This may lead to the discovery of a subtype with an unusual effector mechanism sensitive to novel therapeutic targets.

5.7 Cross-talk between mGlu II and GABA_B receptors

Cross-talk between different members of the G-protein coupled receptors family has been demonstrated (Fernandes et al., 2011; Pacey et al., 2011). For example, in audiogenic seizures model, a selective antagonist of mGlu5 receptors and baclofen each produced a reduced incidence of seizures. When co-applied each at lower doses that had no effect on the seizures, the mGlu5 antagonist and baclofen reduced seizure incidence, implicating cross-talk between the intracellular signaling pathways of their respective receptors (Pacey et al., 2011). Functional cross-talk between mGlu1 and GABA_B receptors also has been demonstrated in cortical neurons and in transfected HEK cells when both receptors form mGlu1-GABA_B oligomerization (Rives et al., 2009).

Based on the above reports, we expected that LY341495 would block the antiallodynic effects of baclofen *in vivo*. We could not show cross-talk between group II mGluRs and GABA_B receptors using the PGE₂ model of allodynia. Given the limitations of the von Frey assay (cf. page 94), we cannot rule out the possibility that there may be crosstalk between the two receptor groups. Further studies using more robust assays are warranted to test for such potential crosstalk.

5.8 Isovaline proposed mechanism of actions

In this thesis we have shown that isovaline decreased GABA and glutamate release by activating mGlu II and GABA_B receptors of nerve terminals impinging on ventrobasal neurons. The involvement of these receptor systems is consistent with the susceptibility of isovaline analgesia to mGlu II and GABA_B antagonists. Different mechanisms have been suggested for inhibition of

transmitter release: (1) Inactivation of voltage dependent Ca^{2+} channels due to activation of presynaptic receptors (e.g., GABA_B or mGlu II receptors) may lead to inhibition of Ca^{2+} -dependent release of transmitter and propagation of action potentials (Guyon et al., 1995; Dutar et al., 2000); (2) Shunting of action potentials due to increase in K^+ and/or Cl^- channel conductance thus preventing action potentials from reaching the active zone of nerve terminal and subsequently decreasing release of transmitter (Wilson, 1995; Ries and Puil, 1999); (3) Direct inhibition of voltage-gated Ca^{2+} channels on presynaptic terminals. For example, the effects of analgesic gabapentin (which has structural similarity with isovaline) have been demonstrated in DRG neurons (Sutton et al., 2002; Zhu et al., 2017). This effect was due inhibition of N- or L-type Ca^{2+} channels which are expressed in ventrobasal thalamus (Kammermeier et al., 1997; Hsu et al., 2010). There is still an ongoing debate concerning the relative contribution of the three mechanisms to the inhibition of transmitter release (cf. Guo and Hu, 2014). Other possible mechanisms include inhibition of cAMP- dependent Protein kinase A (Kubista and Boehm, 2006), ATP-binding proteins (Hosaka and Sudhof, 1998) and vesicular GABA and glutamate transport (Gasnier, 2000; Takamori, 2006). Thus, these presynaptic mechanisms may help to explain the mechanism of isovaline and identification of new targets for isovaline-like analgesics.

The incomplete antagonism of the effects of isovaline on evoked synaptic currents by GABA_B and mGlu II antagonists suggests that isovaline may activates additional metabotropic receptor(s). The existence of new subtype of GABA_B receptors has been suggested previously based on the observations that GABA_B antagonist (2-hydroxysaclofen) produced presynaptic antagonism of CGP35348-insensitive GABA_B receptors or some other metabotropic receptor

type (Emri et al., 1996). Another possibility is that isovaline activates heterodimers of inhibitory metabotropic receptors with distinct pharmacological profile or perhaps, less sensitivity to these antagonists. In the guinea pig ileum, isovaline decreased the amplitude of ileal muscle contractions by an action that was independent of GABA_B receptors in ileal muscle.

Future experiments may define a specific action of isovaline on variants of the GABA_B or mGlu II receptor subtypes. An expression system which expresses both receptors could be used to verify this hypothesis. This multiple receptor mechanism of action could be exploited to develop new analgesic agents similar to isovaline.

5.9 Significance of two receptor mechanisms of isovaline

An analgesic with a multiple mode of receptor action could be a promising strategy for relief of clinical pain. Analgesics that activate different target receptors tend to be more efficacious in treatment of pain with improved side effects profile (Singh et al., 2013; Fowler et al., 2014). Systemic administration of group II mGluR agonists and prototypical GABA_B receptor agonist, baclofen produces centrally mediated side effects whereas isovaline has no detectable central side effects owing to its peripheral distribution (Whitehead et al. 2012; see also Introduction). By combining the activation of peripheral metabotropic GABA_B and group II mGluRs, the dual agonist isovaline can both improve the analgesic effects and minimize the side effects caused by activating either type of receptors alone. Thus, isovaline emerges as a promising analgesic with unique mechanism to block transmission of nociceptive signals.

5.10 Concluding remarks

The experiments described in this thesis demonstrate that peripheral receptors mediate isovaline analgesia. In pathophysiological circumstances, isovaline may gain entry into the CNS, and act on metabotropic mGlu II and GABA_B receptors in thalamus to elicit analgesia. Given the dual, and possibly multiple receptor mechanism of action demonstrated in this thesis, isovaline may serve as a prototypical member of a new class of analgesics.

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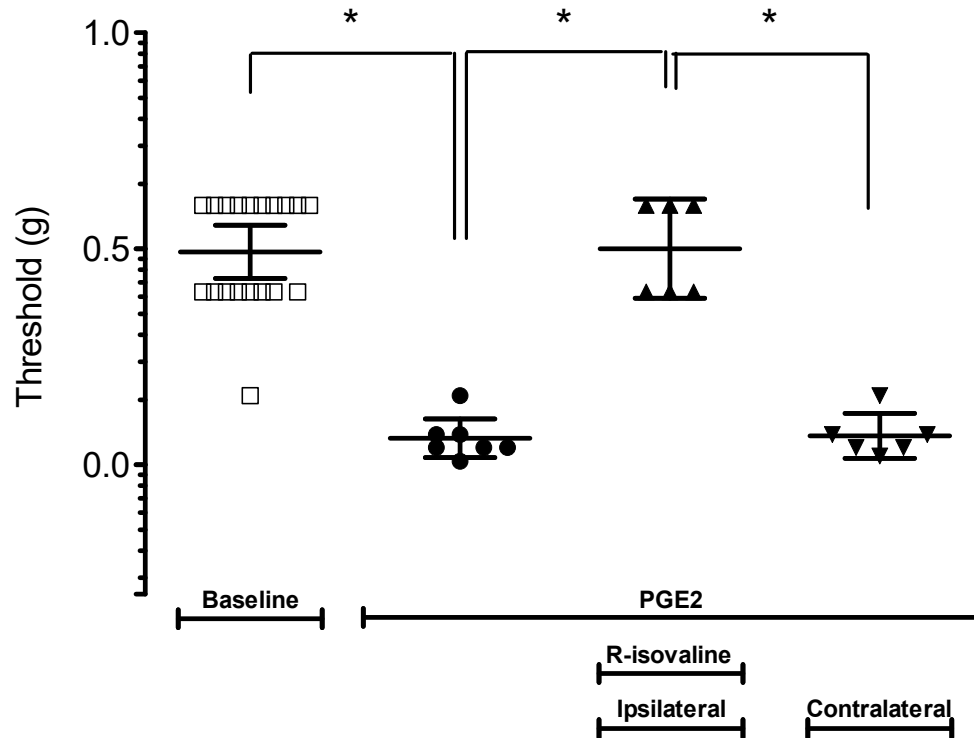
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Appendix A



Analgesic effect of isovaline was localized to the ipsilateral hindpaw in naïve mice. Each treatment cluster of data represents threshold force (g) exerted by von Frey filaments (means \pm 95% CI). Isovaline (14 μ mol) produced a significant reduction in allodynia induced by PGE₂ injection (0.002 pmol) in ipsilateral paw. Each cluster shows responses at 15 min after PGE₂ intraplantar injection in both paws. * $P < 0.05$; one-way ANOVA followed by Bonferroni's Multiple Comparison Test (n = 6-24).