# SCREENING FOR NOVEL MODULATORS OF $GABA_A$ RECEPTORS

by

Ling Zhang

B.Sc., Peking University, 2009

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

October 2013

## Abstract

$GABA_A$ (γ-amino butyric acid, type A) receptors are a family of ligand gated ion channels that play essential roles both in normal brain functions and psychiatric conditions. While the classical $GABA_A$ receptor modulators benzodiazepines have been in clinical use for decades and are still among the most widely prescribed drugs for the treatment of certain disorders, their limitations and side effects have been driving the search for alternative solutions. Recent studies from our lab have demonstrated that glutamate can act as positive allosteric modulators of $GABA_A$ receptors, and the suggested binding sites are on the interface of alpha and beta subunits. This surprising finding drove us to pursuit two objectives: (1) screen glutamate-like molecules that can compete with glutamate and prevent the potentiation of $GABA_A$ receptors, so we can study the intrinsic functions of the modulating effects of glutamate; (2) screen glutamate-like molecules that can mimic glutamate and potentiate $GABA_A$ receptors but do not produce any other physiological effects, so we can utilize them as candidates for clinical treatment as alternatives of benzodiazepines.

Experiments are performed on hippocampal neuron cultures and HEK 293 cells transfected with $GABA_A$ receptor subunits. Virtual screening and electrophysiology recording are employed. For objective (1), we identified 10 compounds that can inhibit glutamate from potentiating $GABA_A$ receptor. Unfortunately, none of them can completely block the potentiation. For objective (2), we identified one compound, 2-methyl aspartic acid (2-MAA), as the final candidate for further study. We found that 2-MAA shares the binding sites with glutamate and potentiates $GABA_A$ receptors in a dose-dependent manner, while it does not affect normal neuronal activities. In

addition, although it shows no significant effect on tonic GABA current amplitude, it does increase the frequency of mini-IPSCs.

In the future, for objective (1), it will require screening for additional candidates and/or modification of the 10 compounds. For objective (2), we can continue testing the anti-epilepsy effect of 2-MAA on both *in vitro* and *in vivo* models. It will also be applicable to modify the compound or to screen similar compounds so as to get a better candidate with bigger potentiation at a lower concentration.

## Preface

This thesis is conceived and planned by Ling Zhang and Dr. Yu Tian Wang. Ling Zhang carried out the experimental work with assistance from Mei Lu, Yan Li and Peter Axerio-Cilies. Ling Zhang carried out the statistical analyses and writing of the thesis under the supervision of Dr. Yu Tian Wang.

## Table of Contents

## List of Figures

## List of Abbreviations

**2-MAA**: 2-Methyl-L-Aspartic Acid

**AMPA**: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

**AP5**: D-2-amino-5-phosphonovaleric acid

**CNQX**: 6-cyano-7-nitroquinoxaline-2,3-dione

**GABA**: γ-amino butyric acid

**KA**: kainate or kainic acid

**NMDA**: N-methyl-D-aspartate

**CNS**: Central nervous system

**HEK 293 cell**: Human Embryonic Kidney 293 cell

**ECS**: Extra-cellular solution

**ICS**: Intra-cellular solution

## Acknowledgements

First and foremost I offer my sincerest gratitude to my supervisor Dr. Yu Tian Wang who has been supportive throughout my study in UBC with his patience, knowledge and passion. I would like to thank Dr. Lidong Liu and Dr. Dongchuan Wu for instructions on some experiment in the thesis. I also would like to thank Dr. Yuping Li for preparing cultured neurons and thank Mei Lu, Yan Li and Peter Axerio-Cilies for assistance on some experiments in the thesis.

I would like to thank my supervisory committee members Dr. Ann Marie Craig, Dr. Weihong Song and Dr. William Jia for their support and helpful suggestion.

In addition, I would like to thank Dr. Jie Lu, Dr. Lorren Oschipok and Agnes Kwok for all their kind help and support during my study in UBC. I also would like to thank for the support from my other lab mates Wuyang Jin, Shally Fan, Ya Wen, Donovan Ashby, Kimberly Girling, Bo Gong, Joon-Hyuk Imm, Dr. Andrea Titterness, Dr. Allen Chan and Dr. Steven Connor, as well as Dr. Ted Lai, Dr. Thomas Bartlett and Dr. Taesup Cho who already left the Wang lab. Without them, my life here could not have been so happy and fruitful.

Finally, I would like to thank my wife Lin Luo. This work could never be done without her love and support.

# Chapter 1: Introduction

A stable basal level neuronal activity is essential for normal brain functions, which is achieved by precise regulation of the balance between excitatory and inhibitory synaptic transmission. Malfunction of either excitatory or inhibitory synaptic transmission would result in the impairment of brain functions and subsequent neural disorders, and requires specific treatment to bring brain activity to normal. This thesis will focus on the regulation of inhibitory γ-amino butyric acid type A ($GABA_A$) receptors by novel modulators, which could be used as alternative clinical treatment for disorders involving $GABA_A$ receptor impairment, and could be used to reveal the mechanisms under the mutual regulation between inhibitory and excitatory systems.

## 1.1 $GABA_A$ receptors

In the mammalian brain, γ-amino butyric acid (GABA) receptors are the primary inhibitory receptors that are essentially involved in almost all aspects of neuronal functions. GABA receptors can be divided into two sub-families, ionotropic type A GABA receptors ($GABA_A$ receptors) and metabotropic type B GABA receptors, the former of which are the dominant inhibitory receptors on the synapses and will be the focus of this study.

### 1.1.1 Structure of $GABA_A$ receptors

$GABA_A$ receptors belong to Cys-loop pentameric ligand-gated ion channel (LGIC) superfamily (Olsen and Sieghart, 2008, 2009). Each $GABA_A$ receptor contains five subunits and every subunit consists of an extracellular N-terminal domain followed by four transmembrane domains (TM1-TM4) and ends with an extracellular C-terminal region.

In mammalian nervous system, there are 19 $GABA_A$ receptor subunits, including α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3 that are encoded by 19 different genes (Simon et al., 2004). As a result, the composition of $GABA_A$ receptors is highly diverse. The majority of $GABA_A$ receptors are heteropentameric receptors containing two α subunits, two β subunits, and one another subunit, such as γ, δ, or ε (Sieghart and Sperk, 2002; Olsen and Sieghart, 2008). The other homomeric or heteropentameric compositions include homomeric β3, homomeric ρ, and heteromeric α and β compositions, which are mainly reported in overexpression systems (Cutting et al., 1991; Qian and Dowling, 1993; Wang et al., 1994, 1995; Connolly et al., 1996; Wooltorton et al., 1997; Enz and Cutting, 1999; Taylor et al., 1999).

The minimal requirement for composing the functional $GABA_A$ receptors is α/β (Malherbe et al., 1990a; Sigel et al., 1990), which, due to the lack of γ subunit, only lacks benzodiazepine sensitivity comparing with native $GABA_A$ receptors (Levitan et al., 1988). The αβγ composition shows almost the full properties of native $GABA_A$ receptors (Sigel et al., 1990). In mammalian nervous system, specifically, the αβγ2 composition accounts for more than 90% of the native $GABA_A$ receptors, and the α1β2γ2 composition accounts for around 60% of the native $GABA_A$ receptors in the central nervous system (Möhler, 2006).

### 1.1.2 Distribution of $GABA_A$ receptors

Basically, $GABA_A$ receptors exist ubiquitously through the whole mammalian central nervous system and 20-30% of CNS neurons are GABAergic (Rudolph and Knoflach, 2011). Revealed by radioactivity binding assay in rodent brains, they are expressed the most in frontal cortex, cerebellum granule cell layer, olfactory bulb, thalamic medial geniculate, hippocampus

molecular layer, and the external (1-4) layers of the cortex. In basal ganglia, pons, medulla and brainstem, $GABA_A$ receptors are also expressed at a lower level (Palacios et al., 1981; Bowery et al., 1987).

Specific distribution of different $GABA_A$ receptors varies with their subunit compositions and displays distinct patterns, which are revealed by *in situ* hybridization in rodent brains (Wisden et al., 1992, 1991; Persohn et al., 1991; MacLennan et al., 1991; Zhang et al., 1990; Ymer et al., 1990; Seeburg et al., 1990; Malherbe et al., 1990b; Lüddens et al., 1990; Kato, 1990; Hironaka et al., 1990; Shivers et al., 1989; Lolait et al., 1989; Khrestchatisky et al., 1989, 1991).

In cortex, α1 subunits are expressed in layer 2-6. α2 subunits are mainly expressed in layer 2. α3 subunits are predominantly expressed in layer 5. α4 subunits are more expressed in layer 2 and 3. α5 subunits are expressed through all layers at a lower level. α6 subunits are unique that they are only expressed in cerebellum but not any other brain regions. As for β subunits in cortex, β1 subunits are expressed at a very low level if any. β2/3 subunits are expressed in layer 2-6, a similar pattern with α1. In addition, γ1 and γ3 subunits are expressed through all layers. γ2 subunits are expressed in layer 2-6, a similar pattern with α1 and β2/3. δ subunits are mainly expressed in layer 2 while in other layers there are also lower expression.

In hippocampus, α1, α2 and α4 subunits are expressed throughout the CA1, CA3 and dental gyrus, α3 subunits are mainly expressed in granule cells of dental gyrus, and α5 are predominantly expressed in CA1 and CA3 regions. β1-3 subunits are all expressed throughout

the hippocampus. γ1 subunits are not express in hippocampus but γ2 subunits are expressed at a high level. δ subunits are mainly expressed in dentate gyrus.

In caudate nucleus and nucleus accumbens, α subunits are mainly α2 and α4, β subunits are mainly β3, γ subunits expression is very low, and δ subunits are expressed at a moderate level. In amygdala, α subunits are mainly α2, β1-3 subunits are all expressed at a low to moderate level, γ subunits are mainly γ1, and no δ subunits are expressed. In thalamus, α subunits are mainly α1 and α4, β subunits are mainly β2, γ subunits expression is very low, and δ subunits are only expressed in several nucleuses.

In cerebellum, α subunits are mainly α1 and α6. α1 subunits are mainly expressed in stellate/ basket cells Purkinje cells and granule cells, and α6 subunits are only expressed in granule cells. β subunits are mainly β2 and β3, both of which are expressed in Purkinje cells and granule cells. γ subunits are γ2 that are expressed in Purkinje cells and granule cells. δ subunits are only expressed in granule cells.

On cellular level, the distribution of $GABA_A$ receptors also varies with the subunit compositions. Overall, α1β2/3γ2, α2β2/3γ2 and α3β2/3γ2 are the predominant receptor subtypes, α4βxδ, α6βxδ and α5βxγ2 are predominantly or exclusively extrasynaptic (Farrant and Nusser, 2005); however, no subunit has been found to have an exclusively synaptic location, even for α1β2/3γ2 receptors there are more outside than inside synapses. In granule cells of cerebellum, $GABA_A$ receptors with α6β2/3δ subunits are distributed in extra-synaptic areas, while $GABA_A$ receptors with α1β2/3γ2, α6β2/3γ2, and α1/α6β2/3γ2 subunits are distributed in synaptic areas (Nusser et al.,

1995, 1998). In hippocampus, $GABA_A$ receptors containing α1 subunits are predominant in inhibitory synapses of pyramidal neurons, and $GABA_A$ receptors containing α2 subunits are mainly located in synapses on axon initial segments (Nusser et al., 1996).

### 1.1.3 Functions of $GABA_A$ receptors

As the primary inhibitory receptors in mammalian brains, native $GABA_A$ receptors are ligand-gated chloride channels. Under normal conditions, regulated by $Cl^-$ extruding $K^+/Cl^-$ co-transporters (KCC2), extracellular $Cl^-$ concentration is higher than intracellular and the $Cl^-$ equilibrium potential is negative comparing to neuronal resting potential (Payne et al., 2003). Activation of $GABA_A$ receptors by binding with GABA opens the channel and increases the channel's chloride conductance, which decreases the membrane potential towards $Cl^-$ equilibrium potential, resulting in hyperpolarization and inhibition of the neurons' activity.

Interestingly, during the early nervous development stage, the intracellular $Cl^-$ concentration is higher than extracellular due to lack of KCC2 and the $Cl^-$ equilibrium potential is positive comparing to neuronal resting potential. In this case, activation of $GABA_A$ receptors would depolarize the neurons and induce excitatory effect, which is quite opposite to the normal condition in adult brains (Farrant and Nusser, 2005; Rivera et al., 2005).

The inhibitory regulations of $GABA_A$ receptors include fast "phasic" synaptic inhibition and slow or consistent "tonic" extrasynaptic inhibition (Farrant and Nusser, 2005). The fast phasic inhibitory current is induced on postsynaptic $GABA_A$ receptors by GABA released transiently and massively from synaptic vesicles. The consistent tonic current, however, is induced by

extrasynaptic $GABA_A$ receptors by low concentration of ambient GABA. Impairment of $GABA_A$ receptors function causes imbalance between excitatory and inhibitory regulation, and contributes to various psychiatry conditions such as seizure, epilepsy, insomnia and schizophrenia (French and Faught, 2009; Tabuchi et al., 2007; Yee et al., 2005). Clinically, to treat these disorders, $GABA_A$ receptors modulators are widely used, among which the most typical are benzodiazepines which could potentiate GABA currents.

### 1.2 Benzodiazepines and the limitations

Benzodiazepines are a family of chemicals with a fusion structure of a benzene ring and a diazepine ring. It was discovered in 1950s to exhibit anxiolytic, sedative, muscle relaxing and seizure suppressive effects (Sternbach, 1979).

Benzodiazepines are allosteric agonists of $GABA_A$ receptors, on which the binding site for benzodiazepines (BZ site) is located at the interface of α and γ subunits. In α subunits, a conserved histidine residue is critical for the binding (α1-H101, α2-H101, α3-H126, or α5-H105). α1-5 containing $GABA_A$ receptors will lose diazepam sensitivity if this histidine is replaced, say, by arginine (Benson et al., 1998; Wieland et al., 1992). In γ subunits, residue M57, Y58, F77, A79, T81 and M130 are important for the binding (Buhr and Sigel, 1997; Buhr et al., 1997; Kucken et al., 2000; Sigel et al., 1998; Wingrove et al., 1997). The $GABA_A$ receptor modulation by benzodiazepines has two special aspects (Rudolph and Knoflach, 2011). First, benzodiazepines alone do not stimulate $GABA_A$ receptors; they need GABA to open the channel.

Second, the potentiation of GABA current by benzodiazepines can also be achieved by increasing the dose of GABA.

During the past decades, benzodiazepines have been the most widely prescribed drugs for the treatment of $GABA_A$ receptors involved conditions, such as insomnia, epilepsy and anxiety disorders. However, limitations of benzodiazepines are also quite obvious (Rudolph and Knoflach, 2011). When anxiolysis is expected for the daytime application, sedation is always a side effect of benzodiazepines even in lower doses. Besides, addiction can be easily developed when benzodiazepines are used for a long time, so abuse of benzodiazepines is a major concern. In addition, physical dependence and tolerance, as well as cognitive impairment, also occur during benzodiazepine treatment.

As a result, searching alternative $GABA_A$ receptor modulators has been a task of researchers for many years. While some novel modulators are under developing, our lab discovered that glutamate and certain glutamate-like molecules can allosterically potentiate $GABA_A$ receptor which may create a new path for screening the desirable compounds while at the same time reveal more secrets of $GABA_A$ receptors.

### 1.3 Potentiation of $GABA_A$ receptors by glutamate-like molecules

The regulatory mechanisms in the nervous system can be divided into two major categories, one is excitatory regulation, and the other is inhibitory. As in the brain, GABA is the primary inhibitory neurotransmitter and glutamate is the primary excitatory neurotransmitter. Although reciprocal interactions between excitatory and inhibitory systems have been well documented

(Patenaude et al., 2003; Puyal et al., 2003; Windhorst, 2007), these interactions are all above cellular level and none is at molecular level, except for glycine binding to NMDA receptors and functioning as the coagonist (Kleckner and Dingledine, 1988).

More than twenty years ago it has been shown that neuronal GABA induced chloride current can be potentiated by glutamate (Stelzer and Wong, 1989), but the mechanism was not revealed. Just recently, our lab discovered that glutamate actually can allosterically bind to $GABA_A$ receptors endogenously expressed in hippocampal neuron cultures and those overexpressed in transfected Human Embryonic Kidney 293 (HEK 293) cells (below are all unpublished data), increase the channel conductance, and potentiate chloride current induced by GABA. In addition, not only glutamate, certain glutamate-like molecules including N-methyl-D-aspartate (NMDA), D-2-amino-5-phosphonovaleric acid (AP5), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainate (KA) can also allosterically potentiate $GABA_A$ receptors. The competitive AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), however, does not potentiate $GABA_A$ receptors. Rather, it can block glutamate from potentiating $GABA_A$ receptors, indicating that all these molecules may share the same binding site(s).

Our lab found that the potentiation by glutamate only requires the presentation of α1 and β2 subunits of $GABA_A$ receptors and further identified that the glutamate binding site is at the interfaces of α1 and β2 subunits. While several residues have been demonstrated to contribute to glutamate binding, E181 is one of the most important. Replacement of E181 by glycine (E181G) could completely abolish the potentiation by glutamate, while having no influence on the normal function of $GABA_A$ receptors.

These discoveries revealed a new family of modulators and a new modulatory binding site on $GABA_A$ receptors, which is completely different from benzodiazepines and benzodiazepines site, especially in the way that glutamate is an endogenous molecule and the primary excitatory neurotransmitter in the brain. It will be very intriguing to know the physiological function of this interaction, as well as the potential of developing novel glutamate-like $GABA_A$ receptor modulators for clinical use to replace benzodiazepines.

### 1.4 Screening for novel glutamate-like $GABA_A$ receptor modulators

For the purpose of investigating the physiological function of the interaction between glutamate and $GABA_A$ receptors, we need a compound whose only physiological effect would be competitively blocking glutamate from binding to $GABA_A$ receptors, similar with CNQX but without the ability as the antagonist or agonist of any receptors.

For the purpose of developing novel compounds to clinically potentiate $GABA_A$ receptors, we need compounds that can mimic glutamate to potentiate $GABA_A$ receptors but have no any other side effects such as stimulating neurons, affecting normal GABA or glutamate receptor function, nonreversible binding, etc.

This thesis will mainly focus on screening and testing for the above compounds in *in vitro* systems, as preparation for *in vivo* experiments in the future.

# Chapter 2: Materials and Methods

## 2.1 Hippocampal neuron cultures

Cultured hippocampal neurons were prepared from the brains of E18 fetal Wister rats. Tissues were digested with a 0.25% trypsin solution for 25 min at 37 ℃, and then mechanically dissociated using a fire-polished Pasteur pipette. The cell suspension was then centrifuged at 2500 ×g for 50 s and the cell pellets were re-suspended in DMEM containing 10% Fetal Bovine Serum (Sigma-Aldrich). Cells were seeded on poly-D-lysine coated 24-well coverslips at a density of 2.5 $\times 10^5$ cells/well. Cultures were maintained in a humidified incubator with 5% $CO_2$ at 37 ℃. After 24 hours, plating medium was changed to Neurobasal medium supplemented with B-27 supplement and L-glutamine and the media was changed twice weekly thereafter. Cultured neurons were used for electrophysiological recordings 14-21 days after plating.

## 2.2 HEK 293 cell culture and transfection

HEK 293 cells were cultured in DMEM supplemented with 10% FBS. Cells were grown to 20% confluence and transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. Cells were transfected with three pBK-CMV NB-200 expression vectors respectively containing a rat recombinant $GABA_A$ receptor α1, β2 and γ2 subunits. The transfection ratio with α1, β2 and γ2 plasmids was 2:2:1. pcDNA3-GFP was also co-transfected as a transfection marker, in order to facilitate the visualization of the transfected cells during electrophysiological experiments. 12 hours following transfection, cells were re-plated on glass coverslips and cultured for an additional 12-24 hrs before whole-cell patch-clamp recordings.

## 2.3 Whole-cell patch-clamp recording

Whole-cell recordings were performed under voltage-clamp mode using an Axopatch 200B or 1D patch-clamp amplifier (Molecular Devices). Whole-cell currents were recorded at a holding potential of -60 mV, and signals were filtered at 2 kHz, digitized at 10 kHz (Digidata 1322A). Recording pipettes (3-5 MΩ) were filled with the intra-cellular solution (ICS) that contained (mM): CsCl 140, HEPES 10, Mg-ATP 4, QX-3145 and BAPTA 10 mM, pH 7.20; osmolarity, 290-295 mOsm. The coverslips were immersed in the extra-cellular solution (ECS) containing (mM): NaCl 140, KCl 5.4, HEPES 10, $MgCl_2$ 1.0, $CaCl_2$ 1.3, glucose 20, pH 7.4; osmolarity, 305-315 mOsm. For recording on cultured neurons, TTX (0.5 μM) was added to the ECS in order to inhibit action potentials. A computer-controlled multi-barrel fast perfusion system (Warner 177 Instruments) was employed to control what stimulation the cells would get. Two glass pipettes (A and B) were paralleled back to back to give treatment.

For recording of GABA currents in HEK 293 cells or cultured neurons (ECS for neuronal recording also contained CNQX 10 μM and TTX 0.5 μM), pipette A was placed right against the target cell on the coverslips, and ECS or ECS + Tested Compounds was continuously delivered through pipette A towards the target cell. Pipette B was connected with solution of ECS + GABA or ECS + GABA + Tested Compounds, and the solution was continuously delivered but did not reach the target cell. When recording is triggered, pipette B would shift its position to that of pipette A for 2000ms and then shift back. During this period $GABA_A$ receptors were stimulated by GABA from pipette B and phasic GABA current was recorded. For recording of mIPSCs and tonic GABA currents in cultured neurons, the ECS also contained CNQX (10 μM)

and TTX (0.5 μM). Only pipette A was used, to continuously deliver ECS or ECS + Tested Compounds. All experiments were performed at room temperature.

### 2.4 Chemicals

NMDA, AMPA, AP5, CNQX and kainate were purchased from Tocris. Glutamate and glycine were purchased from Sigma-Aldrich. Bicuculline was purchased from Alexis Biochemicals. TTX was purchased from Almone Labs. All tested compounds were obtained from National Cancer Institute (NCI) database, except that 2-Methyl-L-Aspartic Acid (2-MAA) was also purchased from Sigma-Aldrich.

### 2.5 Data analysis

Values are expressed as mean ± SEM (n = number of experiments). One-way ANOVA or a two-tailed Student's test was used for statistical analysis and P values less than 0.05 were considered statistically significant. Dose response curves were created by fitting data to Hill equation: $I = I_{max} / (1+EC50 / [A]^n)$, where I is the current, $I_{max}$ is the maximum current, [A] is a given concentration of agonist, n is the Hill coefficient.

# Chapter 3: Results

## 3.1 Virtual screening of glutamate-like compounds

We started the compound screening with computer based virtual screening and this part of work is mostly done by another student, Peter Axerio-Cilies. The National Cancer Institute (NCI) open compounds database was chosen as the chemical pool. The Molecular Operating Environment (MOE), software platform of Chemical Computing Group, was used to perform the virtual screening.

The purpose of the virtual screening is to narrow down the range of glutamate-like candidates that may share the same binding site of glutamate on the interface of $GABA_A$ receptor α1 and β2 subunits. We first constructed the virtual binding model of glutamate with α1 and β2 subunits and obtained data of the following six aspects regarding the binding: 1) size of the cavity; 2) receptor/ligand flexibility; 3) energy of hydrogen bonds; 4) hydrophobic interactions; 5) free energies; 6) docking consistency. Then we did a high throughput virtual screening that every compound in the NCI database was tested to fit into the glutamate binding site and the binding data of the above six aspects was recorded and compared to that of glutamate binding.

Around 200 compounds that showed similar binding data like glutamate were selected. Considering the availability and purity of these compounds, finally 78 compounds were chosen as the initial candidate pool for real screening.

### 3.2 Screening for glutamate competitors

In order to screen for a glutamate competitor whose only physiological effect is competitively blocking glutamate from binding to $GABA_A$ receptors, the following three screening criteria was employed: The compound 1) alone does not stimulate any reaction or have any known function in neurons, 2) does not potentiate GABA current when co-applied with GABA to $GABA_A$ receptors, and, 3) can block the potentiation of GABA current by glutamate.

For criterion 1), the above mentioned 78 compounds (Pool A) were tested one by one whether they could stimulate any current on cultured neurons. Whole cell voltage-clamp recordings were performed on hippocampal neuron cultures in ECS (containing 0.5 μM TTX) at holding potential of -60mV and a candidate compound (200 μM in ECS, 2s) was fast perfused to the patched neuron. All compounds that induced current were eliminated from the list, and the rest compounds (Pool B) went on to the next screening step, criterion 2).

For criterion 2), whole cell voltage-clamp recordings were first performed on hippocampal neuron cultures in ECS (containing 0.5 μM TTX) at holding potential of -60mV and GABA (1 μM in ECS, 2s) was fast perfused to the neuron. After a stable baseline GABA current had been obtained, a candidate compound from Pool B was added to the solutions of the fast perfusion system until its concentration reached 200 μM. As a result, the neurons were pre-treated with 200 μM candidate compound (the purpose is to pre-saturate the binding site of $GABA_A$ receptors, as what we did when we discovered that glutamate can potentiate $GABA_A$ receptors). Then GABA (1 μM in ECS, 2s) containing 200 μM candidate compound was fast perfused to the neuron and a stable test GABA current was recorded. If the test current and the

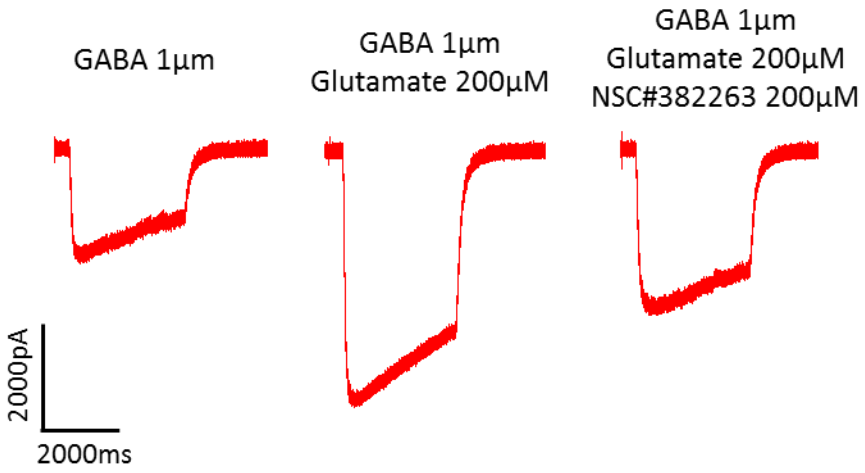
baseline currents were the same, the compound did not affect GABA current and it would be categorized into Pool C. However, if the test current was bigger than baseline current, the compound potentiated GABA current just like glutamate did, and it would be categorized into Pool D. The rest compounds were eliminated.

For criterion 3), whole cell voltage-clamp recordings were first performed on HEK 293 cells transfected with GFP and $GABA_A$ receptor α1β2 subunits at holding potential of -60mV, and GABA (1 μM in ECS, 2s) was fast perfused to the patched cell (the reason to use HEK 293 cells is they do not express glutamate receptors). After a stable baseline GABA current had been obtained, 200 μM glutamate was added into the system and as a result the cells were pre-treated with 200 μM glutamate. Then GABA (1 μM in ECS, 2s) containing 200 μM glutamate was fast perfused to the patched cell and a stable potentiated GABA current was recorded. Thereafter, 200 μM of a candidate compound from Pool C was added into the system and as a result the cells were pre-treated with 200 μM glutamate and 200 μM candidate compound. Finally, GABA (1 μM in ECS, 2s) containing 200 μM glutamate and 200 μM candidate compound was fast perfused to the cell and a stable test GABA current was recorded. If the test current was smaller than the potentiated currents, the compound could reduce the glutamate potentiation and it would be categorized into Pool X. The rest compounds were eliminated.

Eventually, Pool X contained 11 compounds which could block the glutamate potentiation of GABA currents. However, after a close examination, none of these compounds can completely block the glutamate potentiation (Figure 1). In this case, modification of these compounds may be needed to achieve the goal.

**Figure 1: Screening for glutamate competitors.**

Below is the recording results for NSC#382263, a representative compound in Pool X. Whole cell voltage-clamp recordings were performed on HEK 293 cells transfected with GFP and $GABA_A$ receptor α1β2 subunits at holding potential of -60mV. The GABA currents were recording under treatment of GABA, GABA + glutamate, or GABA + glutamate + NSC #382263, in which 200 μM glutamate potentiated GABA current by ~150% and co-applied 200 μM NSC #382263 reduced the potentiation to ~50%. Same with other compounds in Pool X, NSC#382263 cannot completely block the potentiation by glutamate.



### 3.3 Screening for novel $GABA_A$ receptor modulators

In order to screen for glutamate-like novel $GABA_A$ receptor modulators that have the potential of clinical application, the following three screening criteria was employed: The compound 1) alone does not stimulate any reaction or have any known function in neurons, 2) can potentiate GABA current when co-applied with GABA to $GABA_A$ receptors, and, 3) does not affect glutamate currents when co-applied to glutamate receptors.

For criteria 1) and 2), the screening had been done in the above Section 3.2 and the selected compounds were all included in Pool D, which was used for screening of criterion 3).

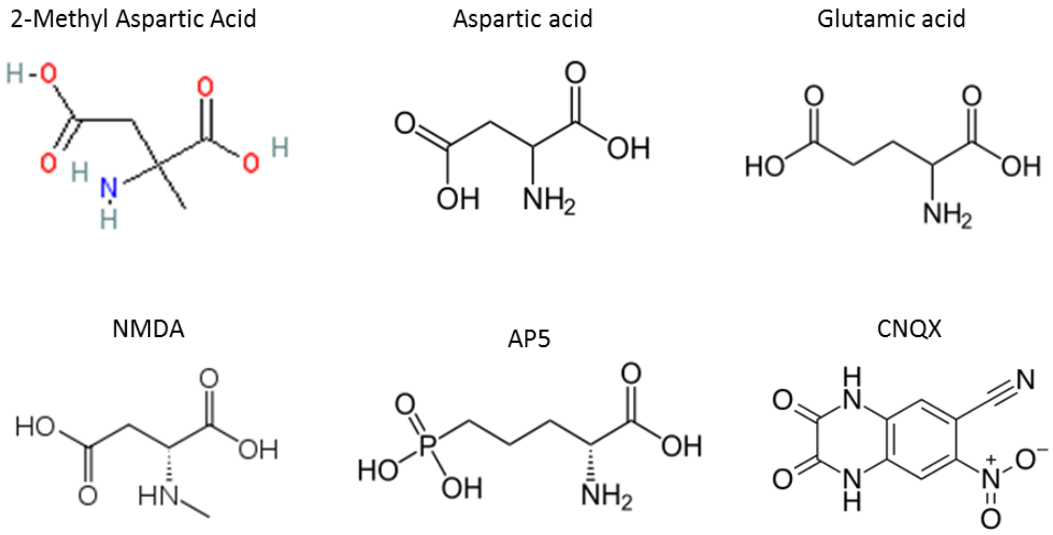
For criterion 3), whole cell voltage-clamp recordings were first performed on hippocampal neuron cultures in ECS (containing 0.5 μM TTX) at holding potential of -60mV and glutamate (2 μM in ECS, 2s) was fast perfused to the neuron. After a stable baseline glutamate current was obtained, a candidate compound from Pool D was added into the system and as a result the neurons were pre-treated with 200 μM candidate compound. Then glutamate (2 μM in ECS, 2s) containing 200 μM candidate compound was fast perfused to the neuron and a test glutamate current was recorded. If the test current was the same with baseline, the compound did not affect glutamate current and would be categorized into Pool Y. Compounds that affected glutamate current were eliminated. Finally, 10 compounds were included in Pool Y and the scale of their potentiation effect varied.

In all the above experiments, consumption of those candidate compounds was comparatively small. However, to further study how the compounds in Pool Y could potentiate $GABA_A$

receptors, experiments including dose responsive curve and tonic GABA current recording would be necessary and in these experiments the consumption of candidate compound would be much larger, which could not be satisfied by supply from NCI database. In this case, considering the commercial availability and the potentiation scale of every compound in Pool Y, only one was chosen for further experiments. The NSC ID of this compound is 14963 and its chemical name is 2-methyl-L-aspartic acid (2-MAA) (Figure 2). In all experiments below 2-MAA was purchased from Sigma-Aldrich.

**Figure 2: Structure of 2-methyl-L-aspartic acid.**

The structure of 2-methyl-L-aspartic acid (2-MAA) is compared with glutamate/glutamic acid and five well-known glutamate-like molecules. Except CNQX, the other five are highly similar especially in their polar groups. The chemical formula of 2-MAA, glutamic acid and NMDA are the same. The minor differences determine their distinct physiological functions.



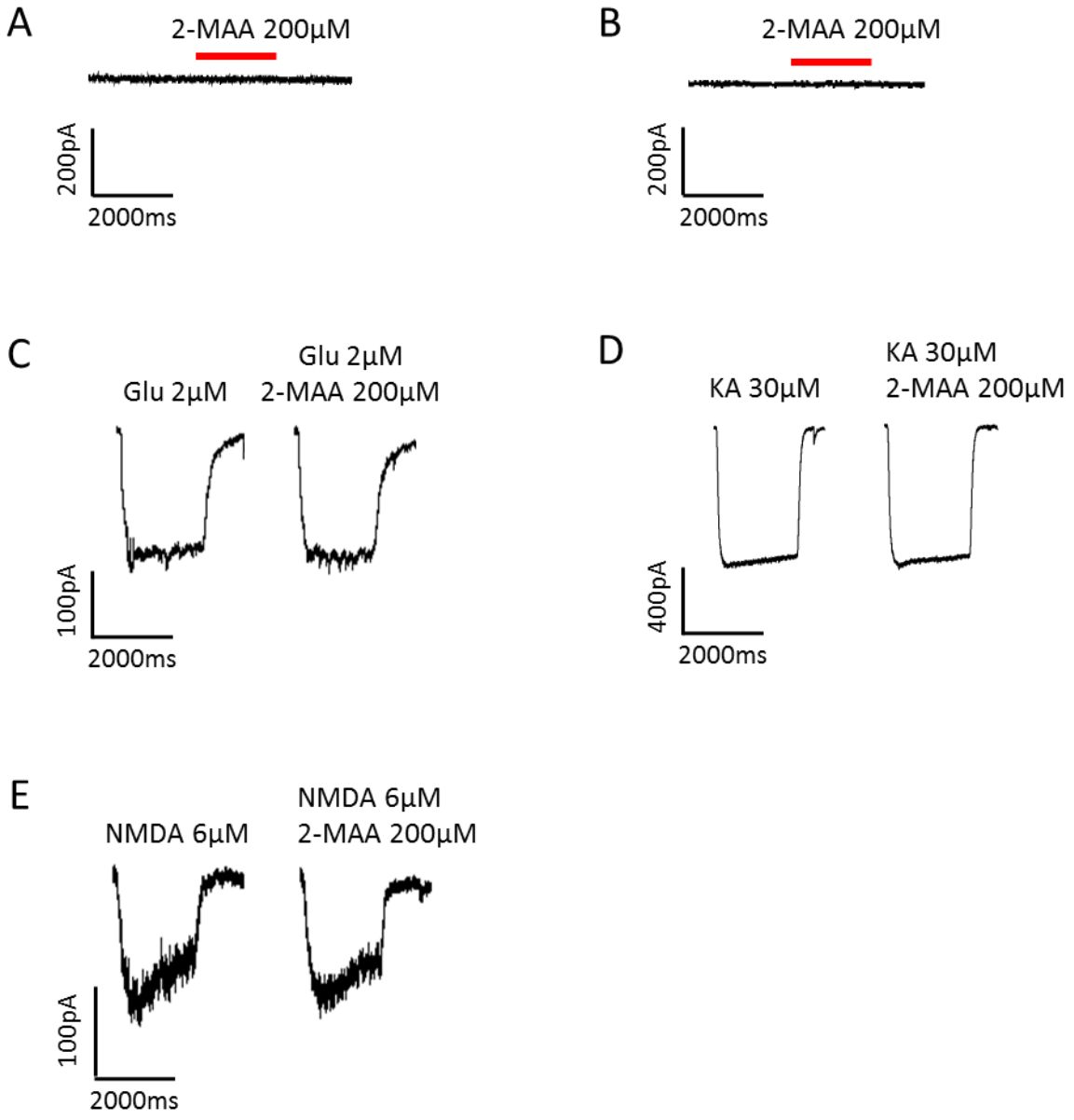
### 3.4 2-MAA does not stimulate neurons or affect glutamate receptors

To test whether 2-MAA from Sigma-Aldrich was same with 2-MAA provided by NCI database, the screening experiments following the three criteria in Section 3.3 were repeated. Indeed, 2-MAA from Sigma-Aldrich did not stimulate cultured neurons (Figure 3A). We also tested whether 2-MAA alone could induce any current in HEK 293 cells transfected with GFP and $GABA_A$ receptor $\alpha1\beta2$ subunits, and no current could be recorded (Figure 3B). Next we confirmed that 2-MAA from Sigma-Aldrich also did not affect glutamate current on cultured neurons (Figure 3C). Nonetheless, because neuronal glutamate current is mainly composed of AMPA and NMDA currents, in order to confirm that 2-MAA has no effect on either kind of glutamate current, separated recordings are required.

To test whether 2-MAA could affect AMPA current, whole cell voltage-clamp recordings were first performed on hippocampal neuron cultures in ECS (containing 0.5 μM TTX) at holding potential of -60mV and kainate (KA, 30 μM in ECS, 2s) was fast perfused to the neuron. After a stable baseline AMPA current was obtained, 2-MAA was added into the system and as a result the neurons were pre-treated with 200 μM 2-MAA. Then kainate (30 μM in ECS, 2s) containing 200 μM 2-MAA was fast perfused to the neuron, and we found the recorded current was same with the baseline AMPA current, indicating that 2-MAA did not affect AMPA current (Figure 3D).

**Figure 3: 2-MAA does not stimulate neurons or affect glutamate receptors.**

A and B: 2-MAA was applied to hippocampal neuron cultures (A) and HEK 293 cells transfected with GFP and $GABA_A$ receptor α1β2 subunits (B), and no current was induced. **C**: 2-MAA was applied with glutamate (Glu) to hippocampal neuron cultures, and it did not affect the general glutamate current. **D**: 2-MAA was applied with kainate (KA) to hippocampal neuron cultures, and it did not affect KA induced AMPA receptor current. **E**: 2-MAA was applied with NMDA to hippocampal neuron cultures, and it did not affect NMDA induced NMDA receptor current.



To test whether 2-MAA could affect NMDA current, whole cell voltage-clamp recordings were first performed on hippocampal neuron cultures in ECS ($Mg^{2+}$ free, containing 0.5 μM TTX, 1 μM glycine) at holding potential of -60mV and NMDA (10 μM in ECS, 2s) was fast perfused to the neuron. After a stable baseline NMDA current was obtained, 2-MAA was added into the system and as a result the neurons were pre-treated with 200 μM 2-MAA. Then NMDA (10 μM in ECS, 2s) containing 200 μM 2-MAA was fast perfused to the neuron, and we found the recorded current was same with the baseline NMDA current, indicating that 2-MAA did not affect NMDA current either (Figure 3E).

The above experiment indicated that the physiological function of 2-MAA is likely restricted to potentiating $GABA_A$ receptors, and it alone does not affect neuronal activity or glutamate receptors.

### 3.5 2-MAA potentiates phasic GABA current

In the above screening experiments, 2-MAA was shown to potentiate GABA current in neuron cultures (Figure 4A). To test whether 2-MAA can mimic glutamate and potentiate $GABA_A$ receptor with only α1 and β2 subunits or with α1, β2, and γ2 subunits, whole cell voltage-clamp recordings were performed on HEK 293 cells transfected with GFP and $GABA_A$ receptor α1β2 subunits or α1β2γ2 subunits in ECS at holding potential of -60mV. GABA (1 μM in ECS, 2s), GABA + 2-MAA (100 μM), or GABA + 2-MAA (200 μM) was applied to the cells and GABA currents were recorded (cells were pre-treated with corresponding concentration of 2-MAA as in previous sections, and in all following experiments cells will be pre-treated with 2-MAA before

recording currents). Results showed that 2-MAA potentiated GABA current in both sets of HEK 293 cells and higher dose triggered bigger potentiation (Figure 4B, 4C), indicating that the potentiation by 2-MAA, like glutamate, is also merely dependent on $GABA_A$ receptor α and β subunits, and suggesting that the potentiation of 2-MAA is dose dependent.

Dose-response curve is important for understanding the kinetics of a compound's effect. We first studied the dose-response curve of GABA on $GABA_A$ receptors with or without co-application of 2-MAA (100 μM or 200 μM). Since dose-response curve is compound/receptor specific, and $GABA_A$ receptors with α1β2γ2 subunits are the most typical $GABA_A$ receptors in the brain, here we chose HEK 293 cells transfected with GFP and $GABA_A$ receptor α1β2 subunits or α1β2γ2 subunits to investigate the kinetics of 2-MAA on potentiating GABA current.

The baseline dose-response curve of GABA was drawn with GABA concentrations (μM) being 0.01, 0.1, 1, 5, 20, 100 and 1000 (Figure 5A, 5B). $GABA_A$ receptors were saturated by 1000 μM GABA so as the maximal GABA current induced by 1000 μM GABA was defined as 100%, and the currents induced by other concentrations of GABA were normalized as percentage of the maximal current. The baseline dose-response curve showed the GABA $EC_{50}$ was 6.3 μM on $GABA_A$ α1β2 receptors and 6.0 μM on $GABA_A$ α1β2γ2 receptors. We then co-applied 2-MAA (100 μM or 200 μM) with GABA to the cells and recorded the dose response curves under 2-MAA potentiation (Figure 5A, 5B). The results showed that, for $GABA_A$ α1β2 receptors the GABA $EC_{50}$ was shifted (from 6.3 μM) to 4.0 μM by 100 μM 2-MAA and to 2.4 μM by 200 μM 2-MAA; for $GABA_A$ α1β2γ2 receptors the GABA $EC_{50}$ was shifted (from 6.0 μM) to 1.7 μM by 100 μM 2-MAA, which were all significant changes ($P < 0.05$, $n = 5$).

**Figure 4: 2-MAA potentiates GABA current in neurons and transfected HEK 293 cells.**

**A**: GABA or GABA + 2-MAA (200 μM) was applied to hippocampal neuron cultures, and GABA current was greatly potentiated by 2-MAA. **B** and **C**: GABA or GABA + 2-MAA (100 or 200 μM) was applied to HEK 293 cells transfected with GFP and $GABA_A$ receptor α1β2 subunits (B) or transfected with GFP and $GABA_A$ receptor α1β2γ2 subunits (C), and GABA currents were greatly potentiated by 2-MAA.

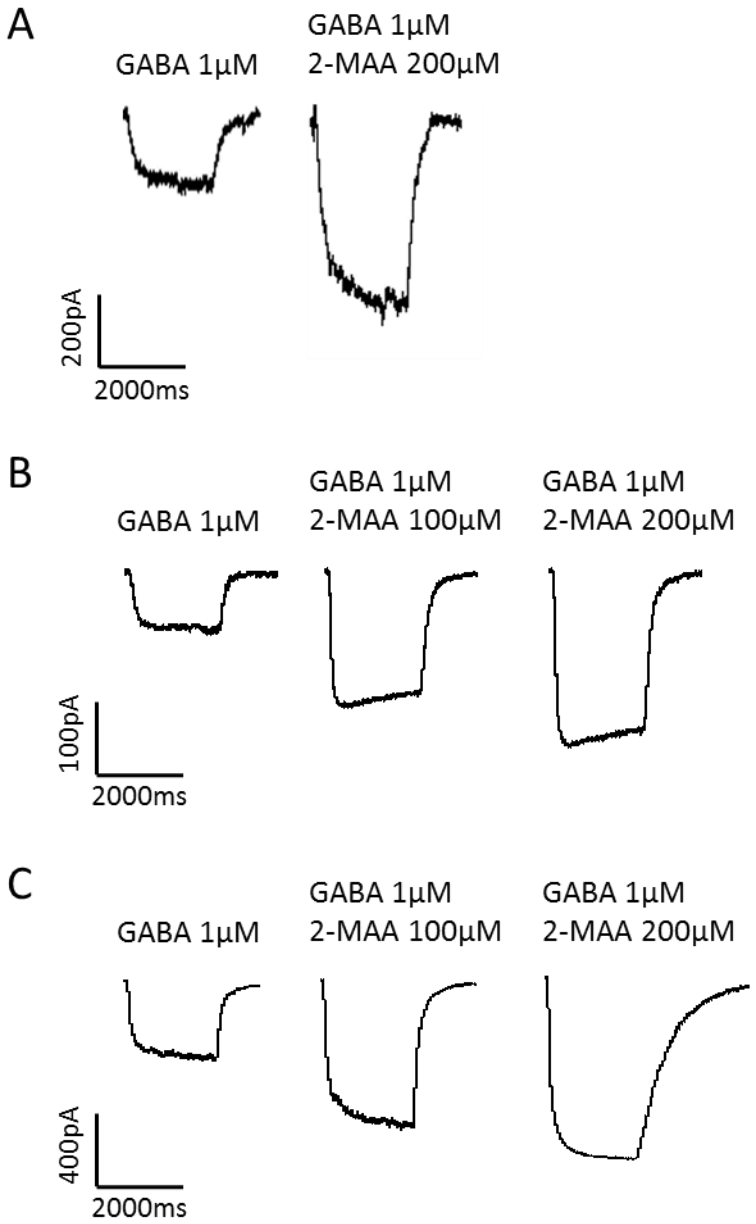
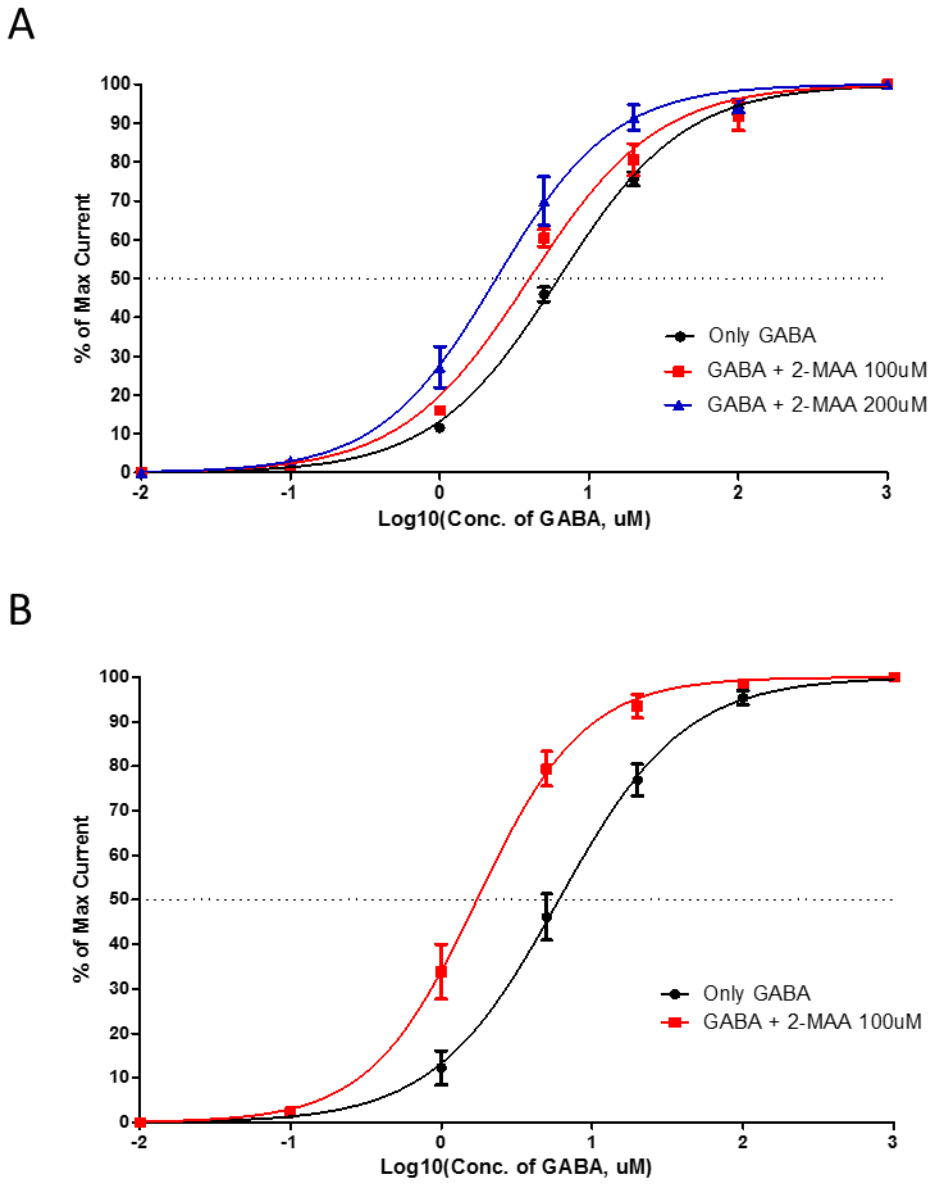


**Figure 5: Dose-response curve of GABA on $GABA_A$ receptors is left-shifted by 2-MAA.**

**A**: Dose-response curve of GABA was recorded on HEK 293 cells transfected with GFP and $GABA_A$ receptor α1β2 subunits. Cells were under treatment of 2-MAA (0, 100 or 200 μM). n=5. **B**: Dose-response curve of GABA was recorded on HEK 293 cells transfected with GFP and $GABA_A$ receptor α1β2γ2 subunits. Cells were under treatment of 2-MAA (0 or 100 μM). n=5.



We then recorded the dose-response curve of 2-MAA to see at what concentration the potentiation would be saturated. HEK 293 cells transfected with GFP and $GABA_A$ receptor $\alpha1\beta2\gamma2$ subunits were used for recording. GABA concentration was 0.5 µM and 2-MAA concentrations were 0.1, 1, 10, 50, 200 and 1000 µM. GABA currents induced by 0.5 µM GABA were used as baseline and the potentiation by 2-MAA was normalized. This dose-response curve showed that the potentiation of GABA current was around 50% with 50 µM 2-MAA, was around 150% with 200 µM 2-MAA and reached the plateau at 180% with 1000 µM 2-MAA (Figure 6).

Next we investigated whether 2-MAA could affect mIPSC, as AP5 could increase the amplitude of mIPSC (unpublished data). Whole cell voltage-clamp recordings were performed on hippocampal neuron cultures in ECS at holding potential of -60mV (20 µM CNQX and 0.5 µM TTX were added into ECS to inhibit mEPSC and action potentials). mIPSC was recorded with or without 100 µM 2-MAA, and the frequency, amplitude and decay time of mIPSC were analyzed and normalized as percentage of control (without 2-MAA). Results showed that with 2-MAA, the frequency of mIPSC was increased significantly by 22% ($P < 0.05$, $n = 10$), while the amplitude and decay time were not changed (Figure 7).

### 3.6 2-MAA does not affect tonic GABA current

Tonic GABA current is regulated by extrasynaptic $GABA_A$ receptors whose compositions are different than synaptic ones. It will be intriguing to know whether 2-MAA could also affect tonic GABA current. To test this, whole cell voltage-clamp recordings were performed on hippocampal neuron cultures in ECS at holding potential of -60mV (20 µM CNQX and 0.5 µM TTX were added into ECS to inhibit mEPSC and action potential). After the baseline was stable,

20 μM Bicuculline ($GABA_A$ receptors competitive antagonist) was added into the ECS. The elevation of the baseline represented the scale of tonic GABA current (Figure 8). We found that with 100 μM 2-MAA, tonic current of the same neuron did not change, indicating that 2-MAA did not affect tonic GABA current.

### 3.7 2-MAA shares the glutamate binding site on $GABA_A$ receptors

Based on all the above results, it is quite likely that 2-MAA shares the same binding site with glutamate on $GABA_A$ receptors. We already knew that residue E181 on β2 subunit is critical for glutamate binding, and the mutation on this residue (E181G) could completely eliminate the potentiation by glutamate while preserve the receptors' normal GABA sensitivity (unpublished data). Therefore, to test whether 2-MAA and glutamate shares the same binding site, whole cell voltage-clamp recordings were performed on HEK 293 cells transfected with GFP and $GABA_A$ receptor α1β2(E181G)γ2 or α1β2(Wild Type)γ2 subunits in ECS at holding potential of -60mV. GABA (1 μM in ECS, 2s) or GABA + 2-MAA (200 μM) was applied to the cells and GABA currents were recorded (Figure 9). We found that for the wild type $GABA_A$ receptors 2-MAA could potentiate GABA current by 165%, as expected; however, for $GABA_A$ receptors containing the mutant β2 (E181G) subunit, the potentiation by 2-MAA dramatically dropped to 32% ($P < 0.0001$, $n = 4$), strongly indicating that this E181 residue on the β2 subunit is also very important for 2-MAA. Considering the similarity in structure of 2-MAA and glutamate, the results strongly suggests that they share the same binding site on $GABA_A$ receptors. The reason that β2 (E181G) mutant could not completely eliminate the potentiation by 2-MAA is probably due to the structure difference between 2-MAA and glutamate so that they do not interact with the same residues located at the binding site in exactly the same manner.

**Figure 6: Dose-response curve of 2-MAA with fixed GABA concentration.**

Dose-response curve of 2-MAA was recorded on HEK 293 cells transfected with GFP and $GABA_A$ receptor α1β2γ2 subunits. GABA concentration was 0.5 μM. 2-MAA concentration range included 0.1, 1, 10, 50, 200 and 1000 μM. n=4.

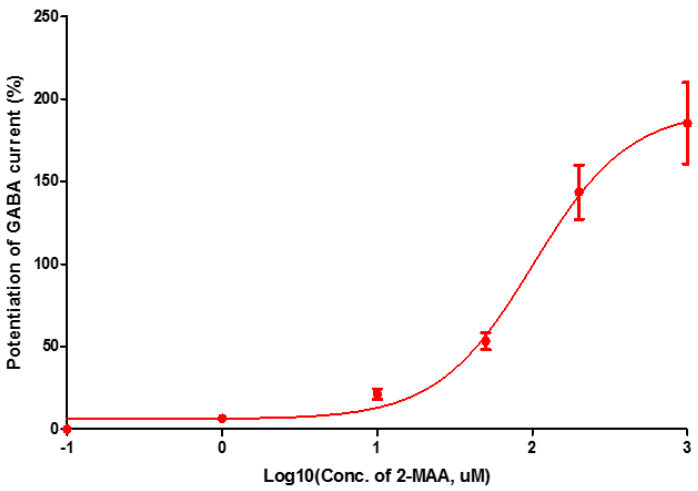



**Figure 7: 2-MAA significantly increases frequency of mIPSC.**

mIPSC was recorded on hippocampal neuron cultures with or without the treatment of 100 μM 2-MAA. 2-MAA significantly increased the frequency of mIPSC by 22% ($P < 0.05$, n=10). The amplitude and decay time of mIPSC were not affected.

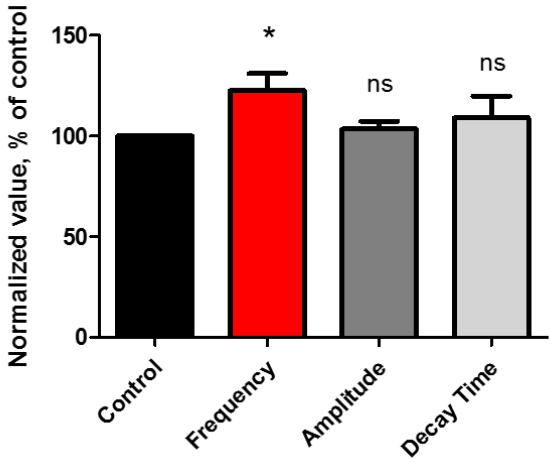


**Figure 8: 2-MAA does not affect tonic GABA current.**

Tonic GABA current was recorded on hippocampal neuron cultures with or without the treatment of 2-MAA. With 20 μM CNQX and 0.5 μM TTX to inhibit mEPSC and action potential, the baseline activity of one neuron was first recorded and Bicuculline (Bicu, 20 μM) was applied to inhibit $GABA_A$ receptors. The elevation of the baseline represented the amplitude of tonic GABA current. Then Bicuculline was washed out, baseline was recovered, and 100 μM 2-MAA was continuously applied to the neuron. Again Bicuculline (20 μM) was applied and the amplitude of tonic GABA current with 2-MAA treatment was recorded. The results indicated that 2-MAA did not affect the amplitude of tonic GABA current.

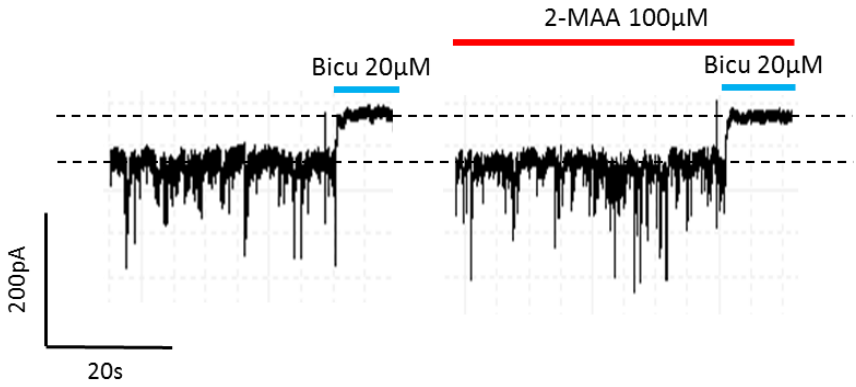
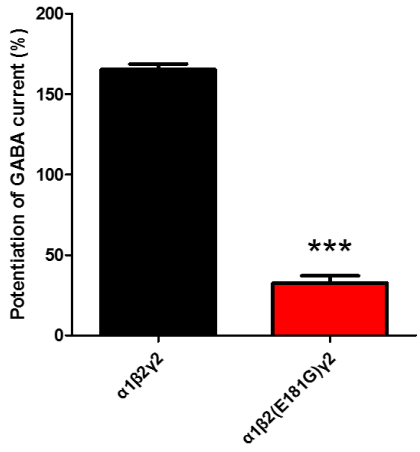



**Figure 9: 2-MAA shares the same GABAA receptor binding site with glutamate.**

Potentiation of GABA current (GABA 1 μM) by 2-MAA (200 μM) was recorded on HEK 293 cells transfected with GFP and $GABA_A$ receptor α1β2γ2 or α1β2 (E181G) γ2 subunits. β2 (E181G) mutation significantly decreased the potentiation by 2-MAA from 165% to 32% ($P < 0.0001$, $n = 4$).



## Chapter 4: Discussion

Our lab previously discovered that glutamate could allosterically bind to and potentiate $GABA_A$ receptors. Here we tried to screen two categories of compounds that (1) can help us investigate the physiological function of this potentiation, or (2) can serve as alternative $GABA_A$ receptor modulators to replace benzodiazepines in clinical treatment.

For category (1), we got 10 compounds that can inhibit glutamate from potentiating GABA current while having no other effect on neurons and $GABA_A$ receptors. However, none of them can completely block the potentiation, thus none of them can be used in *in vivo* experiments. Future direction on this would be to modify the structure of these compounds and to further screen the expected glutamate competitors.

For category (2), we got one compound, 2-MAA, which almost mimicked glutamate to potentiate GABA current and has the potential for further *in vivo* experiments. 2-MAA is highly similar with glutamate in structure, although it does not stimulate or affect glutamate current. This is probably because the methyl group in 2-MAA prevents it from fitting to glutamate binding site in glutamate receptors. 2-MAA alone does not stimulate neuron or $GABA_A$ receptors. When applied with GABA, 2-MAA can potentiate GABA current in both neurons and HEK 293 cells transfected with $GABA_A$ receptors in dose dependent manner. It also shares the same binding site(s) with glutamate on $GABA_A$ receptors.

The results about 2-MAA affecting mIPSC and tonic GABA current is interesting. Another glutamate like molecule AP5 which also potentiates GABA current has been shown to increase the amplitude of tonic GABA current and mIPSC, but does not affect mIPSC frequency (unpublished data). 2-MAA, on the other hand, only increases mIPSC frequency but does not change amplitude of mIPSC and tonic GABA current. This difference may be due to the specific difference in the compound structure and the distribution of $GABA_A$ receptors within and outside synapses (as discussed in Chapter 1). It might be the case that AP5 but not 2-MAA can bind to the extrasynaptic $GABA_A$ receptors. Future experiment on HEK 293 cells transfected with $GABA_A$ receptor α4βxδ, α6βxδ or α5βxγ2 subunits may provide explanation.

Nonetheless, the characteristics 2-MAA showed as a novel $GABA_A$ receptor modulator indeed indicate that in the future, *in vitro* cell models or animal models, of epilepsy for example, could be used to further study 2-MAA's potential for clinical application on treatment of disorders where temporary enhancement of GABA current is required.

## References


Benson, J.A., Löw, K., Keist, R., Mohler, H., and Rudolph, U. (1998). Pharmacology of recombinant gamma-aminobutyric acidA receptors rendered diazepam-insensitive by point-mutated alpha-subunits. FEBS Lett. *431*, 400–404.

Bowery, N.G., Hudson, A.L., and Price, G.W. (1987). GABAA and GABAB receptor site distribution in the rat central nervous system. Neuroscience *20*, 365–383.

Buhr, A., and Sigel, E. (1997). A point mutation in the gamma2 subunit of gamma-aminobutyric acid type A receptors results in altered benzodiazepine binding site specificity. Proc. Natl. Acad. Sci. U. S. A. *94*, 8824–8829.

Buhr, A., Baur, R., and Sigel, E. (1997). Subtle changes in residue 77 of the gamma subunit of alpha1beta2gamma2 GABAA receptors drastically alter the affinity for ligands of the benzodiazepine binding site. J. Biol. Chem. *272*, 11799–11804.

Connolly, C.N., Wooltorton, J.R., Smart, T.G., and Moss, S.J. (1996). Subcellular localization of gamma-aminobutyric acid type A receptors is determined by receptor beta subunits. Proc. Natl. Acad. Sci. U. S. A. *93*, 9899–9904.

Cutting, G.R., Lu, L., O'Hara, B.F., Kasch, L.M., Montrose-Rafizadeh, C., Donovan, D.M., Shimada, S., Antonarakis, S.E., Guggino, W.B., and Uhl, G.R. (1991). Cloning of the gamma-aminobutyric acid (GABA) rho 1 cDNA: a GABA receptor subunit highly expressed in the retina. Proc. Natl. Acad. Sci. U. S. A. *88*, 2673–2677.

Enz, R., and Cutting, G.R. (1999). GABAC receptor rho subunits are heterogeneously expressed in the human CNS and form homo- and heterooligomers with distinct physical properties. Eur. J. Neurosci. *11*, 41–50.

Farrant, M., and Nusser, Z. (2005). Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat. Rev. Neurosci. *6*, 215–229.

French, J.A., and Faught, E. (2009). Rational polytherapy. Epilepsia *50 Suppl 8*, 63–68.

Hironaka, T., Morita, Y., Hagihira, S., Tateno, E., Kita, H., and Tohyama, M. (1990). Localization of GABAA-receptor alpha 1 subunit mRNA-containing neurons in the lower brainstem of the rat. Brain Res. Mol. Brain Res. *7*, 335–345.

Kato, K. (1990). Novel GABAA receptor alpha subunit is expressed only in cerebellar granule cells. J. Mol. Biol. *214*, 619–624.

Khrestchatisky, M., MacLennan, A.J., Chiang, M.Y., Xu, W.T., Jackson, M.B., Brecha, N., Sternini, C., Olsen, R.W., and Tobin, A.J. (1989). A novel alpha subunit in rat brain GABAA receptors. Neuron *3*, 745–753.

Khrestchatisky, M., MacLennan, A.J., Tillakaratne, N.J., Chiang, M.Y., and Tobin, A.J. (1991). Sequence and regional distribution of the mRNA encoding the alpha 2 polypeptide of rat gamma-aminobutyric acidA receptors. J. Neurochem. *56*, 1717–1722.

Kleckner, N.W., and Dingledine, R. (1988). Requirement for glycine in activation of NMDA-receptors expressed in Xenopus oocytes. Science *241*, 835–837.

Kucken, A.M., Wagner, D.A., Ward, P.R., Teissére, J.A., Boileau, A.J., and Czajkowski, C. (2000). Identification of benzodiazepine binding site residues in the gamma2 subunit of the gamma-aminobutyric acid(A) receptor. Mol. Pharmacol. *57*, 932–939.

Levitan, E.S., Blair, L.A., Dionne, V.E., and Barnard, E.A. (1988). Biophysical and pharmacological properties of cloned GABAA receptor subunits expressed in Xenopus oocytes. Neuron *1*, 773–781.

Lolait, S.J., O'Carroll, A.M., Kusano, K., and Mahan, L.C. (1989). Pharmacological characterization and region-specific expression in brain of the beta 2- and beta 3-subunits of the rat GABAA receptor. FEBS Lett. *258*, 17–21.

Lüddens, H., Pritchett, D.B., Köhler, M., Killisch, I., Keinänen, K., Monyer, H., Sprengel, R., and Seeburg, P.H. (1990). Cerebellar GABAA receptor selective for a behavioural alcohol antagonist. Nature *346*, 648–651.

MacLennan, A.J., Brecha, N., Khrestchatisky, M., Sternini, C., Tillakaratne, N.J., Chiang, M.Y., Anderson, K., Lai, M., and Tobin, A.J. (1991). Independent cellular and ontogenetic expression of mRNAs encoding three alpha polypeptides of the rat GABAA receptor. Neuroscience *43*, 369–380.

Malherbe, P., Sigel, E., Baur, R., Persohn, E., Richards, J.G., and Mohler, H. (1990a). Functional characteristics and sites of gene expression of the alpha 1, beta 1, gamma 2-isoform of the rat GABAA receptor. J. Neurosci. Off. J. Soc. Neurosci. *10*, 2330–2337.

Malherbe, P., Sigel, E., Baur, R., Persohn, E., Richards, J.G., and Mohler, H. (1990b). Functional characteristics and sites of gene expression of the alpha 1, beta 1, gamma 2-isoform of the rat GABAA receptor. J. Neurosci. Off. J. Soc. Neurosci. *10*, 2330–2337.

Möhler, H. (2006). GABA(A) receptor diversity and pharmacology. Cell Tissue Res. *326*, 505–516.

Nusser, Z., Roberts, J.D., Baude, A., Richards, J.G., and Somogyi, P. (1995). Relative densities of synaptic and extrasynaptic GABAA receptors on cerebellar granule cells as determined by a quantitative immunogold method. J. Neurosci. Off. J. Soc. Neurosci. *15*, 2948–2960.

Nusser, Z., Sieghart, W., Benke, D., Fritschy, J.M., and Somogyi, P. (1996). Differential synaptic localization of two major gamma-aminobutyric acid type A receptor alpha subunits on hippocampal pyramidal cells. Proc. Natl. Acad. Sci. U. S. A. *93*, 11939–11944.

Nusser, Z., Sieghart, W., and Somogyi, P. (1998). Segregation of different GABAA receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J. Neurosci. Off. J. Soc. Neurosci. *18*, 1693–1703.

Olsen, R.W., and Sieghart, W. (2008). International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol. Rev. *60*, 243–260.

Olsen, R.W., and Sieghart, W. (2009). GABA A receptors: subtypes provide diversity of function and pharmacology. Neuropharmacology *56*, 141–148.

Palacios, J.M., Wamsley, J.K., and Kuhar, M.J. (1981). High affinity GABA receptors-autoradiographic localization. Brain Res. *222*, 285–307.

Patenaude, C., Chapman, C.A., Bertrand, S., Congar, P., and Lacaille, J.-C. (2003). GABAB receptor- and metabotropic glutamate receptor-dependent cooperative long-term potentiation of rat hippocampal GABAA synaptic transmission. J. Physiol. *553*, 155–167.

Payne, J.A., Rivera, C., Voipio, J., and Kaila, K. (2003). Cation-chloride co-transporters in neuronal communication, development and trauma. Trends Neurosci. *26*, 199–206.

Persohn, E., Malherbe, P., and Richards, J.G. (1991). In situ hybridization histochemistry reveals a diversity of GABAA receptor subunit mRNAs in neurons of the rat spinal cord and dorsal root ganglia. Neuroscience *42*, 497–507.

Puyal, J., Grassi, S., Dieni, C., Frondaroli, A., Dem êmes, D., Raymond, J., and Pettorossi, V.E. (2003). Developmental shift from long-term depression to long-term potentiation in the rat medial vestibular nuclei: role of group I metabotropic glutamate receptors. J. Physiol. *553*, 427–443.

Qian, H., and Dowling, J.E. (1993). Novel GABA responses from rod-driven retinal horizontal cells. Nature *361*, 162–164.

Rivera, C., Voipio, J., and Kaila, K. (2005). Two developmental switches in GABAergic signalling: the K+-Cl- cotransporter KCC2 and carbonic anhydrase CAVII. J. Physiol. *562*, 27–36.

Rudolph, U., and Knoflach, F. (2011). Beyond classical benzodiazepines: Novel therapeutic potential of GABAA receptor subtypes. Nat. Rev. Drug Discov. *10*, 685–697.

Seeburg, P.H., Wisden, W., Verdoorn, T.A., Pritchett, D.B., Werner, P., Herb, A., L üddens, H., Sprengel, R., and Sakmann, B. (1990). The GABAA receptor family: molecular and functional diversity. Cold Spring Harb. Symp. Quant. Biol. *55*, 29–40.

Shivers, B.D., Killisch, I., Sprengel, R., Sontheimer, H., K öhler, M., Schofield, P.R., and Seeburg, P.H. (1989). Two novel GABAA receptor subunits exist in distinct neuronal subpopulations. Neuron *3*, 327–337.

Sieghart, W., and Sperk, G. (2002). Subunit composition, distribution and function of GABA(A) receptor subtypes. Curr. Top. Med. Chem. *2*, 795–816.

Sigel, E., Baur, R., Trube, G., Möhler, H., and Malherbe, P. (1990). The effect of subunit composition of rat brain GABAA receptors on channel function. Neuron *5*, 703–711.

Sigel, E., Schaerer, M.T., Buhr, A., and Baur, R. (1998). The benzodiazepine binding pocket of recombinant alpha1beta2gamma2 gamma-aminobutyric acidA receptors: relative orientation of ligands and amino acid side chains. Mol. Pharmacol. *54*, 1097–1105.

Simon, J., Wakimoto, H., Fujita, N., Lalande, M., and Barnard, E.A. (2004). Analysis of the set of GABA(A) receptor genes in the human genome. J. Biol. Chem. *279*, 41422–41435.

Stelzer, A., and Wong, R.K. (1989). GABAA responses in hippocampal neurons are potentiated by glutamate. Nature *337*, 170–173.

Sternbach, L.H. (1979). The benzodiazepine story. J. Med. Chem. *22*, 1–7.

Tabuchi, K., Blundell, J., Etherton, M.R., Hammer, R.E., Liu, X., Powell, C.M., and Südhof, T.C. (2007). A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. Science *318*, 71–76.

Taylor, P.M., Thomas, P., Gorrie, G.H., Connolly, C.N., Smart, T.G., and Moss, S.J. (1999). Identification of amino acid residues within GABA(A) receptor beta subunits that mediate both homomeric and heteromeric receptor expression. J. Neurosci. Off. J. Soc. Neurosci. *19*, 6360–6371.

Wang, T.L., Guggino, W.B., and Cutting, G.R. (1994). A novel gamma-aminobutyric acid receptor subunit (rho 2) cloned from human retina forms bicuculline-insensitive homooligomeric receptors in Xenopus oocytes. J. Neurosci. Off. J. Soc. Neurosci. *14*, 6524–6531.

Wang, T.L., Hackam, A.S., Guggino, W.B., and Cutting, G.R. (1995). A single amino acid in gamma-aminobutyric acid rho 1 receptors affects competitive and noncompetitive components of picrotoxin inhibition. Proc. Natl. Acad. Sci. U. S. A. *92*, 11751–11755.

Wieland, H.A., Lüddens, H., and Seeburg, P.H. (1992). A single histidine in GABAA receptors is essential for benzodiazepine agonist binding. J. Biol. Chem. *267*, 1426–1429.

Windhorst, U. (2007). Muscle proprioceptive feedback and spinal networks. Brain Res. Bull. *73*, 155–202.

Wingrove, P.B., Thompson, S.A., Wafford, K.A., and Whiting, P.J. (1997). Key amino acids in the gamma subunit of the gamma-aminobutyric acidA receptor that determine ligand binding and modulation at the benzodiazepine site. Mol. Pharmacol. *52*, 874–881.

Wisden, W., Herb, A., Wieland, H., Keinänen, K., Lüddens, H., and Seeburg, P.H. (1991). Cloning, pharmacological characteristics and expression pattern of the rat GABAA receptor alpha 4 subunit. FEBS Lett. *289*, 227–230.

Wisden, W., Laurie, D.J., Monyer, H., and Seeburg, P.H. (1992). The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J. Neurosci. Off. J. Soc. Neurosci. *12*, 1040–1062.

Wooltorton, J.R., McDonald, B.J., Moss, S.J., and Smart, T.G. (1997). Identification of a Zn2+ binding site on the murine GABAA receptor complex: dependence on the second transmembrane domain of beta subunits. J. Physiol. *505 ( Pt 3)*, 633–640.

Yee, B.K., Keist, R., von Boehmer, L., Studer, R., Benke, D., Hagenbuch, N., Dong, Y., Malenka, R.C., Fritschy, J.-M., Bluethmann, H., et al. (2005). A schizophrenia-related sensorimotor deficit links alpha 3-containing GABAA receptors to a dopamine hyperfunction. Proc. Natl. Acad. Sci. U. S. A. *102*, 17154–17159.

Ymer, S., Draguhn, A., Wisden, W., Werner, P., Keinänen, K., Schofield, P.R., Sprengel, R., Pritchett, D.B., and Seeburg, P.H. (1990). Structural and functional characterization of the gamma 1 subunit of GABAA/benzodiazepine receptors. EMBO J. *9*, 3261–3267.

Zhang, J.H., Sato, M., Noguchi, K., and Tohyama, M. (1990). The differential expression patterns of the mRNAs encoding beta subunits (beta 1, beta 2 and beta 3) of GABAA receptor in the olfactory bulb and its related areas in the rat brain. Neurosci. Lett. *119*, 257–260.

.