EFFECTS OF AMBD AND ISOVALINE ON GABAERGIC TRANSMISSION IN THALAMIC NEURONS

by

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Abstract

In central neurons, the endogenous amino acid γ-aminobutyric acid (GABA) exerts synaptic inhibition mediated through ionotropic GABA\(_A\)-, or metabotropic GABA\(_B\)-receptors. These receptors exist on both pre- and postsynaptic membranes. The synthetic structural analogues of GABA, 6-aminomethyl-3-methyl-4H-1,2,4-benzothiadiazine-1,1-dioxide (AMBD) and R-isovaline have received little study on synaptic inhibition in the mammalian thalamus. AMBD was originally proposed as a taurine antagonist whereas R-isovaline is a non-biogenic amino acid that increases postsynaptic K\(^+\) conductance of thalamocortical neurons. The aim of this work was to assess the prediction that AMBD and R-isovaline would affect presynaptic release of GABA onto neurons of ventrobasal nuclei.

AMBD and R-isovaline were applied by perfusion of thalamic slices obtained from juvenile Sprague-Dawley rats (P10 -13). During whole-cell patch clamp recording from thalamocortical neurons, we voltage-clamped neurons at a holding potential of -70 mV. Miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of tetrodotoxin (TTX). Kynurenic acid and internal Cs\(^+\) were used to block postsynaptic glutamate receptors and K\(^+\) conductances. We used the GABA\(_A\) antagonist bicuculline to identify GABAergic mIPSCs, without affecting a possible presynaptic GABA\(_B\)-component.

Applied alone at 250 µM, AMBD had no effect on the passive and active membrane properties of neurons. In the range of 10 µM to 1 mM, AMBD had no effect on amplitude or decay time constant of GABAergic mIPSCs. Acting with an IC\(_{50}\) of 232 µM, AMBD reversibly reduced the frequency of GABAergic mIPSCs. The above observations implied that AMBD reduced
presynaptic release of GABA. In a range of 25 to 200 µM, R-isovaline had no effect on the holding current or frequency, amplitude and decay time constant of GABAergic mIPSCs. Hence, R-isovaline did not affect release of GABA and did not affect receptors on nerve terminals.

In summary, AMBD reversibly decreased the presynaptic release of GABA, likely by an action on nerve terminals while having no effects on postsynaptic membrane properties that could account for the reduced frequency of GABAergic mIPSCs. The exact mechanism whereby AMBD decreased GABA release remains unclear. R-isovaline had no effect on GABA release in ventrobasal nuclei.
Preface

The Animal Care Committee of University of British Columbia has approved the use of animals in the following experiments. The animal care certificate number was A06-0155.
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Chapter 1. Introduction

1.1 Scope of the thesis

This thesis studies the effects of 6-aminomethyl-3-methyl-4H,1,2,4-benzothiadiazine-1,1-dioxide (AMBD, also referred to as TAG) and the non-biogenic amino acid isovaline on GABAergic synaptic inhibition in thalamic neurons of the central nervous system (CNS).

AMBD is a conformationally restricted analogue of taurine whereas isovaline is an analogue of glycine and γ-aminobutyric acid (GABA). These endogenous amino acids mediate inhibitory synaptic transmission in the CNS. In ventrobasal nuclei of the thalamus, GABA is a major neurotransmitter, acting at type A (GABA$_A$) and type B (GABA$_B$) receptors. These receptors have well-established roles in mediating inhibition. Previous studies have shown that AMBD has differential effects in antagonizing the actions of GABA and other amino acids in different regions of mammalian CNS (Okamoto et al., 1983; Yarbrough et al., 1981; Curtis et al., 1982). The first objective of this study was to resolve such ambiguities of AMBD actions, by determining the interactions of AMBD and GABA on GABA$_A$ receptors.

In ventrobasal neurons, R-isovaline causes inhibition of evoked firing by increasing potassium conductance (Cooke et al., 2009). This action of isovaline has been suggested to be mediated by GABA$_B$ receptors. Indeed, regulation of transmitter release is an important role of GABA$_B$ receptors on nerve terminals of central neurons (Nicoll et al., 1990). Electrophysiological and biochemical experiments have shown that activation of presynaptic GABA$_B$ autoreceptors results in reduction of neurotransmitter release (Deisz and Prince, 1989). Hence, the second objective of
this thesis was to determine the presynaptic effects of AMBD and isovaline on GABAergic mIPSCs in the ventrobasal nuclei.

1.2 Background

1.2.1 Ventrobasal nuclei of thalamus

Ventral (VB) nuclei can be defined as aggregations of neurons that relay somatosensory signals to a structurally and functionally distinct area(s) of the cerebral cortex. VB nuclei can be subdivided into ventral posterior medial (VPM) and ventral posterior lateral (VPL) nuclei (Jones and Yang, 1985). VB nuclei receive information on pressure, proprioception and touch through the medial lemniscus pathway and on nociception and temperature through the trigemino-spinothalamic pathway (Tsumoto, 1974; Akers and Killakey; 1979; Jones, 1991). The VB neurons project the somatosensory information to the primary somatic sensorimotor cortex, where their terminals distribute mainly to layer IV, V and VI, with a few fibers terminating in the other cortical layers (Hirai et al., 1988; Lu and Lin, 1993). From primary somatic sensorimotor cortex layer VI, corticothalamic neurons provide feedback to ventrobasal neurons by providing them with excitatory inputs (Alitto and Usrey, 2003). The reciprocal interaction between thalamic and cortical neurons is essential for processing nociceptive information and maintaining consciousness (Steriade, 1993; Steriade et al., 1994; Steriade, 2005). Several pathological manifestations may result from lesions or abnormal neuronal activities at the thalamic level. For example, changes in nociceptive input may lead to pain states (Rausell et al., 1992a, b; Ralston and Ralston, 1994). Alteration of transient Ca$^{2+}$ current in thalamic neurons may lead to epileptic states (Huguenard and Prince, 1994).
There are two types of VB thalamic neurons: thalamocortical (TC) relay neurons whose axons project beyond the thalamus and local circuit interneurons whose axons are confined to the thalamus (Yen and Jones, 1983). TC relay neurons represent the great majority of ventrobasal neurons (Harris and Hendrickson, 1987). TC relay neurons are characterized by large somata and dendrites that radiate out in all directions from the soma to create a bushy dendritic field. In contrast, interneurons have much smaller somata with fewer, less branched dendrites. TC relay neurons are further classified into two types based on their morphology; type I and type II. Type I TC neurons are more common and are characterized by larger somata, larger dendritic fields and a lack of dendritic appendages (Yen et al., 1985; Turner et al., 1997).

Electrophysiologically, all TC neurons are characterized by low-threshold Ca$^{2+}$ spikes (LTS). The LTS is generated by Ca$^{2+}$ current activated at membrane potentials negative to -65 mV. Type I and type II TC neurons can be further characterized by their electrophysiological properties. Type I TC neurons have lower input resistance ($R_i$) than type II TC neurons and exhibit smaller afterhyperpolarizations. Furthermore, thalamic interneurons can be distinguished from TC relay neurons by more positive resting membrane potentials and a failure to fire at frequencies higher than 150 Hz (Pape and McCormick, 1995; Turner et al., 1997).

Ventrobasal nuclei receive excitatory glutamatergic inputs from medial lemniscus and spinothalamic pathways (Salt and Eaton, 1996; Blomqvist et al., 1996). They also receive inhibitory GABAergic inputs from the nucleus reticularis (nRt), mostly mediated by ionotropic GABA$_A$ receptors, and zona incerta (Cox et al., 1997; Zhang et al., 1997; Bartho et al., 2002).
Recently, glycinergic transmission has also been demonstrated in VB nuclei (Ghavanini et al., 2005).

1.2.2 Receptors for inhibitory amino acids in ventrobasal nuclei

In VB nuclei, three types of receptors mediate the inhibitory neurotransmission: ionotropic $\gamma$-aminobutyric acid type A (GABA$_A$), metabotropic GABA$_B$ receptors and ionotropic strychnine-sensitive glycine receptors.

1.2.2.1 GABA$_A$ receptors in ventrobasal nuclei

GABA$_A$ receptors are widely distributed and the most important inhibitory transmitter receptors in the CNS. About 20% to 50% of the synapses in the CNS contain GABA$_A$ receptors (cf., Nutt et al., 2001). GABA$_A$ receptors are members of the ligand-gated ion channel superfamily. They are composed of five protein subunits which join to form an annular-like structure, the chloride ion channel. GABA$_A$ receptors are formed by co-assembly of pentamers selected from at least 16 different subunits ($\alpha_{1-6}, \beta_{1-3}, \gamma_{1-3}, \delta, \varepsilon, \pi$ and $\theta$), encoded by unique genes (Cherubini and Conti, 2001; Whiting, 2003). This results in a high level of heterogeneity of functional GABA$_A$ receptors. The most commonly expressed subunits are the $\alpha_1$ and $\gamma_2$ subunits and these subunits occur in a high proportion of GABA$_A$ receptors. Based on the presence of 16 subunits more than 2000 different GABA$_A$ receptor subtypes could exist. But, because the subunit combinations (subtypes) are restricted to those containing two $\alpha$, two $\beta$ and one other subunit, the number of expressed subtypes is limited. Indeed, studies on native GABA$_A$ receptors suggest that there may be less than twenty GABA$_A$ receptor subtypes, with the major combinations being $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_2$ and $\alpha_3\beta_3\gamma_2$ (Barnard et al., 1998; Mohler, 2002; Sieghart and Sperk, 2002; Mohler et al.,
Depending on their subunit composition, receptor subtypes can exhibit distinct pharmacological and electrophysiological properties.

$\text{GABA}_A$ receptors can be divided into two functional types: synaptic and extrasynaptic receptors. Synaptic receptors mainly consist of $\alpha_1$, $\beta_2$ and $\gamma_2$ subunits (Somogyi et al., 1996; Essrich et al., 1998). This receptor type mediates phasic inhibition resulting from intermittent activation by high concentrations of GABA released from presynaptic terminals (Kaila, 1994; Mody and Pearce, 2004; Farrant and Nusser, 2005). Extrasynaptic receptors mainly consist of $\alpha_4$, $\beta$ and $\delta$ subunits (Persohn et al., 1992; Fritschy and Mohler, 1995; Pirker et al., 2000), and mediate tonic inhibition resulting from continuous activation by low concentrations of ambient GABA. These receptors have a higher affinity for GABA, since the extracellular concentrations of GABA are low due to efficient operation of GABA transporters (e.g., GAT-1 and GAT-3). In addition, extrasynaptic receptors have slower desensitization rates compared to synaptic receptors (Christensen, 1962; Brickley et al., 1999; Nusser and Mody, 2002; Yeung et al., 2003). Tonic inhibition mediated by extrasynaptic receptors plays an important role in neuronal excitability and in relaying and processing sensory information (Semyanov et al., 2004; Mody, 2005; Farrant and Nusser, 2005).

In the mature brain, GABA binds to the $\text{GABA}_A$ receptors and opens the chloride channel. Subsequent $\text{Cl}^-$ influx results in a shift of the membrane potential towards the $\text{Cl}^-$ equilibrium potential ($E_{\text{Cl}}$). This process causes postsynaptic inhibition or inhibitory postsynaptic potentials (IPSPs) which hyperpolarize the membrane potential away from the firing threshold. The increase in membrane conductance caused by opening of $\text{Cl}^-$ channels shunts the firing of
thalamocortical neurons (Ries and Puil, 1999). In mature brain, GABA is an inhibitory neurotransmitter. However, during early neuronal development, GABA\textsubscript{A} receptors mediate excitatory responses. During the first postnatal week, the GABAergic responses switch from excitatory (depolarizing) to inhibitory (hyperpolarizing) consequences (Ben-Ari et al., 1989; Cherubini et al., 1991). The shift depends on the concentration of intracellular chloride ions [Cl\textsubscript{i}], which in turn affects the $E_{Cl}$. In immature neurons, [Cl\textsubscript{i}] is high due to the action of the Na\textsuperscript{+}/K\textsuperscript{+}/Cl\textsuperscript{-} co-transporter (KNCC1) which accumulates Cl\textsuperscript{-} inside neurons. Upon activation of GABA\textsubscript{A} receptor a depolarization ensues. In mature neurons, [Cl\textsubscript{i}] is low due the action of the K\textsuperscript{+}/Cl\textsuperscript{-} co-transporter (KCC2) which extrudes Cl\textsuperscript{-} the neurons, leading to hyperpolarization upon activation of GABA\textsubscript{A} receptors (Claudio et al., 1999; Karunesh et al., 2001; Xie et al., 2003).

Inhibitory postsynaptic currents (IPSPs) mediated by GABA\textsubscript{A} receptors are characterized by a faster time course compared to those mediated by GABA\textsubscript{B} receptors (Nicoll et al., 1990; Nakayasu et al., 1995; Ghavanini et al., 2006).

GABA is the major endogenous transmitter agonist at GABA\textsubscript{A} receptors. Other endogenous amino acids, e.g., β-alanine and taurine, activate GABA\textsubscript{A} receptors as well (Okamoto and Sakai, 1980; Simmonds, 1983; Horikoshi et al., 1988). Muscimol and gadoxadol (also referred to as THIP) are selective GABA\textsubscript{A} receptor agonists (Smith and Olsen, 1995; Frolund et al., 2002). There are several GABA\textsubscript{A} antagonists, including picrotoxin, gabazine and bicuculline. Picrotoxin blocks all ligand-gated Cl\textsuperscript{-} channels, acting as a non-competitive antagonist. Gabazine (SR-95531) is a competitive GABA\textsubscript{A} receptor antagonist. At a concentration of 10 µM, gabazine completely blocks GABA\textsubscript{A} receptors mediated currents (Wermuth et al., 1987; Michaud et al., 1986). Bicuculline is also a competitive antagonist at GABA\textsubscript{A} receptors. It blocks GABA\textsubscript{A}
receptors at concentrations in the range of 10 to 20 µM (Curtis et al., 1970; Takhashi et al., 1994). At concentrations higher than 50 µM, bicuculline also blocks strychnine-sensitive glycine receptors in hippocampal neurons (Shirasaki, 1991).

**1.2.2.2 GABA<sub>B</sub> receptors in ventrobasal nuclei**

GABA<sub>B</sub> receptors are metabotropic transmembrane receptors for GABA that are coupled to inhibitory Ga<sub>i</sub> and Ga<sub>o</sub> proteins (Morishita et al., 1990; Greif et al., 2000). These receptors are bicuculline-insensitive and widely distributed in the central and peripheral nervous systems (Bowery et al., 1981). Functional GABA<sub>B</sub> receptors are formed by heterodimerization of GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits by linking their intracellular C-termini (Jones et al., 1998). GABA<sub>B</sub> receptors are directly coupled to G-protein-activated inwardly rectifying K<sup>+</sup> channels (GIRK) and by βγ subunits of G proteins. GABA<sub>B</sub> receptors are negatively coupled to voltage dependent Ca<sup>2+</sup> channels. Activation of GABA<sub>B</sub> receptors increases the K<sup>+</sup> conductance and inhibits Ca<sup>2+</sup> influx via blocking voltage-dependent calcium channels. GABA<sub>B</sub> receptors activation decreases adenylyl cyclase activity which converts ATP to cyclic AMP. Cyclic AMP activates several target molecules, such as cyclic AMP-dependent protein kinase (PKA). PKA regulates various cellular functions such as synaptic plasticity, gene transcription and cellular metabolism. (Mott and Lewis, 1994; Greif et al., 2000). GABA<sub>B</sub> receptors occur pre- and postsynaptically in central synapses. Postsynaptic GABA<sub>B</sub> receptors inhibit excitatory transmission by hyperpolarization mainly via activation of K<sup>+</sup> channels (Luscher et al., 1997). In contrast, the primary function of presynaptic GABA<sub>B</sub> receptors is to inhibit neurotransmitter release by inhibition of voltage-dependent Ca<sup>2+</sup> channels (Dittman & Rogehr, 1996; Harayama et al., 1998).
Activation of postsynaptic GABA\textsubscript{B} receptors results in IPSPs that are much slower and longer in latency than fast, GABA\textsubscript{A}ergic IPSPs (Nicoll et al., 1990; Malouf et al., 1990). In ventrobasal nuclei, activation of synaptic GABA\textsubscript{B} receptors results in low threshold spiking (i.e., burst firing) in TC relay neurons due to de-inactivation of T-type currents ($I_T$)(Sanchez and McCormick, 1997). Regulating transmitter release is one of the main functions of GABA\textsubscript{B} receptors. Electrophysiological studies have shown that activation of presynaptic GABA\textsubscript{B} receptors is indeed capable of reducing GABA release (Deisz and Prince, 1989; Nicoll et al., 1990).

In ventrobasal neurons, application of R-isovaline (25 µM) inhibits firing of action potentials and increases membrane conductance mainly by activating rectifying and possibly leak K\textsuperscript{+} currents (Cooke et al., 2009). It has been suggested that these effects of R-isovaline may be mediated by postsynaptic GABA\textsubscript{B} receptors (Cooke, 2010; PhD Thesis, University of British Columbia).

GABA is the only known endogenous agonist at GABA\textsubscript{B} receptors although taurine can activate GABA\textsubscript{B} receptors (Kontro and Oja, 1990; Smith and Li, 1991). GABA\textsubscript{B} receptors are pharmacologically distinguished from GABA\textsubscript{A} receptors by their insensitivity to bicuculline and their selective activation by (R)-baclofen (Bowery et al., 1981). There are several known antagonists of the GABA\textsubscript{B} receptor, including CGP35348 and CGP55845. In the thalamus, CGP35348 selectively antagonizes postsynaptic GABA\textsubscript{B} receptors at nanomolar concentrations (Tennigkeit et al., 1998).
1.2.3 GABA

GABA is an endogenous inhibitory amino acid (Figure 1.1). It is the most common inhibitory neurotransmitter in the brain. It has been estimated that about 17 to 20 % of all brain neurons are GABAergic (cf. Somogyi et al., 1998). It is synthesized from glutamate by the enzyme glutamic acid decarboxylase (GAD), which also is used as a marker for GABAergic processes (Petroff, 2002; Schousboe and Wagepetersen, 2007). GABA is transported from the extracellular space into the cytosol by GABA transporters (GATs) (Hirunsatit et al., 2009; Madsen et al., 2010).

![Chemical structures of GABA, glycine, R-isovaline, β-alanine and taurine.](image)

Figure 1.1 Chemical structures of GABA, glycine, R-isovaline, β-alanine and taurine.

1.2.4 R-isovaline: a non-biogenic amino acid with chemical similarity to GABA and glycine

Isovaline (Figure 1.1) is an exogenous α-amino acid that resembles endogenous amino acids, especially glycine and alanine. It is a non-proteinogenic amino acid found originally in carbonaceous meteorites (Kvenvolden et al., 1970; Zhao and Bada, 1989; Pizzarello and Weber,
Recently, several species of filamentous fungi have been found to synthesize isovaline (Bruckner et al., 2009). Isovaline has a single chiral center, which gives raise to two enantiomers, R- and S-isovaline. Both enantiomers can be absorbed from the gastrointestinal tract by utilizing transporters for endogenous amino acids (Christensen, 1962; Evered et al., 1967).

When applied intrathecally or intravenously in mice, RS-isovaline produces analgesic effects in a dose-dependent manner. These effects of isovaline occur without detectable sedation or respiratory depression (MacLeod et al., 2010).

1.2.5 AMBD

AMBD (6-aminomethyl-3-methyl-4H,1,2,4-benzothiadiazine-1,1-dioxide) was first developed as a competitive antagonist of taurine actions, hence the name TAG (Girard et al., 1982). AMBD is a benzothiadiazine compound with a rigid and planar conformation that contains an acidic centre and a basic group. The chemical structure of AMBD shares some similarities with endogenous amino acids (Figure 1.2). For example, the distance between the basic group and the acidic centre is almost identical to the spacing between the carboxyl and amine groups of GABA. More interestingly, with rotation of aminomethyl group toward C-5, the distance between the acidic and the basic groups closely resembles the spacing between the amino and sulfonic acid groups of taurine. Indeed, receptors activated by taurine may recognize the sulfonamide group of AMBD as part of the structure of taurine, contributing to pharmacological action on these receptors (Girard et al., 1982).
In 1982, Girard et al., demonstrated that AMBD specifically antagonized the inhibitory effects of taurine application in cerebellar Purkinje neurons. Taurine, GABA as well as AMBD were applied using iontophoretic techniques. AMBD did not antagonize the inhibitory effects of GABA on these neurons.

Okamoto et al. (1982) performed electrophysiological studies on guinea pig cerebellar slices to investigate the role of taurine as a neurotransmitter using iontophoretic application of taurine, glycine, GABA and β-alanine hyperpolarized the membrane potential. The effects of AMBD (200 µM) on the hyperpolarizations evoked by iontophoretically applied agonists were studied. The results showed that AMBD reversibly antagonized the action of taurine, but had a little effect on glycine action, and no effect on GABA action. AMBD delayed the onset of the hyperpolarization induced by β-alanine. At higher concentrations (400 µM), AMBD antagonized the actions of taurine, glycine and β-alanine, but not that of GABA. The effects of AMBD were also investigated on amino acid-induced decreases in neuronal membrane resistance.

Figure 1.2 The chemical structures of AMBD, taurine and GABA.
Iontophoretically applied taurine decreased the input resistance, and AMBD blocked this effect. In contrast, the GABA-induced decrease in input resistance was little affected by AMBD (400 µM). AMBD (200 and 400 µM) shifted the dose-response curve for taurine to the right in parallel manner. These data suggest that AMBD is a competitive and selective antagonist of taurine.

Qualitative studies investigated the effects of AMBD in rat somatosensory cortical neurons, cerebellar Purkinje neurons and amphibian spinal cord. Using iontophoretic techniques, the results showed that the hyperpolarizing actions of taurine and β-alanine on membrane potential were selectively and reversibly antagonized by AMBD. AMBD did not antagonize the action of GABA (Yarbrough et al., 1981). Thus, these results suggested that AMBD acts as a selective β-amino acid antagonist in the mammalian CNS.

The effects of AMBD on actions of taurine, β-alanine, GABA and glycine and synaptically dorsal root potentials (DRPs) were investigated. In these experiments, the agonists were applied by bath application techniques. Taurine and β-alanine evoked depolarizing responses in the dorsal roots which were reversibly antagonized by AMBD (100 - 250 µM). In contrast, AMBD had no effect on the depolarizations evoked by GABA or glycine. The effect of AMBD on the ventral root-evoked dorsal root potential (VR-DRP) was studied. Acting in concentration-dependent manner, AMBD depressed the VR-DRP, which was proposed to be mediated by taurine. These results suggested that AMBD selectively antagonizes the actions of β-amino acids (Padjen et al., 1988).
In dissociated mouse spinal cord neurons, AMBD (100 – 200 µM) blocked the actions of glycine and taurine at glycine A receptors. AMBD showed a greater antagonistic potency when taurine was employed as the agonist (Mathers, 1993). In the cat spinal cord interneurons, AMBD antagonized the inhibition of firing caused by application of taurine, β-alanine, glycine and GABA to an equal extent. All agents were applied using ionophoretic techniques. These data indicate that AMBD is a non-selective antagonist antagonist of β-amino acids (Curtis et al., 1982).

In ventrobasal thalamic neurons, AMBD reduced the peak amplitude of electrically evoked IPSCs with mixed GABAergic and glycinergic components as well as purely glycinergic IPSCs. These effects of AMBD were reversible and concentration-dependent. AMBD did not affect the majority of purely GABAergic IPSCs. These results suggest that AMBD has antagonistic actions distinct from GABAergic and glycine-receptor antagonists (Mathers et al., 2009).

Although many studies demonstrated AMBD as a specific β-amino acid antagonist which had no effect on GABA actions, other studies suggested that AMBD lacks this specificity and it could antagonize the action of GABA (Curtis et al., 1982). The specificity of AMBD has been investigated using different preparations (e.g., cerebellum, spinal cord and somatosensory cortex) of different animals (e.g., mouse, rat, guinea pig, cat and frog). Furthermore, some studies used ionophoretic techniques which have a technical disadvantage that the concentration of the drug at the effector site is not known. These differences may explain this ambiguity of AMBD actions.
To resolve these issues, further research using more quantitative drug delivery techniques are warranted.

1.3 Experimental rationale, objectives and hypothesis

In central synapses, the endogenous amino acid GABA mediates inhibitory actions through GABA\textsubscript{A} as well as GABA\textsubscript{B} receptors. In ventrobasal nuclei, GABA\textsubscript{A} and GABA\textsubscript{B} receptors have a well-establish role in mediating inhibitory transmission. The GABA\textsubscript{B} receptors exist on both presynaptic and postsynaptic sites at central synapses (Harrison, 1990; Thompson and Gahwiler, 1992). Activation of presynaptic GABA\textsubscript{B} autoreceptors reduces the release of GABA (Deisz and Prince, 1989; Nicoll et al., 1990; Feuvre et al., 1997). The effects of AMBD and isovaline on synaptic inhibition in mammalian thalamus have received little study.

Previous research investigating the effect of AMBD on the actions of GABA and other inhibitory amino acids revealed more controversy about its specificity. Differences in experimental techniques and preparations used in those experiments may explain the controversy. The first objective of this thesis was to resolve the ambiguity of AMBD actions by determining the effects of AMBD on responses mediated by postsynaptic GABA\textsubscript{A} receptors.

Action potential-independent inhibitory postsynaptic currents (mIPSCs) are result of spontaneous release of synaptic vesicles or quanta from the presynaptic terminal (Fatt and Katz, 1952; Isaacson and Walmsley, 1995). Changes in mIPSC amplitude are believed to reflect changes in postsynaptic sensitivity to neurotransmitter or decrease in vesicular content, whereas changes in mIPSC frequency are due to changes in the release frequency of single vesicles from
the presynaptic terminals (Thompson et al., 1993). Thus, studying the effects of AMBD on mIPSCs properties can reveal the site of action of AMBD besides its effects on responses of endogenously released GABA. The second objective was to determine the presynaptic effects of AMBD on GABA\textsubscript{A}ergic mIPSCs.

Previous studies have revealed the anti-allodynic and anti-nociceptive properties of isovaline. These properties of isovaline have been demonstrated in rodent pain models, without detectable systemic toxicity (MacLeod et al., 2010). In ventrobasal thalamic neurons, R-isovaline produced a long-lasting inhibition by increasing K\textsuperscript{+} conductance. This action of isovaline may be mediated by metabotropic GABA\textsubscript{B} receptors. GABA\textsubscript{B} receptors occur presynaptically as auto-receptors in ventrobasal neurons (Ulrich and Huguenard, 1996). In ventrobasal nuclei, application of GABA\textsubscript{B} receptors agonist baclofen decreased the frequency of GABA\textsubscript{A}ergic mIPSCs (Ulrich et al., 1996; Feuvre et al., 1997). The third objective of this thesis was to investigate the presynaptic effects of R-isovaline on GABA\textsubscript{A}ergic mIPSCs in the ventrobasal thalamus by studying the effects of R-isovaline on GABA\textsubscript{A}ergic mIPSCs in ventrobasal neurons.

We first hypothesized that AMBD would have no effect on responses to GABA at postsynaptic GABA\textsubscript{A} receptors. The second hypothesis was that AMBD would affect the release of GABA. The third hypothesis was that R-isovaline would decrease GABA release by activating presynaptic GABA\textsubscript{B} receptors. We sought to investigate these hypotheses by studying the effects of AMBD and R-isovaline on GABA\textsubscript{A}ergic mIPSCs in ventrobasal neurons.
Chapter 2. Materials and Methods

The Animal Care Committee of University of British Columbia approved the use of animals in these experiments.

2.1 Whole-cell patch clamp recording

2.1.1 Slice preparation

Thalamic slices were prepared as previously described (Ghavanini et al., 2006). Briefly, 10 to 13 day-old Sprague-Dawley or Wistar rat pups of either sex were decapitated while under deep halothane anesthesia. The brain was rapidly removed from the cranium and transferred into ice-cold (4°C) artificial cerebrospinal fluid (aCSF), perfused with 95% O₂ 5% CO₂. The aCSF contained (in mM): 124 NaCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 2 MgCl₂, 2 CaCl₂, and 15 dextrose, with pH of 7.3-7.4. Two brain tissue blocks were obtained by sectioning the brain along the interhemispheric fissure. The medial surface of the block was glued onto a Teflon stage of a Vibroslicer (Campden Instruments Ltd., London, England). The tissue containing thalamus was cut into parasagittal slices of 230-250 µm thick (Paxinos and Watson, 1987). The slices were incubated in a chamber containing oxygenated aCSF (pH 7.3-7.4) at 23-25 °C for at least 1 h prior to recording. The aCSF used in the holding chamber and for perfusion had an osmolarity of 312-317 mOsm.

2.1.2 Electrophysiological recording

After incubation for 1 h in aCSF, slices were transferred into a Perspex chamber with a ~ 2 ml volume. They were immobilized with a polypropylene mesh and perfused with oxygenated aCSF (23-25 °C) at a rate of 1.5-2 ml / min. Ventrobasal nuclei of thalamus were visually localized
using a stereotaxic atlas of the rat brain (Paxinos and Watson, 1987). The neurons were visually identified using a differential interference contrast (DIC) microscope equipped with a 400X water immersion lens (Axioscope II, Zeiss, Germany). The criterion for visual identification of thalamocortical neurons was a large soma with a diameter of 20-30 µm and exhibited low threshold Ca\(^{2+}\) spikes (LTSs) (Leresche, 1992).

Recording microelectrodes were pulled from thin-wall borosilicate glass tubing (World Precision Instruments, Sarasota, USA) using a Narishige micropipette puller. When filled with intracellular solution, pipette resistances ranged between 4 and 7 MΩ.

Whole-cell patch clamp recordings were obtained at 23 C˚ using a List EPC-7 amplifier (HEKA, Lambrecht, Germany) in both the current- and voltage-clamp modes. Signals were filtered at 3 kHz, digitized at 10 kHz with a 16-bit data acquisition system (Digidata 1322A; Axon Instruments) and stored on a personal computer for later analysis using pClamp 8.0 software (Axon Instruments). All neurons had stable membrane potentials and exhibited LTSs with burst firing when depolarized from -75 mV. Membrane potentials were corrected for a junction potential of -11 mV. Recordings were obtained from a single neuron within a given slice.

2.1.3 Current-clamp recording

Membrane input resistance (R\(_i\)) and time constant were calculated from small voltage responses (usually <5 mV) evoked by hyperpolarizing current pulses. Decay phase of the voltage responses were fitted with single exponential function. The voltage-current (V-I) relationships were obtained with a slow voltage-ramp protocol (400 ms at – 40 mV, followed by a ramp to – 100
mV). Action potential threshold was defined as the voltage where current clamp trace shows a maximal rate of change with respect to the baseline (cf., Sekerli et al., 2004).

In these recordings, microelectrodes were filled with an intracellular solution containing (in mM): 133 KOH, 10 EGTA, 12 KCl, 4 NaCl, 10 HEPES, 0.5 CaCl\(_2\), 2.7 Na\(_2\)-phosphocreatine, 3 MgATP, 0.3 Na\(_2\)GTP (osmolarity, ~ 290 mOsm). The pH was adjusted to 7.3-7.4 using 25% gluconic acid or KOH. With this solution, the Nernst potentials for Cl\(^-\) and K\(^+\) were -53 mV and -104 mV, respectively.

### 2.1.4 Voltage-clamp recording

To record miniature inhibitory postsynaptic currents (mIPSCs), the intracellular solution contained (in mM): 16.5 CsOH, 10 EGTA, 128.5 CsCl, 4 NaCl, 10 HEPES, 0.5 CaCl\(_2\), 2.7 Na\(_2\)-phosphocreatine, 3 MgATP and 0.3 Na\(_2\)GTP. The pH was adjusted to 7.3–7.4 using 25% gluconic acid or CsOH. With this solution, the Nernst potential for Cl\(^-\) was 0 mV. Neurons were voltage-clamped at a holding potential, \(V_h = -70\) mV. Therefore, GABA\(_A\) receptor-mediated mIPSCs appeared as inward currents. Series resistance was checked repeatedly during each experiment and ranged from 8 to 26 M\(\Omega\). The data were discarded if the series resistance increased by more than 20%.

All external solutions routinely contained 1 mM kynurenic acid to block ionotropic glutamate receptors. 1 \(\mu\)M tetrodotoxin (TTX) was also co-applied to block voltage dependent Na\(^+\) channels and multi-quantal IPSCs. Cs\(^+\) replaced K\(^+\) in the intracellular solution to block K\(^+\) currents. Guanosine triphosphate (GTP) and adenosine triphosphate (ATP) were added to the intracellular solution immediately prior to recording.
Bicuculline (20 µM) was used to identify the GABA<sub>A</sub>ergic mIPSCs. Inhibition of more than 95% of mIPSCs frequency indicated purely GABA<sub>A</sub>ergic events. Complete recovery was defined as regaining 90% of control values (R<sub>i</sub> or mIPSCs frequency). At least 10 min were allowed for the internal contents of the recording electrode to equilibrate with the cell contents.

Stationarity can be defined as the quality of a process in which the statistical parameters (mean and variance) of the process do not change with time. The purpose of testing for mIPSCs stationarity is to ensure that the frequency does not change with time and avoid detecting changes due time-dependent trends. To test for stationarity of data, we divided the recording duration into time intervals of equal lengths (1 min). Then we computed the mean value and the standard error of mean for each interval and compared the mean values of all intervals for fluctuations that reflect non-stationarity trends.

### 2.2 Drugs

Drugs were bath applied at a rate of 1.5-2 ml per minute. Drug stock solutions were prepared in distilled water or dimethyl sulfoxide (DMSO) and diluted in aCSF. The final concentration of DMSO was < 0.01%. DMSO up to concentration of 0.1% had no effects on mIPSCs or holding current (Belelli et al., 2005; Peden et al., 2007; Joksovic et al., 2009). Drug solutions were perfused with 95% O<sub>2</sub> - 5% CO<sub>2</sub>. Kynurenic acid and GABA were purchased from Sigma-Aldrich Inc. (St. Louis, USA). Bicuculline methiodide was purchased from Sigma Chemical Company. AMBD was synthesized by Biofine International Inc. (Vancouver, Canada). All drug solutions were applied for a minimum of 7 min. After each application, slices were perfused with control solution for drug wash-out.
2.3 Data analysis

Analysis of electrophysiological data was performed using pClamp software (Clampfit, Axon Instruments), CorelDraw software (Ottawa, Canada), Microsoft Excel and Prism GraphPad software (San Diego, USA). Voltage-current relationships were constructed from voltage responses to depolarizing and hyperpolarizing current pulses range from -150 pA to 150 pA at holding potential of -60 mV. Slope resistance was calculated from a linear segment of voltage-current curves between -60 and -70 mV. Reversal potentials for drug actions were determined from the intersections of the control and drug curves.

2.3.1 Analysis of mIPSCs

Miniature IPSCs (mIPSCs) were detected and analysed off-line using Mini Analysis software (Synaptosoft, Decature, GA). Frequency of mIPSCs was determined by counting the number of events over 5 or 10-min epoch. The threshold for detection was set at –12 pA or to three times the root-mean-square baseline noise, which was measured for each epoch of recording in segments with no visible events. Area threshold was set at –200 pA·ms. Thus, the area of each event is further compared to the area threshold to distinguish the peaks from noise contributions. The area threshold was determined by trying out different sets of values until the most of the peaks due to noise were eliminated. All automatically detected events were visually inspected for validity. Accepted events were analyzed for peak amplitude, 10-90% rise time and decay time constant. The decay time constant ($\tau_d$) was measured for events which were judged to be single mIPSCs with no overlapping events. The $\tau_d$ was determined by fitting the falling phase of mIPSCs with a monoexponential function:

$$y = Ae^{-\nu \tau_d}$$
where A is peak amplitude and t was time.

A minimum of 200 accepted events was required for each data segment to be included in the analysis. In each single neuron, changes in interevent interval (frequency) or peak amplitude was determined by comparison of their cumulative distributions, with the Kolmogrov-Smirnov test. For group analysis of variations in frequency and peak amplitude of mIPSCs in all neurons were evaluated using the two-tailed Student’s t-test or ANOVA.

### 2.3.2 Concentration-response analysis

The percentage reductions in mIPSC frequency at given concentrations of AMBD were used to construct a concentration-response curve. AMBD was cumulatively applied in a step-wise manner. Each data point represented as an average of multiple measurements in different cells. Mean values in concentration-response curve were fitted by the following (Hill-equation) function:

\[
Y = \frac{100}{1 + 10^{\exp\left((\log IC_{50} - X) \times \text{HillSlope}\right)}}
\]

where IC$_{50}$ was the concentration at half maximal response.

### 2.3.3 Statistical analysis

All data were presented as mean ± standard error of mean (SEM) and n indicated the number of neurons. Prism GraphPad software and Microsoft Excel were used to perform statistical analyses. Data with normal distribution were analyzed with the Student’s t-test for comparing two groups or analysis of variance (ANOVA) for multiple comparisons, and Tukey post hoc for comparing group pairs. Comparison of cumulative amplitude distributions was performed using non-parametric Kolmogrov-Smirnov statistics. The significance level was set at a value of
$P < 0.05$. 
Chapter 3. Results

3.1 Postsynaptic effects of AMBD

In this thesis, we used ~ 170 animals and the average time of recording from each neuron range from 1.5 to 3 hrs.

3.1.1. Effects of AMBD on action potential properties

Initially, we investigated the effects of AMBD (250 µM) on action potential properties. Action potentials were evoked by injecting 120 pA depolarizing current pulses. The 250 µM concentration of AMBD has been chosen based on previous studies, where it was found to antagonize the action of taurine, β-alanine and/or glycine but not GABA (Okamoto et al., 1983; Mathers, 1993; Mathers et al., 2009). Application of AMBD had no significant effects on action potential threshold or amplitude. The threshold and amplitude of action potentials had averages of 40 ± 2 mV and 58 ± 3 mV before AMBD application and 40 ± 1 mV and 60 ± 3 mV after AMBD application (n = 5) (paired t-test, P > 0.05). AMBD (250 µM) did not alter rate of rise, rate of fall or half-width of action potentials (paired t-test, P > 0.05; Figure 3.1 A). We determined whether AMBD (250 µM) had effects on LTSs to determine whether it altered T-type Ca\textsuperscript{2+} channels. LTSs were evoked by injecting hyperpolarizing current-pulses of a -160 pA. The LTSs had a mean amplitude of 18 ± 2 mV in control solution and 18 ± 1 mV after the application AMBD (paired t-test, P > 0.05, n = 5; Figure 3.1 A). Table 3.1 summarizes the effects of AMBD (250 µM) on action potential properties and LTS.

3.1.2 Effects of AMBD on passive membrane properties

As a specific antagonist, AMBD should have no effect on passive membrane properties that might account for its antagonistic action. To investigate that, we examined the effects of AMBD
on passive properties. We determined the effects of AMBD on resting membrane potential \(V_m\), input resistance \(R_i\), membrane time constant \(\tau_m\) and current-voltage relationships. AMBD (250 µM) was applied to 5 previously untreated VB neurons at rate of ~ 1.7 ml/min for 7 min.

### 3.1.2.1 Effects of AMBD on resting membrane potential and input resistance

In five neurons tested, \(V_m\) had an average of – 74 ± 1 mV. Bath application of AMBD (250 µM), had no significant effects on \(V_m\) (– 77 ± 2 mV) of previously untreated VB neurons (\(p > 0.05\), paired Student’s \(t\)-test).

The \(R_i\) had an average of 330 ± 53 MΩ. Application of AMBD (250 µM) had no significant effects on \(R_i\) (328 ± 54 MΩ) (\(p > 0.05\), paired Student’s \(t\)-test) (Figure 3.1).

### 3.1.2.2 Effects of AMBD on membrane time constant and membrane capacitance

We investigated the effects of AMBD on \(\tau_m\). The average \(\tau_m\) was 68 ± 17 ms in control solution and 61 ± 16 ms after application of 250 µM AMBD (\(p > 0.05\), paired Student’s \(t\)-test).

The effects of AMBD on \(C_m\) were examined on calculations from the \(\tau_m\). Application of AMBD (250 µM), had no significant effects of on \(C_m\). The average of \(C_m\) was 206 ± 22 pF in control solution and 185 ± 19 pF after application of 250 µM AMBD (\(p > 0.05\), paired Student’s \(t\)-test).
3.1.3 Effects of AMBD on voltage-current relationship

We assessed the effects of AMBD on the voltage-current relationship. Figure 3.2 shows control and AMBD curves obtained from five VB neurons. Within this range, application of 250 µM AMBD had no effect on the current-voltage relationship.

3.1.4 Summary of AMBD effects on membrane properties

Table 3.2 summarizes the effects of AMBD (250 µM) on $V_m$, $R_i$, $\tau_m$ and $C_m$. Our data indicate that AMBD had no significant effects on membrane properties that might contribute to its antagonistic action. These observations suggest that AMBD had no postsynaptic effects on $\text{Na}^+$, $\text{K}^+$ or $\text{Ca}^{2+}$ channels.
Figure 3.1 AMBD (250 µM) had no effects on properties of action potential or input resistance. (A) AMBD (blue line) did not affect the properties of action potentials during control (grey line). (B) AMBD did not affect hyperpolarizing voltage responses (< 5 mV) evoked by intracellular injections of current (10 pA × 400 ms). (C) Superimposed hyperpolarizing responses to current pulses taken during control (grey) and application of AMBD (blue).
Figure 3.2 AMBD (250 µM) had no effects on voltage-current relationship or input resistance. (A) No significant changes in voltage-current relationship were observed after application of AMBD. (B) In five neurons, AMBD had no effects on input resistance (p > 0.05, paired Student’s $t$-test).
Table 3.1 AMBD had no effects on active membrane properties or LTS.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AMBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshold (mV)</td>
<td>41 ± 2</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>58 ± 3</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>Rate of rise (mV/ms)</td>
<td>63 ± 4</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>Rate of fall (mV/ms)</td>
<td>20 ± 2</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>Half width (ms)</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>LTS (mV)</td>
<td>18 ± 2</td>
<td>18 ± 1</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, p > 0.05, paired Student’s t-test, n = 5.

Table 3.2 AMBD had no effects on passive membrane properties

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AMBD (250 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_m$ (mV)</td>
<td>–74 ± 1</td>
<td>–77 ± 2</td>
</tr>
<tr>
<td>$R_i$ (MΩ)</td>
<td>330 ± 53</td>
<td>328 ± 54</td>
</tr>
<tr>
<td>$\tau_m$ (ms)</td>
<td>68 ± 17</td>
<td>61 ± 16</td>
</tr>
<tr>
<td>$C_m$ (pF)</td>
<td>206 ± 22</td>
<td>185 ± 19</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, p > 0.05, paired Student’s t-test, n = 5.
3.2 Effects of AMBD on GABAergic mIPSCs

We determined effects of AMBD on properties of GABAergic mIPSCs. In these experiments, whole-cell patch clamp recordings were obtained from a total of 19 neurons located within the VB nuclei. Under voltage-clamp conditions, neurons were held at –70 mV and $E_{Cl}$ was set at ~ 0 mV. mIPSCs were pharmacologically isolated by bath application of 1 mM kynurenic acid to block glutamatergic currents and 1 µM tetrodotoxin (TTX) to block voltage dependent Na$^+$ channels and hence prevent action potential-dependent transmitter release. In addition, K$^+$ conductance was suppressed by internal application with CsCl through the patch pipette. All mIPSCs recorded in these experiments were completely blocked by the GABA$\text{A}$ receptor antagonist bicuculline (20 µM). We investigated the effects of AMBD on mIPSCs frequency, peak amplitude, decay time constant, 10 – 90 % rise time and charge transfer of mIPSCs.

3.2.1 Effects of AMBD on holding current

In VB neurons, extrasynaptic GABA$\text{A}$ receptors mediate tonic inhibition as a result from continuous activation by low concentration of ambient GABA (Chandra et al., 2006). The first objective of this thesis was to determine the effects of AMBD on the actions of GABA at postsynaptic GABA$\text{A}$ receptors. To determine whether AMBD had effects on postsynaptic GABA$\text{A}$ receptors, we sought to determine the effects of AMBD on holding current. We recorded the holding current for 10 min before and during the application of AMBD and it was stable for all neurons tested (see Figure 3.7 for stationarity analyses). In control conditions, the magnitude of holding current had a mean of $–117 \pm 10$ pA. This value was not altered by the
3.2.2 Properties of GABAergic mIPSCs

The frequency of these mIPSCs ranged from 0.2 to 4.0 Hz with a mean of value of 1.3 ± 0.1 Hz. For all 43 neurons, mIPSC frequency was stable, as shown by comparing 1 min-interval samples taken during the 5- or 10-min recording period. The variations in frequency were within 10 % of the mean frequency. The peak amplitude ranged from –17 to –54 pA with an average value of –34 ± 2 pA. We further determined the holding current which ranged from –154 to –52 pA with a mean value of –110 ± 10 pA. In all tested neurons, tonic current was stable during the 5 to 10-min recording duration. The 10 – 90 % rise time of these mIPSCs was ranged from 1.1 to 2.9 ms with an average value of 2.1 ± 0.1 ms. We determined the decay time constant (τ_d) of mIPSCs which was defined as the time required for a currents to fall to 1/e (that is, 36.8%) of its maximum peak value. The average τ_d for mIPSCs was 21 ± 1 ms. The charge transfer or area under curve had a mean value of – 613 ± 21 pA·ms. These results are in consistent with results from previous reports (Ghavanini et al., 2006; Rajasekaran et al., 2007; Mathers et al., 2009).

3.2.3 Effects of AMBD on frequency of mIPSCs

Initially, we investigated the effects of AMBD on the frequency of mIPSCs using a bath solution containing 2.5 mM KCl. In two neurons, application of AMBD (250 µM), reversibly decreased the frequency from 3.1 to 1.5 Hz (62 % of control). AMBD had no apparent effects on amplitude or mIPSCs kinetic properties. Next, we increased the external concentration of KCl to 5 mM in order to increase the number of events for analysis. Under these conditions, again AMBD
decreased the frequency from $1.5 \pm 0.3$ to $0.6 \pm 0.1$ Hz (60% of control; $p < 0.05$, ANOVA; $n = 19$) in 17 out of 19 neurons tested (Figure 3.4). This effect of AMBD was evident on the cumulative distribution of inter-event intervals. AMBD caused a reversible rightward shift ($p < 0.05$, Kolmogorov-Smirnov test; Figure 3.5A). In 10 out of 17 neurons tested, recovery was obtained after washing out AMBD for 60 min. The average frequency of mIPSCs at the end of AMBD washout was $1.2 \pm 0.3$ Hz (80% of control). In summary, AMBD (250 $\mu$M) caused a significant increase in mIPSCs inter-event interval (i.e., decreased frequency) ($p < 0.05$, paired $t$-test; Figure 3.5B).
Figure 3.3 AMBD (250 µM) had no effect on holding current of GABAergic mIPSCs (n = 19, P > 0.05, ANOVA).
Figure 3.4 AMBD (250 µM) decreased the frequency of GABAergic mIPSCs. Traces show mIPSCs recorded from a ventrobasal neuron in control conditions (top left). Bath application of 20 µM bicuculline completely abolished mIPSCs (bottom left). Recovery was obtained after washing out bicuculline for about 40 min (top right). Application of AMBD decreased the frequency of mIPSCs (middle right). Recovery was observed after washing out AMBD for 60 min (bottom right). GABAergic mIPSCs were recorded in the presence of TTX, Kynurenic acid and 5 mM KCl. Holding potential was set as –70 mV and $E_{Cl}$ was 0 mV.
Figure 3.5 AMBD (250 μM) decreased the frequency of GABAergic mIPSCs. (A) Cumulative probability histograms of GABAergic mIPSC interval in control conditions and after the application of AMBD to one neuron are shown. Application of AMBD, shifted the cumulative probability curve to the right, indicating that the average time interval between GABAergic mIPSCs increased. The histograms had the following mean values and number of inter-event intervals: control (1175 ± 49 ms; n = 510 intervals), AMBD (2709 ± 189 ms; n = 219 intervals) and recovery (1113 ± 73 ms; 473 intervals). (B) In 17 neurons, 250 μM AMBD significantly decreased the frequency of mIPSCs (* P < 0.05 compared to controls, ANOVA).
3.2.4 AMBD reduced mIPSCs frequency in a concentration-dependent manner

Previous reports suggested that the antagonistic actions of AMBD on membrane channels activated by taurine and glycine as well as evoked IPSCs were concentration-dependent (Mathers, 1993; Mathers et al., 2009). Therefore, we obtained a cumulative concentration-response curve for the depressant effects of AMBD on mIPSCs frequency to determine an IC\textsubscript{50}. Eight concentrations of AMBD were applied to ventrobasal neurons. As shown in Figure 3.6, AMBD application decreased mIPSCs frequency in a concentration-dependent manner (p < 0.05, Repeated measures ANOVA). The curve was fitted by a single Hill function with an IC\textsubscript{50} of 232 ± 21 µM and Hill slope of 1.2 ± 0.1.

3.2.5. Stationarity of mIPSCs frequency

We used stationarity analyses to investigate whether the action of AMBD is stable and not changed with time. Figure 3.7A shows the frequency of mIPSCs recorded over 10 min and binned in 1 min intervals. During control conditions, the frequency of mIPSCs was stable with an average of 1.9 ± 0.1 Hz and there was no significant change during 10 min of recording (p > 0.05, ANOVA). Application of AMBD (250 µM), decreased the frequency of mIPSCs to an average of 1.1 ± 0.1 Hz and this effect of AMBD was stable (i.e., no significant changes in frequency means) during the 10 min of recording (p > 0.05, ANOVA). Furthermore, we sought to analyze possible time-dependent AMBD actions at different concentrations. AMBD was applied at 250, 500 and 1000 µM. On comparing the mean frequency of mIPSCs during control, 250, 500 and 1000 µM AMBD, there was no significant changes in mIPSCs mean during each conditions and the decrease in frequency was concentration dependent (Figure 3.7B).
**Figure 3.6** AMBD decreased the frequency of GABAergic mIPSCs in a concentration-dependent manner with an IC$_{50}$ of 232 ± 21 µM and Hill slope of 1.1 ± 0.1. AMBD was applied in cumulative-stepwise manner. Numbers in parentheses indicates the number of neurons tested for each data point. The dashed line indicates the IC$_{50}$. 
Figure 3.7 Stationarity of frequency of GABAergic mIPSCs. (A) Histogram shows no significant changes in frequency of mIPSCs during control and AMBD (250 µM) (p > 0.05, ANOVA). Control had a total average of 1.9 ± 0.1 Hz and 1.1 ± 0.1 Hz during AMBD (250 µM) application (n = 9). (B) Histogram shows stationarity of GABAergic mIPSCs during application of AMBD at concentration of 250, 500 and 1000 µM (n = 9). mIPSCs had total mean of 1.9 ± 0.4 Hz, 1.0 ± 0.3 Hz, 0.4 ± 0.2 Hz and 0.06 ± 0.4 Hz during control, 250 µM, 500 µM and 1000 µM AMBD (p < 0.05, ANOVA).
3.2.6 Effects of AMBD on mIPSC amplitude

We examined the effects of AMBD on the amplitude of GABAergic mIPSCs. In individual neurons, we compared the cumulative amplitude distribution of mIPSCs before and during application of AMBD (250 µM). The distributions were found to be indistinguishable (p > 0.05, Kolmogorov-Smirnov test; Figure 3.8A). In 19 neurons tested, mIPSCs had mean amplitude of –35 ± 2 pA. Application of AMBD had no significant effect on the mean amplitude which was –34 ± 3 pA (97% of control; p > 0.05, ANOVA). As summarized in Figure 3.8B, AMBD had no significant effect on the peak amplitude of GABAergic mIPSCs.

3.2.7 Effects of AMBD on mIPSC decay time constant

We determined whether AMBD had effects on the decay of GABAergic mIPSCs. The decay of individual mIPSCs was well fitted with a single-exponential function. As illustrated in Figure 3.9A, representative mIPSCs before and during application of AMBD showed no apparent difference in decay kinetics. In 19 neurons examined, GABAergic mIPSCs had an average τ_d time constant of 21 ± 2 ms. Decay time constants were consistent with data in previous reports (cf. Ghavanini et al., 2006; Mathers et al., 2009). Application of AMBD (250 µM), had no significant effect on the τ_d (22 ±1 ms, 104 % of control; P > 0.05, ANOVA; Figure 3.9B). Thus, our data shows that AMBD had no effect on the τ_d of GABAergic mIPSCs.
Figure 3.8 AMBD (250 µM) had no effect on the amplitude of GABAergic mIPSCs. (A) Cumulative probability histograms of GABAergic mIPSC amplitude in control conditions and during the application of AMBD to a ventrobasal neuron. The histograms had the following mean values and number of events: Control (28 ± 0.4 pA; 510 events), AMBD (27 ± 0.5 pA; 219 events) and recovery (28 ± 0.6 pA; 473 events). AMBD did not affect the amplitude-cumulative probability of GABAergic mIPSCs. (B) In 19 neurons, 250 µM AMBD had no significant effect on the mean amplitude of mIPSCs (P > 0.05, ANOVA).
Figure 3.9 AMBD had no significant effect on the decay time constant of GABAergic mIPSCs. (A) Averaged mIPSC traces from a neuron in control and during application of AMBD with identical decay time constants of 50 mIPSCs. Decay phases of mIPSC were fitted with single-exponential function to identify decay time constant ($\tau_d$). (B) In 19 neurons tested, 250 µM AMBD had no significant effect on decay time constant of GABAergic mIPSCs (bottom) ($P > 0.05$, ANOVA).
3.2.8 Effects of AMBD on mIPSC rise time

We further examined the effects of AMBD on the 10–90 % rise time of mIPSCs. This rise time is defined as 10 to 90 % of the time required for current to peak. The recorded mIPSCs had an average rise time of 2.1 ± 0.1 ms. Application of AMBD (250 µM), had no significant effects on the rise time (2.3 ± 0.2 ms) of GABAergic mIPSCs which was 109 % of control (P > 0.05, paired Student’s t-test; Figure 3.10A).

3.2.9 Effects of AMBD on mIPSC charge transfer

We examined the effects of AMBD (250 µM) on charge transfer during mIPSCs. As we anticipated, AMBD did not alter the charge transfer of GABAergic mIPSCs. In 19 neurons tested, the charge transfer mean was – 667 ± 38 pA × ms before the application of AMBD and – 664 ± 35 pA × ms after the application of 250 µM AMBD (99 % of control; P > 0.05, ANOVA; Figure 3.10B).
Figure 3.10 AMBD (250 µM) had no effects on rise time (A) or charge transfer (B) of GABAergic mIPSCs (n = 19, P > 0.05, ANOVA).
3.2.10 Summary of Effects of AMBD on GABA$\textsubscript{A}$ergic mIPSCs

Table 3.3 summarizes the effects of AMBD on mIPSCs. Application of AMBD (250 µM) decreased the mean frequency of mIPSCs, but had no significant effects on peak amplitude, rise time, decay time or charge transfer during these events. These data suggested that AMBD did not block the action of GABA at postsynaptic GABA$\textsubscript{A}$ receptors. Rather, AMBD decreased the release of GABA by a presynaptic mechanism (see Discussion).
**Table 3.3** Summary of effects of AMBD (250 µM) on GABAergic mIPSCs

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AMBD</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frequency (Hz)</strong></td>
<td>1.5 ± 0.3</td>
<td>0.6 ± 0.1 *</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td><strong>Amplitude (pA)</strong></td>
<td>– 35 ± 2</td>
<td>– 34 ± 2</td>
<td>– 37 ± 4</td>
</tr>
<tr>
<td><strong>10-90% rise time (ms)</strong></td>
<td>2.1 ± 0.1</td>
<td>2.3 ± 0.2</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td><strong>Time constant (ms)</strong></td>
<td>21 ± 2</td>
<td>22 ± 1</td>
<td>– 25 ± 1</td>
</tr>
<tr>
<td><strong>Charge transfer (- pA × ms)</strong></td>
<td>– 667 ± 38</td>
<td>– 664 ± 35</td>
<td>– 717 ± 42</td>
</tr>
</tbody>
</table>

Values are means ± SEM. * P < 0.05 compared to controls, ANOVA, n = 19 neurons.
3. 2.11 Effects of Ni\textsuperscript{2+} on GABAergic mIPSCs

It has been established that the release of transmitter is triggered by Ca\textsuperscript{2+} influx into presynaptic terminals (Katz and Miledi, 1970; Wu and Saggau, 1997). At a concentration of 100 µM, Ni\textsuperscript{2+} reduced the frequency of miniature excitatory synaptic currents (mEPSCs) in rodent spinal cord neurons (Bao et al., 1998). This effect of Ni\textsuperscript{2+} was attributed to the blockade of low-threshold (T-type) Ca\textsuperscript{2+} channels (Fox et al., 1987). Thus, we examined whether AMBD decreased the frequency of mIPSCs by blocking presynaptic T-type Ca\textsuperscript{2+} channels (see Table 3.1). We hypothesized that application of Ni\textsuperscript{2+} (100 µM) would occlude the action of AMBD. In control conditions, GABAergic mIPSCs had an average frequency of 1.5 ± 0.4 Hz and amplitude of –35 ± 3 pA (n = 6). Application of Ni\textsuperscript{2+} (100 µM) decreased the frequency of mIPSCs to 0.9 ± 0.3 Hz (56% of control; P < 0.05, ANOVA; Figure 3.11). Furthermore, Ni\textsuperscript{2+} reduced the amplitude of mIPSCs to –29 ± 3 pA (81% of control; P < 0.05, ANOVA; Figure 3.11). We co-applied Ni\textsuperscript{2+} and AMBD (250 µM) to the same neurons. In the presence of Ni\textsuperscript{2+}, AMBD further decreased the frequency to 0.4 ± 0.3 Hz (19% of control; P < 0.05, ANOVA; Figure 3.12A) and the amplitude to –26 ± 3 pA (72% of control; P < 0.05, ANOVA; Figure 3.12B). These results suggested that Ni\textsuperscript{2+} (100 µM) had pre- and postsynaptic effects and had not occluded the effects of AMBD. This finding argues that the inhibitory effect of AMBD on GABA\textsubscript{A}ergic mIPSCs was not conclusively due to blockade of presynaptic T-type Ca\textsuperscript{2+} channels.
Figure 3.11 Ni\textsuperscript{2+} (100 µM) decreased the frequency of GABAergic mIPSCs and did not occlude the action of AMBD (250 µM). Representative traces show mIPSCs recorded from a VB neuron (top). Application of Ni\textsuperscript{2+} (100 µM) decreased the frequency of mIPSCs (middle). Co-application of AMBD (250 µM) with Ni\textsuperscript{2+} further decreased the frequency of mIPSCs.
Figure 3.12 Ni$^{2+}$ (100 µM) decreased the frequency and amplitude of GABAergic mIPSCs. (A) In six neurons, 100 µM Ni$^{2+}$ decreased the frequency of mIPSCs to 56 % of control. Co-application of AMBD (250 µM) with Ni$^{2+}$ further decreased the frequency of mIPSCs to 19 % of control (* p < 0.05, ANOVA). (B) 100 µM Ni$^{2+}$ decreased the amplitude of mIPSCs to 81 % of control. Co-application of AMBD (250 µM) with Ni$^{2+}$ decreased the amplitude of mIPSCs to 72 % of control (* p < 0.05, ANOVA).
3.3 Effects of R-isovaline on GABAergic mIPSCs

The third objective of this thesis was to determine the effects of R-isovaline on GABAergic mIPSCs in ventrobasal neurons.

3.3.1 Effects of R-isovaline on the frequency of GABAergic mIPSCs

Initially, we investigated the effects of R-isovaline on the frequency of GABAergic mIPSCs in 22 VB neurons. In thirteen out of twenty two neurons tested, application of R-isovaline increased the frequency of mIPSCs and decreased in the remaining nine neurons. However, the overall effect of R-isovaline was insignificant compared to control (Figure 3.13). We compared the cumulative inter-event interval distribution of mIPSCs before and during application of R-isovaline (200 µM). The distributions were found indistinguishable (p > 0.05, Kolmogorov-Smirnov test; Figure 3.14 A). In 22 neurons tested, mIPSCs had a mean frequency of 1.2 ± 0.1 Hz. As summarized in Figure 3.14 B, application of R-isovaline (200 µM) had no significant effect on the mean frequency of GABAergic mIPSCs which was 1.3 ± 0.2 Hz (108% of control; p > 0.05, paired Student’s t-test).
Figure 3.13 R-isovaline (200 µM) had no effect the frequency of GABAergic mIPSCs. Traces show mIPSCs recorded from a VB neuron in control conditions (top left). Bath application of 20 µM bicuculline completely abolished mIPSCs (bottom left). Recovery was observed after washing out bicuculline for ~ 40 min (top right). Application of R-isovaline (200 µM) did not affect the frequency of mIPSCs (bottom right).
Figure 3.14 R-isovaline (200 µM) had no effect on the frequency of GABAergic mIPSCs. (A) Cumulative probability histograms of GABAergic mIPSC interval in control conditions and during the application of R-isovaline to one neuron are shown. Application of R-isovaline did not affect the cumulative probability. (B) In 22 neurons, R-isovaline had no significant effect on the mean frequency of mIPSCs (P > 0.05, ANOVA).
3.3.2 Stationarity of GABAergic mIPSCs

To investigate whether the frequency of mIPSCs was stable and did not change with time, we used the stationarity analyses. In Figure 3.15A, the frequency of mIPSCs was recorded over 5 min and binned in 1 min intervals. During control conditions, the frequency of mIPSCs was stable with a mean frequency of $1.3 \pm 0.2$ Hz and there was no significant change during 5 min of recording ($n = 9; p > 0.05$, ANOVA). Application of R-isovaline (200 µM), did not affect the frequency of mIPSCs which had a mean of $1.3 \pm 0.1$ Hz. This effect of R-isovaline was stable during the 5 min of recording ($p > 0.05$, ANOVA). We also analyzed the stationarity of R-isovaline actions at different concentrations. R-isovaline was applied at 25, 100 and 200 µM to 20 neurons. By comparing the mean frequency of mIPSCs during control, 250, 500 and 1000 µM R-isovaline, there was no significant changes in mIPSCs mean during each condition. The changes in frequency of mIPSCs were concentration independent (Figure 3.15B).

3.3.3 Effects of R-isovaline on peak amplitude

We examined whether R-isovaline has any effects on peak amplitude of GABAergic mIPSCs in VB neurons. In individual neurons, application 25, 100, 200 and 400 µM R-isovaline had little effect on the cumulative amplitude distribution of mIPSCs ($p > 0.05$, Kolmogorov-Smirnov test; Figure 3.16A). In 22 neurons examined, GABAergic mIPSCs had an average amplitude of $-32 \pm 1$ pA. Application of R-isovaline, had no apparent effect on the amplitude of mIPSCs which was $-31 \pm 1$ pA (97% of control; $p > 0.05$, paired Student’s $t$-test; Figure 3.16 B).

In summary, R-isovaline had no significant change in mIPSCs amplitude.
Figure 3.15 Stationarity of frequency of GABAergic mIPSCs. (A) Histogram shows no significant changes in frequency of mIPSCs during control and R-isovaline (200 µM) (p > 0.05, ANOVA). Control had a total average frequency of 1.2 ± 0.2 Hz and 1.3 ± 0.2 Hz during R-isovaline (200 µM) application (n = 9). (B) Histogram shows no significant changes in frequency of mIPSCs after application of R-isovaline at concentrations of 25, 100 and 200 µM (n = 20: p > 0.05, ANOVA).
Figure 3.16 R-isovaline (25, 100 and 200 µM) had no effects on the amplitude of GABAergic mIPSCs. (A) Cumulative probability histograms of GABAergic mIPSC amplitude in control conditions and during the application of R-isovaline to a ventrobasal neuron are shown. Application of R-isovaline, did not affect the amplitude cumulative probability of GABAergic mIPSCs. (B) In 22 neurons, R-isovaline had no significant effect on the mean amplitude of mIPSCs (p > 0.05, paired Student’s t-test).
3.3.4 Effects of R-isovaline on decay time constant

The effects of R-isovaline on decay time constant of GABAergic mIPSCs were investigated. Decay phase of each mIPSCs was well fitted with a single-exponential function to determine the time constant. As illustrated in Figure 3.17A, representative mIPSCs before and during the application of R-isovaline (200 µM) showed no apparent difference in decay kinetics. In 22 neurons examined, GABAergic mIPSCs had an average time constant of 20 ± 1 ms. The decay time constants were consistent with data in previous reports (Ghavanini et al., 2006; Mathers et al., 2009). Application of R-isovaline had no significant effect on the decay time constant (21 ± 1 ms; 105 % of control; P > 0.05, paired Student’s t-test; Figure 3.17B). Hence, our data shows that R-isovaline had no effect on the decay time constant of GABAergic mIPSCs.

3.3.5 Effects of R-isovaline on mIPSC rise time

We tested the effects of R-isovaline on the 10–90 % rise time of mIPSCs. In these experiments, application of R-isovaline did not affect the rise time. In 22 neurons, the rise time had an average of 2.0 ± 0.2 ms in control conditions. Application of R-isovaline did not alter the mean rise time which was 2.1 ± 0.1 ms (105 % of control; p > 0.05, paired Student’s t-test; Figure 3.18).
Figure 3.17 R-isovaline had no significant effect on the decay time constant of GABAergic mIPSCs. (A) Averaged mIPSC traces from a neuron in control and during application R-isovaline with identical decay time constants of 100 mIPSCs. (B) Decay phases of mIPSC were fitted with single-exponential functions. In 22 neurons tested, R-isovaline had no significant effect on decay time constant of GABAergic mIPSCs (P > 0.05, paired Student’s t-test).
3.3.6 Effects of R-isovaline on charge transfer

Since R-isovaline had no effect on peak amplitude and decay time constant of GABAergic mIPSCs, we anticipated that it would not affect charge transfer. The recorded mIPSCs had an average charge transfer of $-564 \pm 17 \text{ pA} \times \text{ms}$. Application of R-isovaline, had no significant effect on the mean of mIPSCs charge transfer $-572 \pm 16 \text{ pA} \times \text{ms}$ (101% of control; $p > 0.05$, paired Student’s $t$-test; Figure 3.18).

3.3.7 Effects of R-isovaline on holding current

Effects of R-isovaline (25, 100 and 200 µM) on holding current were also examined. Holding current was recorded for 5 to 10 min before and during the application of R-isovaline. In 22 neurons tested, the holding current had a mean of $-134 \pm 13 \text{ pA}$ before the application of R-isovaline and $-142 \pm 14 \text{ pA}$ after the application of R-isovaline (106% of control; $p > 0.05$, paired Student’s $t$-test; Figure 3.18).
Figure 3.18 R-isovaline (25, 100, 200 and 400 µM) had no effects on rise time (top), charge transfer (middle) or holding current (bottom) of GABAergic mIPSCs (n = 22; P > 0.05, paired Student’s t-test).
3.3.8 Summary of Effects of R-isovaline on GABAergic mIPSCs

We investigated the effects of R-isovaline on GABAergic mIPSCs recorded from 22 VB neurons. Table 3.4 summarizes the effects of R-isovaline on mIPSCs. In 13 out 22 neurons tested, R-isovaline (25, 100 and 200 µM) increased the mean frequency of mIPSCs and decreased it in the remaining 9 neurons. Altogether, R-isovaline had no effect on the frequency of GABAergic mIPSCs. In all tested neurons, R-isovaline had no effects on peak amplitude, decay time constant, tonic current, rise time or charge transfer. These data indicate that R-isovaline had no effect on the release of GABA from presynaptic terminals by a presynaptic mechanism.
Table 3.4 Summary of effects of R-isovaline on GABA\textsubscript{A}ergic mIPSCs

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>R-isovaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency (Hz)</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Amplitude (\text{\textmu}A)</td>
<td>−32 ± 1</td>
<td>−31 ± 1</td>
</tr>
<tr>
<td>10-90% rise time (ms)</td>
<td>2.0 ± 0.2</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Time constant (ms)</td>
<td>20 ± 1</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>Charge transfer (\text{\textmu}A\text{\textmu}s)</td>
<td>−564 ± 17</td>
<td>−572 ± 16</td>
</tr>
<tr>
<td>Holding current (\text{\textmu}A)</td>
<td>−134 ± 13</td>
<td>−142 ± 14</td>
</tr>
</tbody>
</table>

Values are means ± SEM. P > 0.05, paired Student’s \textit{t}-test, \textit{n} = 22.
Chapter 4. Discussion

4.1 Summary of the results

In this thesis, we used patch clamp recording and pharmacological analysis techniques to determine the interactions of bath applied AMBD on postsynaptic GABA<sub>A</sub> receptors and properties, as well as GABA release from nerve terminals of ventrobasal thalamic neurons. AMBD had no effects on the membrane properties or on postsynaptic activation of GABA<sub>A</sub> receptors by spontaneously released GABA. AMBD decreased spontaneous release of GABA through a presynaptic mechanism. In contrast to AMBD, bath application of R-isovaline had no effects on spontaneous GABA release from presynaptic terminals.

4.2 Postsynaptic effects of AMBD

Our first objective was to shed light on AMBD antagonism of inhibition due to GABA (cf. Mathers et al. 2009). We determined the interactions of AMBD and spontaneously released GABA on postsynaptic GABA<sub>A</sub> receptors of neurons. In these studies, internal Cs<sup>+</sup> was used to block K<sup>+</sup> channels which eliminated potential interference by GABA<sub>B</sub> receptor activation by GABA. Prior to this thesis, several studies have shown that AMBD antagonized strychnine-sensitive responses in different regions of mammalian CNS. In thalamocortical neurons, Mathers et al. (2009) showed that AMBD antagonized a strychnine-sensitive component of the mixed IPSCs that were presumably due to the actions of glycine-like amino acids, i.e., under conditions were the GABA<sub>A</sub>-component was blocked by bicuculline. In cortical, cerebellar and spinal neurons, AMBD reduced the responses to taurine and glycine (Curtis et al., 1982; Okamoto et al., 1982), consistent with these findings. However, AMBD did not antagonize the
majority of purely GABAergic IPSCs or the postsynaptic responses to applied GABA (cf. Yarbrough et al., 1981; Okamoto et al., 1982; Mathers et al., 2009). The latter findings are consistent with the results here – bath applications of AMBD did not affect the postsynaptic responses attributable to released GABA. At the same time, AMBD did not cause alterations in postsynaptic properties which might account for presynaptic effects (see below).

4.2.1 Effects of AMBD on membrane properties

AMBD antagonism of mIPSCs did not result from actions on passive or active membrane properties. Application of AMBD at 250 µM had no significant effects on membrane potential, input resistance or membrane time constant. AMBD did not alter properties of action potentials, LTSs or voltage-current relationships. These results are consistent with previous reports (Mathers, 1993; Mathers et al., 2009). Lack of significant effects on membrane properties implies that AMBD action was attributable to receptor antagonism.

4.2.2 Effects of AMBD on holding current

To further assess the effects of AMBD on postsynaptic GABA\(_A\) receptors, we examined the effects of AMBD on holding current. In thalamic neurons, the holding current is mediated by \(\alpha_4\) subunit-containing postsynaptic GABA\(_A\) receptors which are continuously activated by low concentrations of ambient GABA (Cope et al., 2005; Jia et al., 2005; Chandra et al., 2006). Our data indicate that AMBD had no apparent effects on holding current. These findings are in agreement with those of other studies in which AMBD did not affect responses of GABA at GABA\(_A\) receptors in cortical and cerebellar neurons (Yarbrough et al., 1981; Okamoto et al., 2009).
The data also suggest that AMBD had no effect on GABA concentration at the extrasynaptic space.

4.3 Presynaptic effects of AMBD

The second objective of this thesis was to determine the presynaptic effects of AMBD on GABAergic mIPSCs in the ventrobasal nuclei.

Over a wide concentration range, AMBD reversibly and in a concentration-dependent manner reduced the frequency of GABAergic mIPSCs and had no significant effects on amplitude or decay time constant. These data indicate that the AMBD membrane mechanism underlying the reduction of GABAergic mIPSCs comprises a presynaptic mechanism (cf., Thompson et al., 1993).

4.3.1 Comparison of AMBD effects on evoked and miniature IPSCs

We demonstrated that AMBD reduced the frequency of GABAergic mIPSCs in 17 out of 19 ventrobasal neurons. Mathers et al. (2009) reported that GABAergic evoked IPSCs were not altered by AMBD application in 4 out 5 neurons. This disagreement with these present results may be attributable to differences between evoked and spontaneous IPSCs. For example, miniature and evoked IPSCs have different temperature, ionic sensitivities and dependency on LVA Ca$^{2+}$, and not high voltage-activated (HVA) Ca$^{2+}$ channels. In 1998, Bao and colleagues showed that (HVA) Ca$^{2+}$ channel blockers such as Cd$^{2+}$ (50 µM) and ω-CgTx-GVIA (1 µM) can completely block the evoked EPSCs without affecting the frequency of mEPSCs. Furthermore, small depolarizations induced by raising the [K]$_o$ to 10 mM produce a significant increase of
mEPSCs frequency by increase opening of LVA Ca$^{2+}$ channel. This increase was fully blocked by the T-type Ca$^{2+}$ channel blocker, mibefradil (5 µM).

4.3.2 AMBD may inhibit presynaptic Ca$^{2+}$ channels

Our results suggest that the AMBD reduces the release of GABA through a presynaptic mechanism. There are a limited number of agents known to reduce the presynaptic release of transmitter by interacting with various presynaptic sites. The influx of Ca$^{2+}$ into the presynaptic terminals through voltage-gated Ca$^{2+}$ channels is an important trigger for neurotransmitter release (Dittman & Regehr, 1996; Qian et al., 1997). The presynaptic T-type Ca$^{2+}$ channel is one possible target known to modulate the release of neurotransmitter (Pan et al., 2001; cf. Carbone et al., 2006). In neurons of spinal laminae I and II, blockade of presynaptic low-threshold Ca$^{2+}$ channels using Ni$^{2+}$ (≤ 100 µM) reduced the frequency of mEPSCs while leaving the evoked monosynaptic EPSCs unchanged (Bao et al., 1998). Thus we hypothesized that the presynaptic action of AMBD was due to blockade of presynaptic T-type Ca$^{2+}$ channels and the co-application of Ni$^{2+}$ with AMBD would occlude the depressant action of the latter. However, in this thesis we have shown that 100 µM Ni$^{2+}$ decreased the frequency of mIPSCs but did not occlude the effects of AMBD. Thus, we conclude that the blockade of presynaptic low-threshold Ca$^{2+}$ channels is not likely the primary mechanism of action of AMBD. This finding is consistent with postsynaptic studies where AMBD had no effect on the LTS. However, these results do not exclude the possibility that the presynaptic action of AMBD was attributable to interaction with other different type of presynaptic Ca$^{2+}$ channels such as L-type Ca$^{2+}$ channels. For example, in nucleus basalis of Meynert (nBM), the L-type Ca$^{2+}$ channel antagonist nicardipine reduced the
frequency of GABAergic mIPSCs and had no significant effect on the amplitude. In contrast, nicardipine produced no apparent effect on the evoked IPSCs (Rhee et al., 1999).

4.3.3 Proposed mechanisms of action of AMBD

We proposed two possible mechanisms of presynaptic action of AMBD: 1) Activation of presynaptic $K_{\text{ATP}}$ channels and 2) blockade of presynaptic strychnine-sensitive glycine receptors.

4.3.3.1 AMBD may activate presynaptic $K_{\text{ATP}}$ channels

Studying the chemical structure of diazoxide, an opener of ATP-sensitive $K^+$ ($K_{\text{ATP}}$) channels, revealed a striking similarity to the structure of AMBD (Figure 4.1). While $K_{\text{ATP}}$ channels were initially discovered in cardiac myocytes (Noma, 1983), they also have been demonstrated to exist throughout the CNS (Amoroso et al., 1990; Ashford et al., 1990; Ohno-Shosaku et al., 1992). Karschin et al. (1997) demonstrated an evident expression of $K_{\text{ATP}}$ channels mRNA in ventroposterior nuclei. In various regions of CNS, $K_{\text{ATP}}$ channels exist both pre- and postsynaptically (Watts et al., 1995; Matsumoto et al., 2002). Activation of postsynaptic $K_{\text{ATP}}$
channels produces membrane hyperpolarization, while activation of presynaptic $K_{ATP}$ channels can modulate neurotransmitter release from nerve terminals (Crepel et al., 1993; Ye et al., 1997). Previous studies showed that activation of presynaptic $K_{ATP}$ channels reduces the release of GABA from terminals in the rat substantia nigra (Amoroso et al., 1990; Schmid-Antomarchi et al., 1990; Watts et al., 1995), and hippocampal neurons (Crepel et al., 1993; Ohno-Shosaku et al., 1993; Matsumoto et al., 2002). In the present study, we have shown that AMBD decreased the release of GABA through a presynaptic mechanism. Sulfonamides, including AMBD have been demonstrated to alter $K_{ATP}$ channels (Ashcroft and Gribble 2000). These data, therefore, raise the possibility that AMBD may reduce GABA release by activating presynaptic $K_{ATP}$ channels in ventrobasal neurons.

4.3.3.2 AMBD may block presynaptic glycine receptors

Like many ionotropic ligand-gated channels, glycine receptors found on presynaptic nerve terminals modulate neurotransmitter release throughout the CNS (for review see MacDermott et al., 1999; Hussy et al., 2001; Turecek and Trussell, 2001). Unlike the classical action of glycine, presynaptic glycine receptors induce a depolarizing Cl$^-$ current in the presynaptic terminal because the intracellular concentration of Cl$^-$ is relatively high (Hara et al., 1992). The depolarization triggers transmitter release by activating Ca$^{2+}$ channels and elevating intraterminal Ca$^{2+}$ concentrations (Zhou, 2001; Kilb et al., 2002; Laube et al., 2002). In various regions of mammalian CNS, activation of presynaptic glycine receptors facilitates the release of GABA (Ye et al., 2004), glycine (Jeong et al., 2003) and glutamate (Turecek and Trussell, 2001; Lee et al., 2009), and these effects are blocked by strychnine. In ventrobasal nuclei, immunohistochemical studies have shown that glycine receptor $\alpha_1$ and $\alpha_2$ subunits are mainly localized around somata.
and proximal dendrites. The functional nature of these receptors was confirmed by demonstrating the effects of glycine receptors agonists on thalamocortical neurons (Ghavanini et al., 2005). AMBD has been demonstrated to block strychnine-sensitive glycine receptors (Curtis et al., 1982; Mathers, 1993). Hence, we suggest that the presynaptic action of AMBD is attributable to the blockade of presynaptic glycine receptors.

4.4 Presynaptic effects of R-isovaline

The third objective of this thesis was to determine the presynaptic effects of R-isovaline on GABAergic mIPSCs in the ventrobasal nuclei. Previous studies by Cooke et al. (2009) showed that R-isovaline produces long-lasting inhibition through an increase in K+ conductance. They also showed that this action of R-isovaline was blocked by the GABA_B antagonist CGP35348 and the pre-treatment with the positive allosteric modulator of GABA_B receptors, CGP7930, potentiated the R-isovaline-induced inhibition (Cooke et al., 2011). He suggested that the action of R-isovaline was mediated through G-protein coupled GABA_B receptors. In rat ventrobasal neurons, the existence of functional presynaptic GABA_B receptors on the GABAergic terminals has been established where application of the GABA_B receptor agonist baclofen reduced the frequency of GABA_Aergic mIPSCs (Ulrich et al., 1996; Feuvre et al., 1997). Thus, we hypothesized that R-isovaline application would reduce the presynaptic release of GABA through activation of presynaptic GABA_B receptors (cf. Deisz and Prince, 1989). In our experiments, we investigated the effects of R-isovaline on properties of GABA_Aergic mIPSCs. Postsynaptically, R-isovaline did not alter responses to endogenously released GABA at postsynaptic GABA_A receptors. In these experiments, application of R-isovaline (25, 100 or 200
µM) had no significant effect on holding current, amplitude or decay time of GABA\textsubscript{A}ergic mIPSCs. These findings were in agreement with a recent study where application of picrotoxin, a chloride channel blocker, had no effect on the postsynaptic inhibition by R-isovaline (Cooke et al., 2009). Presynaptically, application of R-isovaline had no significant effect on the frequency of GABA\textsubscript{A}ergic mIPSCs. These results were inconsistent with a typical GABA\textsubscript{B} agonist as suggested before. A possible explanation for these discrepancies is that presynaptic GABA\textsubscript{B} receptors are pharmacologically and structurally different from postsynaptic GABA\textsubscript{B} receptors. For example, CGP35348 was ineffective at blocking the baclofen effect on presynaptic GABA\textsubscript{B} receptors in the dorsolateral septal nucleus (Yamada et al., 1999) and in the cerebral cortex (Bonanno and Raiteri, 1992; Deisz et al., 1997), while it blocked the postsynaptic GABA\textsubscript{B} receptors. Postsynaptic GABA\textsubscript{B} receptors have been demonstrated to be coupled solely with potassium channels (Bon and Galvan, 1996; Stevens et al., 1985; Yamada et al., 1999), while the presynaptic GABA\textsubscript{B} receptors mainly coupled to calcium channels (Kamatchi and Ticku, 1990; Doze et al., 1995). In ventrobasal neurons, Cooke et al. (2011) showed that the actions of R-isovaline characterized by slower kinetics compare to those of baclofen. They also showed that some baclofen-sensitive neurons did not respond to application of R-isovaline (Cooke, Ph.D thesis, The University of British Columbia, 2010). Taken together, these data may explain the ineffectiveness of R-isovaline to activate the presynaptic GABA\textsubscript{B} receptors.

4.5 Proposed mechanism of action of R-isovaline

We propose here that R-isovaline has an action which is atypical of GABA\textsubscript{B} receptors activation (see previous section). Our data showed that R-isovaline did not alter the properties of GABA\textsubscript{A}ergic mIPSCs. We suggest that R-isovaline may activate different G-protein-coupled
receptors, for example, group II metabotropic glutamate receptors (mGlurRs). The activation of presynaptic group II mGlurRs reduces the frequency of GABAergic mIPSCs in nucleus basalis of Meynert neurons (Doi et al., 2002) and in thalamocortical neurons (Turner and Salt, 2003), supporting the possible role of R-isovaline as an agonist of group II mGlurRs. This does not preclude the possibility that R-isovaline may interact with distinctive subtypes of GABA<sub>B</sub> receptors.

4.6 Future directions

In this thesis we have shown that AMBD decreased GABA release through a presynaptic action. However, the exact mechanism whereby AMBD decreased GABA release has yet to be determined. Future experiments should be directed to define the specific target of action of AMBD on GABAergic terminals. K<sub>ATP</sub> channel blockers, such as glibenclamide and tolbutamide, should be utilized to determine whether AMBD induces its presynaptic effect by activating presynaptic K<sub>ATP</sub> channel. The demonstration that AMBD blocks strychnine-sensitive glycine receptors (Curtis et al., 1982; Mathers, 1993; Mathers et al., 2009) suggests that AMBD may reduce GABA release by antagonizing the presynaptic glycine receptors (Ye et al., 2004; Jeong et al., 2003). Therefore, strychnine should be used to verify this hypothesis. Moreover, selective T- and L-type Ca<sup>2+</sup> channel antagonists should be used to examine whether the presynaptic action of AMBD was due to blockade of presynaptic T- and/or L-type Ca<sup>2+</sup> channels.

Baclofen as well as selective GABA<sub>B</sub> receptors antagonists should be used to confirm that R-isovaline does not activate the presynaptic GABA<sub>B</sub> receptors on GABAergic terminals in ventrobasal neurons. Like previous reports, our results indicated that R-isovaline was an atypical
GABA\textsubscript{B} receptors agonist (Cooke et al., 2009; Cooke, J., PhD thesis, 2010). Since activation of group II mGluRs was consistent with the postsynaptic actions of R-isovaline (Cooke et al., 2009) it would be sensible to examine the effect of R-isovaline on group II mGluRs.

4.7 Conclusions

To our knowledge, this is the first study to show a presynaptic effect of AMBD. We also have shown that AMBD had no postsynaptic effects that may account for its presynaptic action. Finally, our results demonstrated that R-isovaline had no significant effect on the frequency of GABA\textsubscript{A}ergic mIPSCs in ventrobasal neurons.

There are a limited number of agents known to reduce frequency of mIPSCs. This thesis gave an explanation of the previous finding by Fariello et al. (1986) where application of GABA but not taurine, β-alanine or glycine reversed the epileptic effects induced by cortical superfusion with AMBD in rat. If AMBD acts through activation of K\textsubscript{ATP} channels, it would be difficult to reconcile the disinhibitory effect of AMBD that results from the reduction of GABA release, with the inhibitory effect resulting from activation of postsynaptic K\textsubscript{ATP} channels. However, activation of K\textsubscript{ATP} channels can play a neuroprotective role during cell damaging conditions, such as ischemia (Yamada et al., 2010).

Moreover, our data suggest that R-isovaline had no effects on GABA release. Further investigation of the specific mechanisms of R-isovaline in the nervous system should contribute to our understanding of its analgesic actions and may also contribute to the development of novel antinociceptive therapies.
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