IDENTIFICATION AND CHARACTERIZATION OF A NOVEL ALLOSTERIC GLUTAMATE BINDING SITE ON THE GABA-A RECEPTOR

by

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Abstract

The A type γ-aminobutyric acid receptor ($GABA_AR$) mediates major inhibition to counteract glutamate receptor-mediated excitation in the Central Nervous System (CNS). However, work in this dissertation identified a novel glutamate-binding site at the α+β- interface of the $GABA_AR$. Activation of this glutamate binding site by glutamate and analogues can potentiate both the synaptic $GABA_AR$-mediated phasic responses and the extrasynaptic $GABA_AR$-mediated tonic inhibition. Using systematic mutagenesis analysis, we identified a conserved group of charged amino acid residues including α1K104, α1K155, α1E137 and β2E181, that form this glutamate binding site at the extracellular domain of the $GABA_AR$. Spatial and electrostatic accessibility are both crucial for glutamate binding on this site. Furthermore, through *in-silicon* and electrophysiological screening, we identified that ampicillin, an antibiotic, and BRC640 as novel compounds that can target this newly identified glutamate-binding site, leading to an enhancement of the $GABA_AR$ function. Comparing with traditional benzodiazepine drugs, these two compounds were able to regulate both synaptic and extrasynaptic $GABA_ARs$. In the cerebellum, depolarization of the Purkinje cells induces both dendritic glutamate release and a rebound potentiation of GABA responsiveness. Application of ampicillin occluded the early phase of rebound potentiation, presumably by saturating the glutamate-binding site on the $GABA_ARs$ and preventing the further potentiation induced by dendritically released glutamate. Taken together, our present study demonstrated a novel glutamate-binding site on the $GABA_AR$ that might lead to future development of novel $GABA_AR$-based therapeutics. This type of excitation/inhibition crosstalk may play an essential role in Purkinje cell inhibitory plasticity.

Preface

Chapter 1: All figures in Chapter 1 were reprinted with permission. Please refer to legend for details.

Chapter 3-6: A version of these materials will be assembled into a manuscript for submission. Dr. Dongchuan Wu performed and analyzed the work in section 3.2.3. Mr. Peter Axerio generated the $GABA_AR$ homology model and performed the *in-silicon* screening work in Chapter 4 and 5. I performed and analyzed all the other data presented in Chapter 3-6. Dr. Yu Tian Wang supervised my work and provided thoughtful advice for experimental design. The work presented in this dissertation will likely result in a publication.

Experiments were performed in compliance with regulations at the University of British Columbia. Animal experiments were performed in compliance with institutional requirements at the University of British Columbia and in accordance with guidelines set forth by the Canadian Council on Animal Care. Animals were obtained and handled according to University of British Columbia ethics protocols A13-0139 (Slice and Primary Culture Protocol).

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List of Abbreviations

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AMPAR	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
NMDA	N-methyl-D-aspartic acid
NMDAR	N-methyl-D-aspartic acid receptor
GABA	γ -Aminobutyric acid
$GABA_AR$	A type γ -Aminobutyric acid receptor
GlyR	Glycine receptor
CNS	Central nerves system
E/I	Excitation/inhibition
KA	Kainate acid
KAR	Kainate receptor
APV	(*2R*)-amino-5-phosphonovaleric acid
TTX	Tetrodotoxin
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
MK801	Dizocilpine
mIPSC	Miniature inhibitory postsynaptic currents
ECS	Extracellular solution
Glu	Glutamate
Amp	Ampicillin
PNG	Penicillin-G
BBB	Blood-brain-barrier

PC	Purkinje cell
LGIC	Ligand-gated ion channel
nAChR	Nicotinic acetylcholine receptor
GluCl	Glutamate-gated chloride channel
ELIC	*Erwinia chrysanthem* pentameric ligand-gated ion channel
KCC2	K-Cl co-transporter isoform 2
NKCC1	Na-K-Cl cotransporter isoform 1
nRT	Thalamic reticular nucleus
VB	Ventrobasal nucleus
DBI	Diazepam-binding inhibitor
FLZ	Flumazenil
BZD	Benzodiazapine
PTZ	Pentylenetetrazole
ABHD6	Serine hydrolase α/β-hydrolase domain 6
CB1	Cannabinoid receptor 1
2-AG	2-arachidonoylglycerol
3β-OH steroids	3β-hydroxysteroids
PS	Pregnenolone sulphate
3α,5α-THDOC	3α,5α-tetrahydrodeoxycorticosterone
3α,5β-THPROG	5β-pregnan-3α-ol-20-one
3α,5α-THPROG	5α-pregnan-3α-ol-20-one
mGluRs	Metabolic glutamate receptors
iLTP	Long-term potentiation of inhibition

PAG	Phosphate activated glutaminase
HERV	Human endogenous retroviruses
MAS	Malate-aspartate shuttle
DON	6-diazo-5-oxo-l-norleucine
VGLUT	Vesicle glutamate transporters
VRAC	Volume-sensitive organic osmolyte–anion channel
OGD	Oxygen and glucose deprivation
VGAT	Vesicle GABA transporter
PG	Periglomerular
FSN	Fast-spiking non-accommodating interneurons
GS	Glutamine synthetase
EAAT	Excitatory amino acid transporters
ALS	Amyotrophic lateral sclerosis
ATD	Amino terminal domain
VFD	Venus flytrap domain
DSI	Depolarization-induced suppression of inhibition
RP	Rebound potentiation
DPI	Depolarization-induced potentiation of inhibition
DSE	Depolarization-induced suppression of excitation
DISC	Depolarization-induced slow current
PP-1	Protein phosphatase 1
$GABA_BR$	Metabotropic GABA receptor
GABARAP	$GABA_AR$ associated protein

VOR	Vestibulo-ocular reflex
NEM	N-ethylmaleimide
GDP-β-S	Guanosine 5-[β-thio]diphosphate
BoNT-B	Botulinum neurotoxin light-chain B
Plic-1	Protein that links integrin-associated protein with the cytoskeleton-1
GODZ	Golgi-specific DHHC zinc finger domain protein
NSF	N-ethylmaleimide-sensitive factor
PRIP	Phospholipase C-related catalytically inactive proteins
CAML	Calcium-modulating cyclophilin ligand
HAP1	Huntingtin associated protein-1
PKA	cAMP-dependent protein kinase
PKC	Calcium/phospholipid-dependent protein kinase
CaMKII	Calcium/calmodulin-dependent protein kinase II

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Dedicated to my beloved daughter Evelyn Yachen Zhang and son Elwyn Wengian Zhang.

Chapter 1: Introduction

1.1 Overview of the $GABA_AR$

1.1.1 $GABA_AR$ composition and structure

The A type γ-aminobutyric acid receptor ($GABA_AR$) is the principal inhibitory receptor in the central nervous system (CNS) (Macdonald and Olsen, 1994). It belongs to the Cys-loop superfamily of ligand-gated ion channels (LGICs), which includes nicotinic acetylcholine receptors (nAChRs), $GABA_ARs$, Glycine Receptors and 5-HT_3 receptors (Connolly and Wafford, 2004; Sine and Engel, 2006). Mammalian $GABA_ARs$ are heteropentameric chloride channels constructed from a possible 19 subunits (α1-6, β1-3, γ1-3, δ, ε, π, ρ1-3 and θ) (Enna and Möhler, 2007; Nayeem et al., 1994). Despite the extensive heterogeneity of the $GABA_AR$ subunits, most endogenous $GABA_ARs$ in the brain consist of 2 α subunits, 2 β subunits and 1 γ subunit. The γ subunit can be replaced by either δ, ε, θ or π (Figure 1.1)(Olsen and Sieghart, 2009; Sieghart and Sperk, 2002). These types of α/β-containing receptors are the most abundantly expressed $GABA_ARs$ in brain (consisting more than 95% of all $GABA_ARs$) and among which, α1β2γ2 is the most common isoform consisting 60% of all $GABA_ARs$ (Mohler, 2006). Unlike other subunits, ρ subunits form homomeric ρ-containing $GABA_ARs$, which are previously known as $GABA_CRs$. The ρ-containing $GABA_ARs$ has a much higher sensitivity to GABA and plays a unique role in the signal processing in the retina (Lukasiewicz et al., 2004; Zhang et al., 2001). In a heterologous cell line, such as HEK 293 cells, co-expression of α and β subunits is the minimum requirement for functional receptor expression (Malherbe et al., 1990; Sigel et al., 1990), while the full pharmacological profile of $GABA_ARs$ requires α, β and γ subunits (Sigel et al., 1990). Interestingly, β3 subunits can form homopentameric chloride channels, which are GABA insensitive and spontaneously open, when they are expressed in heterologous cell lines

(Taylor et al., 1999; Wooltorton et al., 1997b). Many studies used this β3 homopentamer as a model to study the structure of heteromeric $GABA_A$Rs (Miller and Aricescu, 2014; Yip et al., 2013).

Most of our knowledge about $GABA_A$R structure come from comparative models based on the structures of nicotinic acetylcholine receptors (nAChRs), acetylcholine-binding protein and some bacterial homologues, such as *Caenorhabditis elegans* glutamate-gated chloride channel (GluCl) and pentameric ligand-gated ion channel from *Erwinia chrysanthem* (ELIC) (Bocquet et al., 2007; Brejc et al., 2001; Ernst et al., 2005; Hibbs and Gouaux, 2011; Hilf and Dutzler, 2008; Karlin and Akabas, 1995). Recently, the first crystal structure of human $GABA_A$R was published (Miller and Aricescu, 2014), which largely enhanced our understanding of the architecture and functional determinants of $GABA_A$Rs. Each of the $GABA_A$R subunits consists of a large extracellular domain (200–250 amino acids), four hydrophobic transmembrane domains (TM1–4) (range between 20-25 amino acids) and an intracellular loop between TM3 and TM4 (85-255 amino acids) (Figure 1.1)(Ernst et al., 2005; Miller and Aricescu, 2014; Sigel and Steinmann, 2012). The transmembrane domain TM2 is believed to line the pore of the channel (Olsen and Tobin, 1990); and protein interactions and post-translational modifications occur in the TM3-TM4 intracellular loop (Brandon et al., 2000; Nani et al., 2013; Saliba et al., 2012). The large extracellular domain comprises an amino-terminal α-helix (α1) followed by ten β sheets with a second α-helix (α2) between β sheets 3 and 4 (Miller and Aricescu, 2014). The signature Cys-loop with a disulfide bridge is located betweenβ sheet 6 and 7 (Ernst et al., 2005). The extracellular domains form a vestibule containing a positively charged ring, which may be responsible for the receptor selectivity for chloride anion (Miller and Aricescu, 2014).

The extracellular domain contains binding sites for the endogenous ligand GABA, as well as various modulators, such as benzodiazepines (BZDs) (Enna and Möhler, 2007; Sigel and Buhr, 1997). For the major subunits isoforms αβγ (consisting 90% of native $GABA_AR$s), extracellular domain of each subunit of the receptor contributes towards a pseudo-symmetrical ring that provides two binding sites for GABA at the α-/β+ interfaces (Ernst et al., 2003; Kash et al., 2004; Lummis, 2009). Similar to other Cys-loop receptors, the binding pocket of $GABA_AR$ is constructed from six loops in the extracellular domain, namely loops A–C on the β subunit and loops D-F on the α subunit (Lummis, 2009). Critical residues for GABA binding have been identified by site-directed mutagenesis, photo-affinity labelling studies, and substituted cysteine accessibility modification (SCAM). These critical residues include aromatic residues (α1F64, β2Y62, β2Y97 and β2Y205), hydroxylated residues (α1S68, β2T160, β2T202, β2S204 and β2S209), and charged residues (α1R120, α1D183, α1R66 and β2R207) (Amin and Weiss, 1993; Boileau et al., 2002; Newell and Czajkowski, 2003; Wagner et al., 2004; Westh-Hansen et al., 1997; 1999). Using similar techniques, the critical residues for GABA binding in ρ-containing $GABA_AR$ have been identified as Y102, R104, Y106, F138 , V140, R158, Y198, F240, T244 and Y247 (Amin and Weiss, 1994; Harrison and Lummis, 2006; Lummis et al., 2005; Zhang et al., 2008). The aromatic residues in the binding sites create an aromatic box, which occludes the water from the binding pocket and form a cation-π interaction with GABA (Lummis, 2009). This is a common feature of all agonist-binding site on the Cys-loop family receptors (Beene et al., 2002; Mecozzi et al., 1996; Mu et al., 2003; Zhong et al., 1998).

1.1.2 $GABA_AR$ distribution

1.1.2.1 Subcellular localization

The localization of $GABA_AR$ is largely dependent on its subunits composition. At the subcellular level, $GABA_ARs$ composed of α (1-3) subunits together with β and γ subunits are primarily localized at the postsynaptic sites (Smart and Paoletti, 2012); while α5βγ and α(4 or 6)βδ receptors are located largely at the extrasynaptic sites (Figure 1.1) (Belelli et al., 2009; Brickley and Mody, 2012; Mody and Pearce, 2004). Clustering of $GABA_ARs$ at synapses depends on their interactions with $GABA_AR$-associated proteins. One important cluster-regulating protein is gephyrin, which is enriched at the inhibitory postsynaptic sites containing α(1-3)β(2,3)γ2 $GABA_ARs$ (Fritschy and Brünig, 2003). The interactions between gephyrin and $GABA_AR$ α (1-3) or γ2 subunits have been indicated (Alldred et al., 2005; Christie et al., 2006; Mukherjee et al., 2011; Tretter et al., 2008; 2011). The gephyrin subdomain III and IV and the $GABA_AR$ α3 subunits residues 368-376 at its intracellular loop have been identified as the key regions for the gephyrin-dependent recruitment of $GABA_ARs$ to the postsynaptic sites (Maric et al., 2014). However, the molecular mechanism of how gephyrin mediates the subunit-specific localization of $GABA_ARs$ is still unknown.

The anchoring of $GABA_ARs$ at the extrasynaptic site is mediated by the actin-binding protein radixin, which is from the ezrin/radixin/moesin (ERM) family (Bretscher et al., 2002; Fehon et al., 2010; Loebrich et al., 2006). Radixin directly binds to α5 containing $GABA_ARs$ and links them to the actin cytoskeleton. The activation of radixin requires the phosphatidylinositol-4,5-bisphosphate (PIP_2)-dependent phosphorylation of T564 residue at the carboxyl terminus (Loebrich et al., 2006), which can be dephosphorylated through a RhoA GTP- and Rho-kinase

(ROCK) mediated pathway in an activity-dependent manner (Hausrat et al., 2015; Matsui et al., 1998). Knocking out radixin in mice lowers the level of extrasynaptic localized α5-containing $GABA_AR$s and consequently impairs the hippocampal-dependent short-term memory and reversal learning of the mice (Hausrat et al., 2015; Loebrich et al., 2006).

1.1.2.2 Cellular localization

$GABA_AR$ is the major inhibitory receptor in brain. The expression pattern of $GABA_AR$s in brain depends on their subunit composition: some $GABA_AR$ subunits have a broader expression while others may be restrictedly expressed in certain cell types. *in situ* hybridization and immunohistochemistry approaches have been used to reveal the mRNA and protein expression patterns of $GABA_AR$s in brain (Fritschy and Mohler, 1995; Khrestchatisky et al., 1991; Laurie et al., 1992a; 1992b; Lolait et al., 1989; MacLennan et al., 1991; Pirker et al., 2000; Wisden et al., 1991; 1992; Zhang et al., 1990). In adult rat brain, the most abundantly expressed subunits include α1, β2, γ2 and they are detected in almost every brain region. Interestedly, α1 is the only α subunit found in cerebellar Purkinje cells (Laurie et al., 1992b). Another synaptic α subunit, α2 subunit, is also widely expressed in CNS except for the thalamus, midbrain and cerebellum regions. The expression is more restricted for α3 subunit, which is enriched in the neocortex and hippocampal CA3 and claustrum of basal nuclei. The α4 subunit is the most widespread extrasynaptic $GABA_AR$ subunit in both neocortex and hippocampus. The α5 subunit is found predominantly in the hippocampus but only in layer V/VI of neocortex. The α6 is the most restrictedly expressed subunit that was only detected in cerebellar granule cells.

The β3 subunit has the similar expression pattern as the β2 subunit in most brain regions except for a lower expression level in the thalamus and midbrain. However, it plays a more predominant role than β2 in the hippocampus and hypothalamus. The β1 subunit is concentrated mostly in the hippocampus and amygdala. The γ1 subunit is detected in the hippocampus, amygdala, septum and hypothalamus, whereas the γ3 subunit is found in the neocortex and basal nuclei. The ε and θ subunits are restrictedly to the locus coeruleus (LC) and hypothalamus (Sinkkonen et al., 2000) and the δ subunit is mostly expressed in the granule cells in the cerebellum (Wisden et al., 1992). The ρ1 subunits are restrictedly expressed in the retina, while ρ2 and ρ3 subunits are present in both retina and other brain regions (Alakuijala et al., 2005; Enz and Cutting, 1999; Mejía et al., 2008).

In the spinal cord, only α(1-3), β3 and γ2 subunits are significantly expressed. α1, α3, β3 and γ2 subunits are widespread while the α2 subunit is restricted to motor neurons and adjacent cells (Persohn et al., 1991; Wisden et al., 1991). $GABA_AR$ α(1-3), β(1-3) and γ2 subunits are also present in the peripheral nervous system, such as in Sciatic nerves and Schwann cells (Magnaghi et al., 2006). Unlike other $GABA_AR$ subunits, the π subunit is not detectable in the brain, but it is present in multiple non-neuronal tissues, including lung, thymus, prostate and particularly abundant in the uterus and mammary gland (Hedblom and Kirkness, 1997; Zafrakas et al., 2006). During development in most brain region, there is an expression switch from the α(2 or 3) subunit that is abundant in embryo to the α1 subunit that is dominant in the postnatal age (Fritschy et al., 1994; Laurie et al., 1992b). Notably, certain neuronal cell types do not exhibit this developmental switch. For example, Purkinje cells express only α1, β2/3, γ2 mRNAs during development (Laurie et al., 1992b).

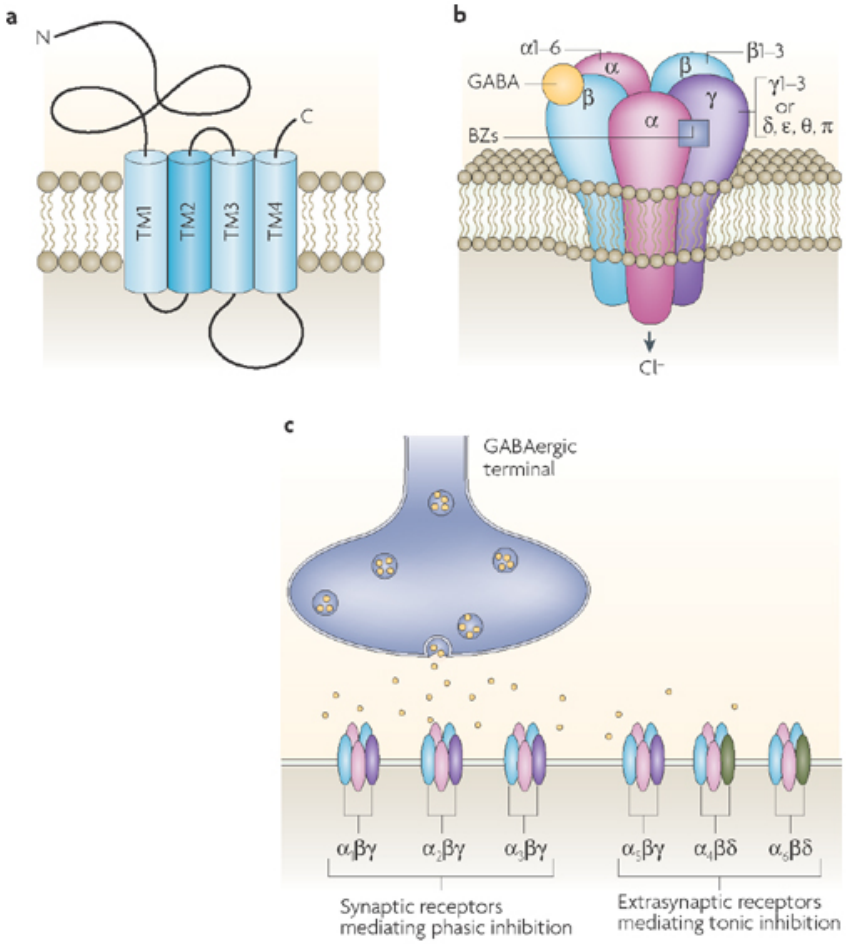

Figure 1.1 $GABA_AR$ structure and subcellular distribution

a) Subunit topology: each subunit of $GABA_AR$ contains a large extracellular N terminal, four transmembrane domains (TM1-4) and a TM3-4 intracellular loop responsible for protein interactions and post-translational modifications. **b)** Structure of pentameric $GABA_AR$: most endogenous $GABA_ARs$ in the brain consist of 2 α subunits, 2 β subunits and 1 γ subunit. The γ subunit can be replaced by the δ, ε, θ or π subunit. The GABA-binding site is located at the α-/β+ interface and the benzodiazepine-binding site is located at the α+/γ- interface. **c)** Subcellular localization of the $GABA_AR$: $GABA_ARs$ composed of α (1-3) subunits with β and γ subunits are primarily localized at the postsynaptic sites, while α5βγ and α(4 or 6)βδ receptors are located largely at the extrasynaptic sites. Reprinted with permission from Jacob, T. C. et al. $GABA_A$ receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat Rev Neurosci.* 9, 331-343 (2008).

1.1.3 $GABA_AR$ trafficking

After translation, $GABA_ARs$ quickly oligomerize in the endoplasmic reticulum (within 5 minutes) (Gorrie et al., 1997). Only the receptors with proper subunit combinations could be transported to the plasma membrane (for proper subunit combinations, see 1.1.1) (Kittler et al., 2002). The N-terminal of the $GABA_AR$ determines the assembly (Gorrie et al., 1997; Jacob et al., 2008; Kittler et al., 2002). Notably, only a small portion of the translated $GABA_ARs$ can oligomerize and reach conformational maturity properly, while the rest are ubiquitinated and degraded via ubiquitin-proteasome system (Bedford et al., 2001; Gorrie et al., 1997). Neuronal activity regulates the $GABA_AR$ ubiquitination level. For example, chronic inhibiting neuronal activity by TTX significantly increases $GABA_AR$ ubiquitination and decrease receptor membrane expression (Saliba et al., 2007). Plic-1 (protein that links integrin-associated protein with the cytoskeleton-1) binds to the α and β subunits of the $GABA_AR$ and stabilizes the ubiquitinated $GABA_ARs$ (Bedford et al., 2001).

After maturation, the $GABA_AR$ is transported to Golgi apparatus and then to plasma membrane. In Golgi apparatus, the γ subunit is palmitoylated by Golgi-specific DHHC zinc finger domain protein (GODZ) (Keller et al., 2004). This process facilitates $GABA_AR$ membrane clustering (Rathenberg et al., 2004). Disrupting it by RNAi knocking down GODZ results in a reduction of $GABA_AR$ mIPSC amplitude (Fang et al., 2006). It is clear that many intracellular proteins facilitate $GABA_AR$ trafficking. For example, Brefeldin A-inhibited GDP/ GTP exchange factor 2 (BIG2), a protein known for its function in membrane budding from Golgi apparatus, interacts with $GABA_AR$ β subunits and facilitates the receptors exiting from the Golgi (Charych et al., 2004). GABA receptor-associated protein (GABARAP) binds to the γ subunits and N-

ethylmaleimide-sensitive factor (NSF) binds to the β subunits (Goto et al., 2005; Wang et al., 1999). Together, they facilitate the transportation of $GABA_A$Rs from Golgi apparatus to the plasma membrane (Chen et al., 2000a; 2007; 2005b; Leil et al., 2004). Phospholipase C-related catalytically inactive proteins (PRIP) promote receptor trafficking either directly by binds to $GABA_A$R β subunits or indirectly by interacting with GABARAP (Kanematsu et al., 2002; Uji et al., 2002). However, despite the above findings, the mechanism of the whole $GABA_A$R trafficking process is still under investigation.

The $GABA_A$Rs are dynamically endocytosed and recycled once been inserted into the plasma membrane. The endocytosis of the $GABA_A$R is mediated by dynamin-clathrin dependent pathway (Comenencia-Ortiz et al., 2014; Jacob et al., 2008). A ten-amino acid motif has been identified on the β subunits critical for clathrin adaptor protein AP2 binding to the $GABA_A$R (Kittler et al., 2005; 2008). Phosphorylation of a serine residue within this motif by protein kinases (PKA, PKB, PKC or CaMKII) inhibits the AP2 binding and therefore stabilizes the membrane $GABA_A$Rs (Brandon et al., 2000; 2002; McDonald and Moss, 1994; McDonald et al., 1998). In contrast, dephosphorylating the serine residues by protein phosphatases PP1α and PP2A allows the AP2 binding and thus triggers the $GABA_A$R internalization (Kittler et al., 2005). PRIP-1, beside its function in trafficking, can inhibit PP1α and prevent $GABA_A$R endocytosis (Terunuma et al., 2004). The similar AP2 binding motifs have also been identified on γ and δ subunits (Gonzalez et al., 2012; Kittler et al., 2005; Smith et al., 2008).

Once endocytosed, $GABA_A$R can either go into lysosomal degradation or be recycled back to plasma membrane (Arancibia-Carcamo et al., 2009; Jacob et al., 2008). Huntingtin associated

protein-1 (HAP1) plays a critical role in determining the fate of internalized $GABA_A$Rs (Kittler et al., 2004; Twelvetrees et al., 2010). It binds to the intracellular domain of the β subunit and reduces $GABA_A$R degradation (Kittler et al., 2004). Overexpression of HAP1 facilitates the receptor recycling and consequently increases the abundance of surface $GABA_A$Rs (Kittler et al., 2004). Similarly as HAP1, calcium-modulating cyclophilin ligand (CAML) interacts with γ subunits of the $GABA_A$Rs and promotes receptor recycling (Yuan et al., 2008). Nevertheless, the details of post-endocytosis receptor sorting are still unknown.

1.1.4 $GABA_A$R channel properties and modulation

1.1.4.1 $GABA_A$R channel properties

$GABA_A$R is activated by the endogenous agonist GABA. The subunit composition of $GABA_A$R largely influences its sensitivity to GABA. δ containing $GABA_A$Rs often have higher GABA sensitivity (represented by lower GABA EC_{50}) (Brown et al., 2002; Feng and Macdonald, 2004), which is consistent with their extrasynaptic localization. For $GABA_A$Rs containing α, β and γ subunits, the GABA sensitivity is strongly affected by the subtype of α subunits. With the same β and γ subunits, $GABA_A$R comprising of the α3 subunit shows the highest GABA EC_{50} while that containing the α6 subunit shows the lowest GABA EC_{50} (Böhme et al., 2004; Fisher and Macdonald, 1997; Knoflach et al., 1996; Minier and Sigel, 2004). The rank order of the GABA EC_{50} is α6<α1<α2<α4<α5<<α3 (Böhme et al., 2004). Overall, the receptor compositions with the lowest EC_{50} are α6β3δ and α4β3δ; whereas α1β3γ2 and α2β3γ2 have the highest EC_{50}.

$GABA_A$R is permeable to chloride ions and other small inorganic anions (Macdonald and Twyman, 1991). By testing the permeability of a series of anions with different sizes, previous

study has shown that anions with a diameter of about 4 Å have the highest permeability. It was also found that the pore diameter of activated $GABA_AR$ was minimally 6 Å in hippocampal neurons(Fatima-Shad and Barry, 1993). $GABA_ARs$ with different subunit compositions exhibit distinct single-channel conductance, although the variation is modest comparing with that of GABA EC_{50}. When expressed in heterologous cell line, the incorporation of either γ or δ subunit into a αβ containing $GABA_AR$ increases the receptor's single-channel conductance from 11~15 pS to 25-32 pS. However, changing the α or β subunits subtypes has little effect on the single-channel conductance (Angelotti and Macdonald, 1993; Brickley et al., 1999; Fisher and Macdonald, 1997; Verdoorn et al., 1990). The native $GABA_ARs$ in neurons show 3 discrete single-channel conductance levels designated as high (25–30 pS), medium (17–19 pS) and low (12–13 pS) (Bormann et al., 1987; Macdonald et al., 1989; Mortensen and Smart, 2006). The existence of the low conductance level, although only accounting for less than 20% of all single-channel events in hippocampal neurons, indicates that the $GABA_ARs$ with αβ composition may be present in the endogenous system (Mortensen and Smart, 2006).

Receptor composition also affects the activation, deactivation and channel open time of $GABA_ARs$. In receptors comprising of αβγ subunits, the activation rate of GABA-induced currents follows the order of α3<α1<α2 (Gingrich et al., 1995; Lavoie et al., 1997; McClellan and Twyman, 1999). Incorporation of the γ subunits into αβ containing $GABA_ARs$ or replacing the δ subunit with the γ subunit produces a 3-fold decrease in the activation time but 2-fold increase in the receptor deactivation time (Boileau et al., 2003; Haas and Macdonald, 1999). α1 containing $GABA_ARs$ exhibit faster deactivation rate than that of the receptors containing other types of α subunits (Bianchi et al., 2002; McClellan and Twyman, 1999). Replacing the γ subunit

with the δ subunit induces a 5-fold decrease in the mean channel open time (Fisher and Macdonald, 1997), which is consistent with the low receptor efficacy of the extrasynaptic $GABA_AR$s.

The desensitization of $GABA_AR$ is important for shaping the inhibitory transmission (Bianchi and Macdonald, 2001; Haas and Macdonald, 1999; Jones and Westbrook, 1995; 1996). Recent study on the crystal structure of $GABA_AR$ reveals that, unlike the regular closed state with a closed gate at the extracellular portion of the pore, the desensitized state of $GABA_AR$ is featured by a closed gate at the bottom of the channel pore (β3A248 position) (Miller and Aricescu, 2014). Subsequent research has specified the region to be the intracellular end of TM3 and the TM1–TM2 linker of $GABA_AR$s (Gielen et al., 2015). $GABA_AR$s with the δ subunit exhibit much slower and less extensive desensitization than that of γ containing receptors (Bianchi and Macdonald, 2002; Saxena and Macdonald, 1996). Similarly as before, the type of α subunits also affects the desensitization of $GABA_AR$s. Replacing the α1 subunit in αβγ containing $GABA_AR$s by α5 negatively impacts the receptor desensitization (Tia et al., 1996). In contrast, substitution of the α1 subunit with the α6 subunit in αβδ $GABA_AR$s increases the receptor desensitization rate (Bianchi et al., 2002).

1.1.4.2 Modulation of $GABA_AR$ channel property

The intracellular domain of the $GABA_AR$ contains several phosphorylation sites that enable $GABA_AR$ to be modulated by cytosolic factors and intracellular signaling pathways. When tested in HEK293 cells, cAMP-dependent protein kinase (PKA) phosphorylates the β1 S409 and β3 S408/409 (McDonald et al., 1998). Phosphorylation of the β1 subunit by PKA reduces GABA-

induced current, while phosphorylation of the two sites on β3 subunit potentiates the GABA current (McDonald et al., 1998). This phenomenon may explain why $GABA_A$Rs from different brain regions respond differently to PKA (Kano and Konnerth, 1992; Kano et al., 1992; Nusser et al., 1997; Poisbeau et al., 1999; Porter et al., 1990; Robello et al., 1993). Single channel recording in spinal cord neurons reveals that PKA phosphorylation compromises the $GABA_A$R-mediated current in these neurons by decreasing the channel opening frequency but not the mean opening time or channel conductance (Porter et al., 1990).

The phosphorylation sites of the calcium/phospholipid-dependent protein kinase (PKC) are β1 S409, β2 S410, β3 S408/409 and γ2 S327/343 (Krishek et al., 1994; Moss and Smart, 1996). In HEK293 cells, PKC phosphorylation depresses the amplitudes of GABA-induced currents but not the time constants for current decay (Krishek et al., 1994). However, PKC's effect on native $GABA_A$R is still unclear. For example, in cultured cortical neurons, activation of PKC compromises the $GABA_A$R function (Brandon et al., 2000). On the contrary, PKC potentiates the mIPSC amplitude recorded in dentate gyrus granule cells (Poisbeau et al., 1999).

Calcium/calmodulin-dependent protein kinase II (CaMKII) is also an important cytosolic factor that modulates $GABA_A$R through phosphorylation. CaMKII activation-induced potentiation of GABA-evoked whole-cell current has been observed in various types of neurons including spinal dorsal horn neurons, CA1 hippocampal pyramidal cells and cerebellar Purkinje cells (Kano et al., 1996; Wang et al., 1995a). Biochemical studies reveal several phosphorylation sites on $GABA_A$R β and γ subunits (β1 S384/409, β2 S410, β3 S383/409 and γ2 S343/348/350) (Houston et al., 2007; McDonald and Moss, 1994). However, when overexpressed in NG108-15

neuroblastoma cell line or in cultured cerebellar granule cells, only β3 containing $GABA_A$Rs but not β2 containing receptors can be potentiated by CaMKII (Houston and Smart, 2006).

Tyrosine kinase Src can phosphorylate the γ2 subunit Y365 and Y367 sites (Brandon et al., 2001; Moss et al., 1995). However, Src also enhances the current of α1β2 $GABA_A$R expressed in HEK293 cells, indicating tyrosine kinase phosphorylation sites on subunits other than γ (Wan et al., 1997). Indeed, mutating of the γ2 Y365/367 sites leads to an increase of phosphorylation at Y384 and Y386 sites (Moss et al., 1995). In HEK293 cells, Src enhances the GABA-evoke current mediated by α1β1γ2 $GABA_A$Rs (Moss et al., 1995). Single channel recording reveals that Src increases the mean open time and channel open probability, but does not affect the channel conductance (Moss et al., 1995).

Besides intracellular kinase, extracellular molecules can also modulate the functions of the $GABA_A$R. These molecules, which usually target the allosteric sites on the $GABA_A$R, bear both physiological and pharmacological significance. I will discuss them in detail in section 1.2.

1.1.5 $GABA_A$R functions

$GABA_A$R is a chloride channel. In the adult CNS, activation of $GABA_A$R leads to an influx of chloride ions and subsequently a hyperpolarization of the postsynaptic membrane, resulting in a decrease in the probability of firing an action potential in the target neuron. The $GABA_A$R-mediated inhibition has been previously categorized as two modes: phasic inhibition and tonic inhibition (Figure 1.2). The former one is a fast and transient inhibition mediated by synaptic $GABA_A$R transmissions (Noebels et al., 2012) and the latter one is a slow but persistent

inhibition achieved by tonic activation of extrasynaptic $GABA_A$Rs (Brickley and Mody, 2012; Farrant and Nusser, 2005). During early development, $GABA_A$R may exert excitatory functions as a result of the reversed chloride membrane gradient (Ben-Ari et al., 1989; Obata et al., 1978; Owens et al., 1996). A developmental switch of $GABA_A$R functions is essential in development (Cherubini et al., 1991; Owens and Kriegstein, 2002).

1.1.5.1 Phasic inhibition

The phasic inhibition contributes to the point-to-point information communication between neurons. Mediated by synaptic $GABA_A$Rs, it rapidly translates the presynaptic activities into postsynaptic inhibitory signals (Farrant and Nusser, 2005; Noebels et al., 2012). Action potential discharge in the presynaptic neuron triggers a massive neurotransmitter release from presynaptic site of the GABAergic synapse. The neurotransmitter GABA diffuses across the synaptic cleft, where its concentration can reach up to the millimolar range, and binds to the postsynaptic cluster of $GABA_A$Rs (Mody et al., 1994). This triggers a simultaneous opening of tens to hundreds of $GABA_A$Rs and results in a large inward chloride flow and an inhibitory postsynaptic potential (IPSP) (Edwards et al., 1990; Nusser et al., 1997). The GABA concentration in the synaptic cleft rapidly decays as a result of either GABA diffusion (Barbour and Häusser, 1997; Kullmann, 2000) or GABA reuptake by the GABA transporter into the presynaptic neurons and adjacent glia cells (Noebels et al., 2012). The time constant of synaptic GABA clearance is ~100 μs (Mozrzymas, 2004; Mozrzymas et al., 2003), ensuring the short time course of GABA exposure and transient activation of $GABA_A$Rs, which are the defining features of phasic inhibition.

A single vesicle spontaneous release from the presynaptic site also contributes to the phasic inhibition. This process elicits a much smaller postsynaptic response that can be represented by the miniature inhibitory postsynaptic currents (mIPSC) during voltage-clamp recording (Bier et al., 1996; Brickley et al., 1999; Nusser et al., 1997). mIPSC recording can be a useful tool to measure the strength of synaptic transmission. The frequency of mIPSC represents the vesicle releasing probability of the presynaptic terminal (Noebels et al., 2012), whereas the amplitude of mIPSC depends on both the presynaptic vesicle size and content and the postsynaptic $GABA_AR$ efficacy (Bier et al., 1996). The rise time of mIPSC reflects the proximity of $GABA_ARs$ to the presynaptic site of GABA release and the activation rate of $GABA_ARs$ (Burkat et al., 2001; Maconochie et al., 1994). The decay rate of mIPSC is an indicator for several factors, such as receptor deactivation and desensitization (Jones and Westbrook, 1995; McClellan and Twyman, 1999).

The essential functions of phasic inhibition include controlling the excitability of neurons as well as regulating network activity. $GABA_AR$-mediated phasic inputs participate in synaptic integration by diminishing the concurrent EPSPs and preventing the membrane potential rising up to the action potential threshold (Gulledge et al., 2005). Consistent with the short duration of synaptic $GABA_AR$ activation, this inhibitory effect is very temporally precise and it counteracts the excitatory inputs within a few milliseconds time window (Staley and Mody, 1992). The phasic inhibition is also spatially restricted. For example, dendritically targeting GABAergic synapses provide inhibition only to the nearby subregions of dendritic trees (Jack et al., 1983; Koch et al., 1990). The location of phasic inhibitory input also affects its role in neuronal information integration. In hippocampal pyramidal cells, the somatically terminating

interneurons produce a much stronger inhibition than that of dendritically targeting ones, and thereby contribute to the precise coincidence detection of excitatory input at the soma (Pouille and Scanziani, 2001). The widespread innervation from some GABA-releasing interneurons can also help to synchronize firing of target cells, contributing to the rhythm oscillation of the neuronal network (Buzsáki and Draguhn, 2004; Cobb et al., 1995; Galarreta and Hestrin, 2001; Jonas et al., 2004).

1.1.5.2 Tonic inhibition

In contrast to the phasic inhibition, the tonic inhibition is mediated by persistent activation of extrasynaptic $GABA_A$Rs. It has been known for a long time that $GABA_A$Rs are widespread on neuronal membranes even at the regions far from the neurotransmitter releasing sites (Kullmann et al., 2005). The tonic $GABA_A$Rs-mediated inhibition was first identified in rat cerebellar granule cells (Kaneda et al., 1995). People found that application of $GABA_A$R blocker bicuculline to granule cells under voltage-clamp configuration did not only prevent the spontaneous GABA currents, but also produced a reduction of background noise and an outward shift of the baseline holding currents, indicating the existence of persistently activated $GABA_A$Rs. *In vivo* recordings from cerebellar granule cells revealed the presence of tonic $GABA_A$R-mediated inhibition in the intact brain (Chadderton et al., 2004). The following studies have confirmed this $GABA_A$R-mediated tonic inhibition to be a common phenomenon in the CNS, existing in many different brain regions such as the hippocampus (Nusser and Mody, 2002; Wlodarczyk et al., 2013), neocortex (Clarkson et al., 2010; Drasbek and Jensen, 2006), amygdala (Herman et al., 2013; Marowsky et al., 2012), striatum (Ade et al., 2008; Janssen et al., 2009), thalamus (Jia et al., 2005; Richardson et al., 2013) cerebellum (Brickley et al., 1996; Wall and

Usowicz, 1997) and spinal cord (Bonin et al., 2011; Maeda et al., 2010; Takahashi et al., 2006; Wang et al., 2008).

Conventionally it is believed that the activation of extrasynaptic $GABA_A$Rs is achieved by ambient GABA present in the extracellular space of the brain (Farrant and Nusser, 2005). The source of GABA has been reported to be both neuron and glia cells (Lee et al., 2010; Rossi et al., 2003; Song et al., 2013). GABA transporters also participate in controlling the extracellular GABA concentration since pharmacological blockade of GABA transporters increase the magnitude of tonic GABA current (Cope et al., 2009; Song et al., 2013). Using microdialysis technique, studies have estimated the ambient GABA concentration to range from 30 nM to 2.9 μM (de Groote and Linthorst, 2007; Glaeser and Hare, 1975; Lerma et al., 1986). Considering the relatively high agonist affinity of the δ containing extrasynaptic $GABA_A$Rs (with GABA EC_{50} of less than 1 μM) (Brown et al., 2002; Feng and Macdonald, 2004), it is possible that the ambient GABA, although at a low concentration, plays a role in the induction of tonic inhibition. However, a recent study has challenged this concept by showing that the spontaneous opening of $GABA_A$Rs is responsible for most of the tonic currents in rat dentate granule cells (Wlodarczyk et al., 2013). This finding suggests a second possible mechanism that maintains a baseline level of tonic inhibition even at the brain region where ambient GABA concentration is low.

Tonic inhibition plays an important role in controlling the basal excitability of neurons by persistently hyperpolarizing the cell membrane. Genetically or pharmacologically reducing the function of extracellular α5 containing $GABA_A$R decreases the amplitude of depolarizing current required to generate an action potential in hippocampal pyramidal neuron (Bonin et al., 2007). In

cerebellar granule cells, tonic GABA current offsets the input-output relationship (shift the input-output relationship to the right), as well as alters the neuronal gain (reduce the slope of input-output relationship). However, in CA1 pyramidal cells, tonic inhibition only affects the offset but not the gain of input-output relationship (Bonin et al., 2007; Pavlov et al., 2009), indicating the involvement of cell-type specific mechanisms of information encoding. In pyramidal cells, tonic inhibitory current increases the threshold of long-term potentiation induction (Martin et al., 2010), which may explain the phenomenon that blocking extrasynaptic $GABA_A$Rs enhances the learning and memory performances in animal models (Chambers et al., 2003; 2004; Collinson et al., 2002; Crestani et al., 2002). Interestingly, tonic inhibition also contributes to the homeostatic regulation of the phasic inhibition of interneurons (Semyanov et al., 2003; 2004). In hippocampal slices from 3-4 week old guinea pigs, $GABA_A$R-mediated tonic currents present solely in interneurons but not in pyramidal cells. Inhibition of this interneuron-specific tonic current with picrotoxin results in a higher action potential firing rate in interneurons and an increase in the frequency of sIPSCs in pyramidal cells (Semyanov et al., 2003).

1.1.5.3 Developmental switch of $GABA_A$R function

One important process of CNS synapse maturation is the developmental switch of the $GABA_A$R-mediated transmission from excitatory to inhibitory. In adult neuronal cells, the membrane chloride gradient is maintained predominantly by K-Cl co-transporter isoform 2 (KCC2), which extrudes chloride ions from the cytoplasm and lowers the intracellular chloride concentration (Chamma et al., 2012; Rivera et al., 1999). However, in immature neurons, there is a delayed expression of KCC2 but early expression of Na-K-Cl cotransporter isoform 1 (NKCC1), which mediates an inward transportation of the chloride ions (Achilles et al., 2007; Dzhala et al., 2005;

Kakazu, 2000; Payne et al., 2003; Yamada et al., 2004). The elevated intracellular chloride concentration depolarizes the reversal potential of the $GABA_A$Rs and results in an excitatory GABA response (Ben-Ari et al., 1989; Cherubini et al., 2011; Obata et al., 1978; Owens et al., 1996; Tyzio et al., 2006; 2008). This excitatory action of $GABA_A$Rs is thought to play an essential role in the generation of giant depolarizing potentials (GDPs) (Ben-Ari et al., 2007; Cherubini et al., 2011; Sipilä et al., 2005) (Ben-Ari et al., 1989)as well as many developmental events, such as cell proliferation, differentiation, migration, and neuronal plasticity (Ben-Ari, 2002; Cherubini et al., 1991; Heck et al., 2007; Khazipov et al., 2004; Owens and Kriegstein, 2002).

The excitatory-to-inhibitory GABA developmental switch is shown to have two phases. The first phase is a transient switch during parturition, which is believed to protect the fetal brain from hypoxic insults at birth (Tyzio et al., 2006) and attenuate autism pathogenesis in the offspring (Tyzio et al., 2014). The second phase is a gradual and permanent switch that happens within the first 5 postnatal days in the rodent brain (Valeeva et al., 2013), which is absent in animal models of autism and fragile X syndrome (Ben-Ari, 2015). Thus, the excitation-to-inhibition GABA developmental switch may play a critical role in preventing neurodevelopmental diseases. Oxytocin, a hypothalamic neurohormone essential for labor, lactation (Yang et al., 2013) and social behavior (Meyer-Lindenberg et al., 2011), was found to participate in the timing of both phases. Oral administration of oxytocin antagonist to pregnant rats prevented the first transient switch of the GABA action in fetal neurons (Tyzio et al., 2006). In the oxytocin receptor knockout mouse strain, the developmental upregulation of KCC2 is delayed and the proper timing of GABA switch is impaired. Treating neurons with oxytocin promotes phosphorylation

of KCC2 at S940 and a subsequent increase in the membrane expression level of KCC2 through an Oxtr/Gq/PKC-dependent pathway in early development (Leonzino et al., 2016).

The excitatory GABA action may also be present in adult brain, especially after various types of neuronal traumatic insults (Nabekura et al., 2002; Toyoda et al., 2003; van den Pol et al., 1996). This so-called secondary excitatory GABA phenotype also exists under many pathological conditions such as ischemia, epilepsy, and pain (Cohen et al., 2002; Dzhala et al., 2010; Huberfeld et al., 2007; Khalilov et al., 2003; 2005; Pallud et al., 2014; Pond et al., 2006; Price et al., 2009). The molecular mechanism of this phenomenon is still under investigation, but there is evidence that the intracellular chloride accumulation after brain injuries and diseases may due to a reduction in KCC2 expression (Rivera et al., 2002; Toyoda et al., 2003) and an elevation of NKCC1 activation (Pallud et al., 2014; Pond et al., 2006). The NKCC1 inhibitor bumetanide has demonstrated neuroprotection effect in various disease models (Dzhala et al., 2010; Huberfeld et al., 2007; Pond et al., 2006), suggesting that neuronal chloride homeostasis might be a novel therapeutic target for neurological disorders (De Koninck, 2007).

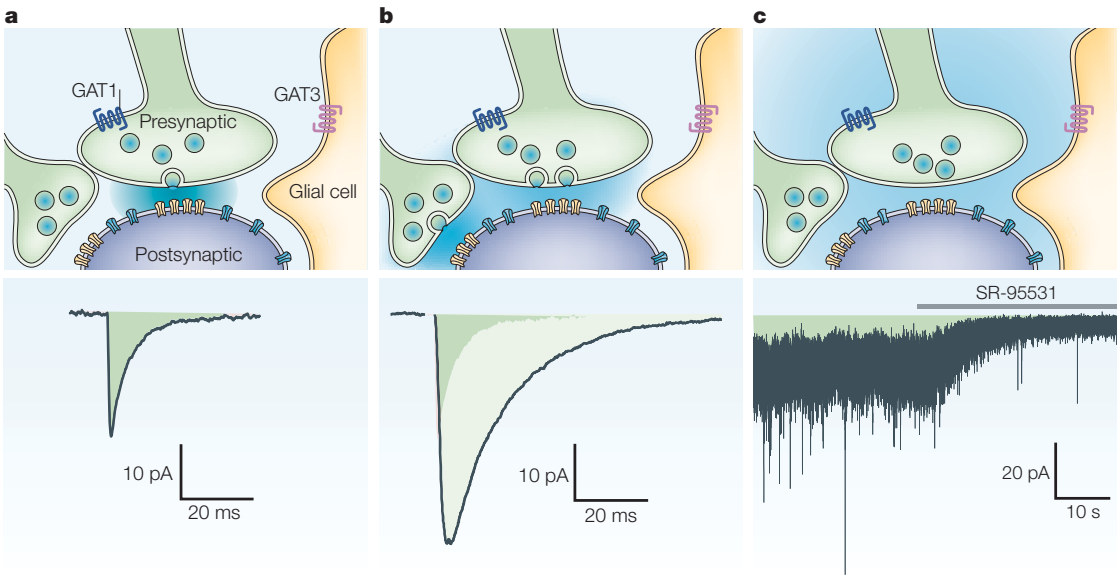

Figure 1.2 $GABA_AR$ mediated phasic and tonic inhibition

a & b. **Phasic inhibition**: **a)** A single vesicle release from presynaptic site activates a small number of synaptic $GABA_ARs$ and evokes a miniature inhibitory postsynaptic current (mIPSC). The representative trace shows the averaged waveform of mIPSCs. **b)** Action potential induces release of multiple vesicles that evokes a large and much slower IPSC as shown by the representative trace. **c)** Tonic inhibition: persistent activation of extrasynaptic $GABA_ARs$ by ambient GABA maintains the tonic inhibitory tone of the neuron. The tonic inhibition can be measured by the baseline holding current change before and after application of $GABA_AR$ antagonists (e.g. SR-95531). Reprinted with permission from Farrant, M. & Nusser, Z, Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nat Rev Neurosci.* 6.3, 215-229 (2005).

1.2 Allosteric modulation of the $GABA_AR$

As the principal inhibitory receptor in the CNS, the $GABA_AR$ has been proposed as the therapeutic target for many neurological disorders. Several types of $GABA_AR$ allosteric modulators, such as benzodiazepines, barbiturates and general anesthetics, have been developed previously and their potency varies depending on their structure and the binding site they target. Many endogenous molecules also allosterically bind on the $GABA_ARs$ and modulate their function. Those molecules include neurosteroid, endocannabinoid 2-arachidonoylglycerol, trace metals, endozepines and most surprisingly glutamate (summary see Table 1.1).

1.2.1 Exogenous allosteric modulators

1.2.1.1 Benzodiazepines

The most well known group of the $GABA_AR$ allosteric modulators is called benzodiazepines. The first drug, chlordiazepoxide (Librium) was found in 1955 because of its effect on reducing animal fear (Shorter, 2005). After that, many drugs have been developed by making minor structural changes on Librium with sedative and anxiolytic actions. The classic benzodiazepine-binding site is located at the α+/γ- interfaces of $GABA_ARs$ in a position homologous to the GABA binding site at the α-/β+ interface (Sigel, 2002; Sigel and Steinmann, 2012). Using site-directed mutagenesis analysis, a conserved histamine residue on α subunits (α1H101, α2H101, α3H126, or α5H105) was found to play an essential role in benzodiazepine binding (Benson et al., 1998; Wieland et al., 1992). Thus $GABA_ARs$ formed by α4 or α6 subunits, which bear an arginine instead of histamine at the corresponding sites, show almost no affinity to benzodiazepines (Wafford et al., 2004). Another two residues on α subunits (α1S205 and α1T206) are found to be important for benzodiazepines positioning (Tan et al., 2009). On the γ

subunit side, several critical residues have also been discovered, such as γ2 subunit M57, Y58, N60, F77, A79, T81, M130 and V190, with A79 and T81 being the most important residues (Buhr and Sigel, 1997; Buhr et al., 1997; Kucken et al., 2000; Middendorp et al., 2014; Sigel and Buhr, 1997; Wingrove et al., 1997). Therefore, $GABA_A$Rs with no γ subunits (e.g. α/β, αβδ or ρ containing $GABA_A$Rs) are almost insensitive to common dose (several nanomolar) of benzodiazepines drugs(Sigel, 2002). Another binding site with much lower benzodiazepine affinity (EC_{50} ~ tens micromolar) has also been suggested to locate within the TM2 transmembrane domains of the of the α, β and γ subunits. The formation of this binding site is independent of the incorporation of γ subunits (Walters et al., 2000). Interestingly, a recent study showed that activation of these two sites by the same drug might lead to entirely opposite modulation effects on the $GABA_A$R (Middendorp et al., 2015).

Based on the benzodiazepines' modulation effects, they can be categorized into 3 classes: benzodiazepine agonists, benzodiazepine inverse agonists, and benzodiazepine antagonist. "Benzodiazepine agonists" are a class of traditional benzodiazepines, such as diazepam (Valium) and alprazolam (Xanax), which are positive allosteric modulators of the $GABA_A$R and provide tranquilizing effect. The binding of benzodiazepine agonists on the $GABA_A$R leads to a conformational change of the receptor and an increase in its agonist (GABA) binding affinity (Johnston, 1996; Sieghart, 1992). Single channel recording revealed that benzodiazepine agonists enhance $GABA_A$R function mainly through increase channel open frequency but not duration (Vicini et al., 1987). The "benzodiazepine inverse agonists" instead are the negative allosteric modulators of $GABA_A$Rs. The inverse agonists induce a pharmacological response on $GABA_A$Rs opposite to that of the classical benzodiazepines although both types have the same

binding site. Examples of benzodiazepine inverse agonists include the anxiogenic drug Ro15-4513 (Becker and Anton, 1989) and a group of proconvulsive drugs known as β-carbolines (Evans and Lowry, 2007; Mehta and Ticku, 1989). The benzodiazepine antagonists can block the effects of the aforementioned two classes of benzodiazepines by competing for the benzodiazepine-binding site. The most well-known benzodiazepine antagonist is RO 15-1788 (or flumazenil, FLZ) (Hoffman and Warren, 1993; Longmire and Seger, 1993), which is often used for treating the overdoses of benzodiazepine agonists such as alprazolam and diazepam (al-Quorain, 1993; Krisanda, 1993).

Recently, a novel drug-binding site has been discovered at the α+/β- interface of $GABA_AR$ for the benzodiazepine drug pyrazoloquinoline (CGS 9895). CGS 9895 was first found as a benzodiazepine antagonist (Katzman and Shannon, 1985). However, when applied at micromolar concentration, CGS 9895 enhanced the $GABA_A$-mediated current even without the presence of γ subunits (Ramerstorfer et al., 2011). Using α1β3 containing $GABA_AR$ as model, people have found β3Q64 and α1V211 as two critical residues at the binding site of CGS 9895 at the α+/β- interface (Ramerstorfer et al., 2011). This binding site is homologous to the classic benzodiazepine-binding site at the α+/γ- interface, thus only receptors containing α1, α2, α3 or α5 subunits bear this novel drug-binding site (Ramerstorfer et al., 2011). Nevertheless, this study has demonstrated the α+/β- interface as a potential target for developing broad-spectrum drugs, since 95% of endogenous $GABA_ARs$ contain α and β subunits (Mohler, 2006). Furthermore, it has been found that patients with prolonged seizures often develop benzodiazepines resistance as a result of the seizure-induced subunit switch from γ containing $GABA_ARs$ to δ containing $GABA_ARs$ (Deeb et al., 2012). Meanwhile, benzodiazepine tolerance has been found in patients

with insomnia and epilepsy after long-term use of benzodiazepine drugs (Browne and Penry, 1973; Schneider-Helmert, 1988), possibly due to uncoupling benzodiazepine site with GABA binding site (Ali and Olsen, 2001) or alternation of $GABA_AR$ subunit expression (Vinkers and Olivier, 2012). Comparing with classic benzodiazepines, this new type of drugs targeting α+/β- interface would presumably overcome those difficulties and might be suitable for long-term treatments for various neurological disorders.

1.2.1.2 Barbiturates

Barbiturates are a group of sedative drugs derived from barbituric acid. The most famous one in this family is pentobarbital. At clinically relevant concentration (<10μM), barbiturates act as $GABA_AR$ positive allosteric modulators. However, when applied at higher concentration (>100 μM), barbiturates also directly activate the $GABA_AR$ (Feng et al., 2004; Fisher and Fisher, 2010; Muroi et al., 2009; Nicoll and Wojtowicz, 1980). Unlike benzodiazepines, barbiturates affect the single channel property of the $GABA_AR$ by increasing the channel opening duration without changing the frequency (Twyman et al., 1989). The dual effects of barbiturates indicate that the $GABA_AR$ contains at least two distinct binding sites for barbiturates (Thompson et al., 1996). However, these binding sites have not yet been well characterized. It has been suggested that $GABA_AR$ β subunits may play an important role in barbiturates binding, since barbiturates directly bind to the homomeric β3 receptors (Davies et al., 1997). Previous studies have identified several residues at the transmembrane domain of β subunits that may participate in barbiturate binding (β1T262, β1S290, β2G219, β3N265 and β3F289) (Birnir et al., 1997; Carlson et al., 2000; Cestari et al., 2000; Chang et al., 2003; Dalziel et al., 1999; Pistis et al., 1999). Using a newly developed photoreactive barbiturate *R*-*m*TFD-MPAB (Savechenkov et al.,

2012), a recent study has located the barbiturate-binding site at the α+/β− and γ+/β− subunit interfaces of the receptor transmembrane domain. The most critical binding residue is M227 at β3 TM1, with A291 and Y294 at α1 TM3 and S301 at γ2 TM3 as the facilitators of barbiturate binding (Chiara et al., 2013). It has also been shown recently that α1S270 and γ2S280, which are at the corresponding positions of β3N265 at the subunit interfaces, contribute to barbiturate binding (Maldifassi et al., 2016). Although the exact location of the binding site is still unknown, it is clear that barbiturates bind to the subunit interfaces but not to any intra-subunit pocket (Chiara et al., 2013; Maldifassi et al., 2016).

1.2.1.3 General anesthetics

The $GABA_AR$ mediates many of the therapeutic actions of the general anesthetics. Many anesthetics, such as propofol, etomidate and isoflurane, positively modulate the $GABA_AR$ function by three distinct effects, including increasing the receptor binding affinity for sub-maximal concentration of GABA, decreasing the rate and extend of desensitization and slowing the receptor deactivation (Krasowski et al., 2001; Nakahiro et al., 1989; Orser et al., 1998; Thyagarajan et al., 1983; Trapani et al., 1998). Similar to barbiturates, anesthetics can also directly activate the $GABA_AR$ when being used at supraclinical concentrations (Garcia et al., 2010). Comparing with that of benzodiazepines, anesthetics-mediated channel modulations are independent of the presence of γ subunits (Harrison et al., 1993; Hill-Venning et al., 1997; Sanna et al., 1995b). The volatile anesthetics, e.g. isoflurane, are likely to act on the α subunits of the receptors (Harrison et al., 1993), while the intravenous anesthetics, e.g. propofol and etomidate, form more potent interaction with the β subunits (Hill-Venning et al., 1997; Sanna et al., 1995b; 1995a). Since the α subunit contributes more in determining the subcellular location and brain

distribution of the $GABA_AR$ (see section 1.1.2), its stronger affinity to α subunits may explain why isoflurane is more selective than those intravenous anesthetics. Based on their independency of γ subunit, general anesthetics has been proposed as the important modulators for extrasynaptic $GABA_ARs$ and the effect on tonic inhibition has been observed with many classes of anesthetics such as propofol (Bieda and Maciver, 2004; Eckle et al., 2015), isoflurane (Caraiscos et al., 2004; Jia et al., 2008), and etomidate (Cheng et al., 2006). Notably, beside the acute potentiation of tonic current, anesthetics, even with a single exposure, can cause a sustain enhancement of tonic inhibition by increasing the cell-surface expression of extrasynaptic α5 containing $GABA_ARs$ (Zurek et al., 2014). The finding of this long-term effect suggested a mechanism underlying the well-known post-anesthetic memory deficits and supported the previous findings that inhibition of α5 $GABA_AR$ may be a feasible way to prevent the anesthetics-induced memory impairment (Martin et al., 2009; Saab et al., 2010; Zurek et al., 2012). However, how a brief exposure to anesthetics leads to the persistent increase of $GABA_AR$ surface expression is still unknown.

Anesthetics are believed to interact with solvent accessible pockets in the $GABA_AR$ transmembrane domain. The binding site of propofol is the best studied one. It has been found that propofol was able to act on homomeric β3 $GABA_ARs$ with a similar affinity as that of αβγ $GABA_ARs$ (Davies et al., 1997; Wooltorton et al., 1997a; Zezula et al., 1996), indicating that the critical residues for propofol binding are located on the β subunits. Based on this assumption, studies have revealed several residues on the β subunits (β1M286, β2G219, β2N265, β2Y444, β2Q185, β3Y143, β3F221, β3Q224, and β3T266) that may involve in propofol binding (Chang et al., 2003; Eaton et al., 2015; Krasowski et al., 1998; Richardson et al., 2007; Siegwart et al., 2003). Using photolabeling techniques, recent studies have shown that H267 residue at the

extracellular end of TM2 of β3 subunits may interact with propofol (Stern and Forman, 2016; Yip et al., 2013). Another residue β3N265 (and its corresponding residue β2N265) has also been found to be critical in propofol binding (Jurd et al., 2003; Maldifassi et al., 2016; Siegwart et al., 2002; Stewart et al., 2014). It has been proposed that the propofol binding site is located at the β+/α- interfaces of the transmembrane domain (Maldifassi et al., 2016). And interestingly, mutational analysis with concatenated receptors reveals that the two β+/α- interfaces actually have different affinities on anesthetics binding (Maldifassi et al., 2016), confirming the pseudosymmetry of the $GABA_AR$ structure.

1.2.1.4 Alcohol

The $GABA_AR$ is the major target of alcohol, especially ethanol in the CNS (Dar and Wooles, 1985; Martz et al., 1983). Electrophysiological experiments have shown that intoxicating concentration of ethanol potentiates the $GABA_AR$ functions in neurons from diverse species (Aguayo, 1990; Nakahiro et al., 1991; Nishio and Narahashi, 1990; Reynolds and Prasad, 1991; Reynolds et al., 1992). Single channel recording reveals that ethanol increases both channel opening frequency and mean open time of the $GABA_AR$ (Tatebayashi et al., 1998). Many alcohol-induced effects, such as anxiolysis, sedation, hypnosis and motor impairment, have been linked to the elevated $GABA_AR$ function (Buck, 1996; Davies, 2003; Grobin et al., 1998; Kumar et al., 2009; Liang and Olsen, 2014). Interestingly, ethanol elicits inhibition instead of potentiation effect on homomeric ρ1 $GABA_AR$ (Mihic and Harris, 1996). By constructing chimeric receptors, researchers have found two critical residues at the TM2 (α2S270) and TM3 (α2A291) for ethanol binding. Mutating homologous residues on β1 subunits (β1S265, β1M286) also reduces the ethanol-induced potentiation effect but to a lesser degree (Mihic et al., 1997).

Following study has found a third residue α2L232 within the same binding pocket that is responsible for defining the boundary of the cavity (Jenkins et al., 2001). This pocket, with the volume of 250–370 Å, has been proposed to be a common binding pocket for a variety of small anesthetic drugs (Jenkins et al., 2001).

The δ containing $GABA_A$Rs show much higher sensitivity to ethanol than other types of $GABA_A$Rs, suggesting low concentration of ethanol may selectively enhance the extrasynaptic $GABA_A$R-mediated tonic inhibition (Herman et al., 2013; Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). The high-affinity binding site on δ containing $GABA_A$Rs is still under investigation. Some studies have shown that benzodiazepine inverse agonist Ro15-4513 competitively inhibits the effect of low-dose ethanol on δ containing $GABA_A$R (Hanchar et al., 2006; Wallner et al., 2006). A possible ethanol/ Ro15-4513 binding pocket comprising α4/6R100 and β3Y66 residues has been recently proposed at the α+/β- interfaces of the αβδ $GABA_A$R extracellular domain (Wallner et al., 2014). However, the reason of why this binding site only exists with the presence of δ subunit but not γ subunit is still unclear. Notably, alcohol exposure can induce rapid alternation of $GABA_A$R subunit assembly (Grobin et al., 1998; Kang et al., 1996). For example, administration of ethanol in rat models significantly reduces the surface expression of δ subunits, which may explain the observed acute alcohol tolerance as well as the cross-tolerance of anaesthetics and benzodiazepines after alcohol exposure (Cagetti et al., 2003; Enoch, 2008; Liang et al., 2004; Olsen et al., 2005; Shen et al., 2011; Whissell et al., 2015).

1.2.1.5 Open channel blockers

Unlike the traditional competitive antagonists, which inhibit the channel function by competing for the same binding site with the agonist, open channel blockers exert their effect through a sequential model: the channel is activated first, followed by the open channel blockers' entry into the channel pore and prevent the current flow or channel closure (Adams, 1976; Neher and Steinbach, 1978). Since their binding sites do not overlap with that of the agonist, the open channel blockers can still be considered as allosteric modulators. The two well-characterized open channel blockers for the $GABA_AR$ are picrotoxin and Penicillin-G.

Picrotoxin is a plant-derived toxin elicits potent inhibition on the $GABA_AR$ (Inoue and Akaike, 1988; Newland and Cull-Candy, 1992; Ticku et al., 1978). At single channel level, picrotoxin shows no effect on $GABA_AR$ single channel conductance, but reduces the channel-opening frequency by stabilization of an agonist-bound closed state (Ikeda et al., 1998; Krishek et al., 1996; Newland and Cull-Candy, 1992; Porter et al., 1992). There is evidence that picrotoxin binds to the channel pore of the $GABA_AR$ but not the agonist site (Etter et al., 1999; Krishek et al., 1996). A recent co-crystallography of the GluCl channel, another member of Cys-loop LGICs, with picrotoxin showed that picrotoxin locates inside the channel pore (Hibbs and Gouaux, 2011). Using point mutation analysis and substituted-cysteine-accessibility method, studies have shown a picrotoxin-binding pocket near the cytoplasmic end of the receptor transmembrane domain comprising 2'- 6' residues of the $GABA_AR$ TM2 helix (Gurley et al., 1995; Perret et al., 1999; Wang et al., 1995b; Xu et al., 1995; Zhang et al., 1995). This deep location in the channel pore overlaps with the recently discovered desensitization gate of the $GABA_AR$ (Gielen et al., 2015). However, several other studies have suggested that picrotoxin

may have more than one binding sites on the $GABA_A$Rs (Perret et al., 1999; Qian et al., 2005; Ramakrishnan and Hess, 2005). A secondary binding site located at the interface of the transmembrane domain and extracellular domain has recently been proposed (Carpenter et al., 2013), but further investigation is still needed.

Penicillin was first discovered in 1928 because of its antibacterial activity (Fleming, 2001). In 1945, penicillin-G (PNG), a prototype of β-lactam antibiotics, was reported to be epileptogenic (WALKER et al., 1945)and this convulsive action had then been confirmed in several *in vitro* models (Dunn and Somjen, 1977; Hochner et al., 1976; Krnjević et al., 1977; Prince, 1968). After decades of investigation, people have found that PNG induces seizures by suppressing the $GABA_A$R-mediated chloride current (Fujimoto et al., 1995; Pickles and Simmonds, 1980). Single channel recording and kinetics analysis reveal that PNG serves as an open channel blocker at millimolar concentration and it shortens the mean open time of the channel without affecting its conductance (Chow and Mathers, 1986; Twyman et al., 1992). Picrotoxin, another open channel blocker, is found to compete with PNG in suppression of the $GABA_A$R function, supporting that PNG's binding site resides within the channel pore (Bali and Akabas, 2007). Recently, using a computational structure model, researchers have predicted the residues at 2', 6' and 9' rings of $GABA_A$R TM2 domain may contribute to PNG-induced channel blockade (Rossokhin et al., 2014). However, the exact site of PNG binding in $GABA_A$R still remains unknown.

1.2.2 Endogenous allosteric modulators

1.2.2.1 Neurosteroids

Neurosteroid is a class of steroids or steroid metabolites that directly modulates neuronal activity through non-genomic mechanisms (Gunn et al., 2015). Neurosteroids are either *de novo* synthesized by neurons and glia cells in the CNS or synthesized in the adrenals or the gonads and subsequently converted into nueroactive forms in the CNS (Lambert et al., 1995; Mellon and Vaudry, 2001; Ugale et al., 2007). The modulation effect of neurosteroids depends on the type of neorosteroids and the subunit composition of the $GABA_AR$. The most well-known neurosteroids, such as progesterone metabolites 5α-pregnan-3α-ol-20-one (3α,5α-THPROG), 5β-pregnan-3α-ol-20-one (3α,5β-THPROG), and the deoxycorticosterone metabolite 3α,5α-tetrahydrodeoxycorticosterone (3α,5α-THDOC) act as positive allosteric modulators at low concentrations, but also directly activate $GABA_ARs$ at higher, often non-physiological concentrations (Belelli et al., 1990; Lambert et al., 1995). The positive allosteric modulating site is located at the α-subunit transmembrane domains, with critical residues of Q241, S240, N407 and Y410 on α1 subunits (Akk et al., 2008; Hosie et al., 2009; 2007; 2006; Li et al., 2007). Activation of this site results in an increase in both channel open frequency and duration of the $GABA_AR$ (Callachan et al., 1987; Puia et al., 1990; Zhu and Vicini, 1997). A distinct binding site on the $GABA_AR$ mediates the agonist effect of neurosteroids. Not as the potentiation site, the agonist binding site is located at the interface between α- and β-subunits. For 3α,5α-THDOC, the critical binding residues include α1T236 and β2Y284 (Hosie et al., 2006). However, the direct activation effect can be enhanced by the neurosteroid binding on the potentiation site (Hosie et al., 2006). Interestingly, some types of δ containing $GABA_ARs$ can be gated effectively by GABA only with the presence of neurosteroids (e.g. 3α,5α-THDOC) (Zheleznova et al., 2008). It

is believed that fluctuations in progesterone-derived neurosteroids in brain modulate the tonic inhibition mediated by δ containing $GABA_A$Rs and alter the susceptibility of seizure and anxiety (Maguire et al., 2005).

Meanwhile, neurosteroids pregnenolone sulphate (PS) and 3β-hydroxysteroids (3β-OH steroids) are found to be negative allosteric modulators of $GABA_A$R (Birzniece et al., 2006; Lundgren et al., 2003; Wang et al., 2002). This antagonist effect is activation dependant, which means that PS and 3β-OH steroids exert stronger inhibition on the $GABA_A$R during agonist binding or channel opening stage (Eisenman et al., 2003; Wang et al., 2002). α1V256 and β2A252 located at the cytoplasmic end of the M2 helix are found to be involved in the binding of PS (Akk et al., 2001; Wang et al., 2006; 2007). PS has been found to have convulsive effect in mice (Kokate et al., 1999; Reddy and Kulkarni, 1998). However, since PS also serves as a positive allosteric modulator of NMDA receptors (Fahey et al., 1995; Guarneri et al., 1998; Wu et al., 1991), the physiological role of the neurosteroid-induced antagonism of $GABA_A$R is still unclear.

1.2.2.2 Endocannabinoid 2-arachidonoylglycerol

The endocannabinoids 2-arachidonoylglycerol (2-AG) is the endogenous ligand for cannabinoid receptors (Di Marzo et al., 2015; Sugiura et al., 1995). Previous researches have shown that cannabinoid receptor 1 (CB1) is widely expressed in the GABAergic presynaptic terminals (Mackie, 2005). Retrograde activation of CB1 by postsynaptically releases 2-AG to produce a tonic suppression of presynaptic GABA release (Lee et al., 2015; Rea et al., 2007; Tanimura et al., 2010). Besides its regulating effect on presynaptic transmission, 2-AG has been found to directly and selectively act on β2 subunit containing $GABA_A$Rs as a positive allosteric modulator.

Two critical residues β_2V436 and β_2F439 for 2-AG binding have been found in the M4 transmembrane domain of the β2 subunit (Baur et al., 2013; Sigel et al., 2011). Based on these results, serine hydrolase α/β-hydrolase domain 6 (ABHD6), the 2-AG hydrolase, has been proposed as a novel therapeutic target for epilepsy. Indeed, a recent research has confirmed that the ABHD6 inhibitor, WWL123, efficiently controls pentylenetetrazole (PTZ)-induced generalized seizure through a $GABA_AR$, but not CB1-dependent pathway. Also in the mouse model of juvenile Huntington's disease, application of WWL123 reduces the incidence and severity of the spontaneous seizures (Naydenov et al., 2014). However, since the physiological functions of this 2-AG/$GABA_AR$ crosstalk have only been observed under the pharmacologically elevated 2-AG concentration, whether this phenomenon exists at the physiological condition is still unknown (Buczynski and Parsons, 2010; Naydenov et al., 2014; Sigel et al., 2011).

1.2.2.3 Trace metals

Several trace metals are present in the CNS, among which, zinc and copper has been shown as two potent inhibitors of the $GABA_AR$. Free zinc in the CNS is concentrated in the presynaptic vesicles of a specialized type of neurons called ‘zinc-containing’ neurons (Danscher, 1996; Franco-Pons et al., 2000), which are rich in regions including the cerebral cortex, the mossy fibers of the hippocampus, the amygdala, and the olfactory bulb (Maret, 2013). Upon excitation of these neurons, zinc is released into the synaptic cleft. The measured concentration of synaptic zinc varies in previous studies from 10 nM to 30 μM, depending on their different detection techniques (Frederickson et al., 2006; Komatsu et al., 2005; Qian and Noebels, 2005; Vogt et al., 2000). The level of zinc-mediated inhibition depends on the subunit composition of the $GABA_AR$. Receptors containing only αβ subunits demonstrated the highest sensitivity to zinc

(with IC_{50} around 0.1 μM) in previous studies (Draguhn et al., 1990; Smart et al., 1991). Incorporation of the γ subunits into the αβ receptors largely prevents the zinc-induced inhibition as it reduces the zinc binding affinity by >3000-fold (Draguhn et al., 1990; Smart et al., 1991). Experiment has shown that this inhibition effect is not through zinc competition with receptor agonist GABA, indicating zinc as an allosteric modulator of $GABA_A$Rs (Barberis et al., 2000; Smart et al., 1994). Studies on αβ containing $GABA_A$R reveal two discrete zinc-binding sites: one high-affinity binding site near the channel pore at the extracellular end of the TM2 domain (with IC_{50} ~0.1μM; ~92% of the overall inhibition) and another low-potency binding site at the subunit interface (with IC_{50} ~100μM; ~8% of total inhibition) (Horenstein and Akabas, 1998; Hosie et al., 2003; Wooltorton et al., 1997a). The critical residues for the former site include β3H267 and β3E270 and for the latter site include α1E137, α1H141 and β3E182 (Horenstein and Akabas, 1998; Hosie et al., 2003). Sequence alignment shows that the γ subunit doesn't contain the residues corresponding to β3E182, H267 and E270. Therefore incorporation of γ subunit leads to a loss of both the high and low affinity binding sites, which may explain the previously observed variances in zinc sensitivity with different subunit compositions (Hosie et al., 2003).

Although it is clear that all $GABA_A$Rs are sensitive to zinc to some degree, the evidence of endogenous zinc modulation on $GABA_A$R under physiological condition is still elusive. Using zinc chelators, a previous study has shown that zinc released from the hippocampal mossy fibre exerts a tonic suppression of $GABA_A$Rs function on the CA3 pyramidal neurons (Ruiz et al., 2004). However, several other researches failed to find this type of endogenous suppression (Lavoie et al., 2007; Molnár and Nadler, 2001). The complexity in revealing endogenous zinc-$GABA_A$R interaction relies on several factors. Firstly, only a small subset (<10%) of endogenous

zinc is colocalized with the GABAergic synapses in the CNS (Frederickson et al., 2000; Tóth, 2011). Secondly, the majority of endogenous $GABA_A$Rs (~90%) contain γ subunits (Mohler, 2006), which is much less sensitive to zinc than the receptors containing only α and β subunits. However, it has been found that in some pathological conditions, there may be zinc overload in the CNS (Frederickson et al., 2005; Sensi et al., 2009; Shuttleworth and Weiss, 2011) or switch in $GABA_A$R subunit expression (Buhl et al., 1996; Gibbs et al., 1997). In these cases, zinc may play an role in $GABA_A$R toning and regulation of neuronal excitability.

Copper is another important metal ion in the CNS and it also exerts suppressive effect on $GABA_A$R-mediated current by decreasing GABA binding affinity (Sharonova et al., 1998). The binding affinity of copper on extrasynaptic δ subunit containing $GABA_A$Rs is much higher than that on synaptic αβγ $GABA_A$Rs (McGee et al., 2013). Interestingly, application of zinc relieves the $GABA_A$R from copper-induced inhibition, indicating the copper binding site and zinc binding site may be conformationally linked and zinc may faciliate the copper turnover (Sharonova et al., 2000). It has been shown that in individuals with autism and depression have elevated plasma levels of copper but lower levels of zinc (Russo, 2011; Russo and Devito, 2011). Furthermore the plasma copper/zinc ration is correlated with the severity of language, attention and motor deficits in autistic individuals (Russo et al., 2012). After zinc therapy, the plasma copper levels decreased significantly and so do the depression and autistic symptoms (Russo, 2011; Russo and Devito, 2011). Thus, the interaction between copper and zinc may play an important role in the pathology of these neuronal diseases, but if it is unclear whether it is through regulation of $GABA_A$Rs.

1.2.2.4 Endozepine

For years, people have been searching for endozepines, the endogenous ligands that exert the benzodiazepine-like effects in brain (Farzampour et al., 2015). The first physiological evidence came from studies with flumazenil (FLZ), the first known benzodiazepine (BZD) inhibitor (Hunkeler et al., 1981; Ramerstorfer et al., 2010). People discovered that the FLZ produces suppressive effects on $GABA_AR$-mediated inhibition in neurons but not in any heterologous cell lines (King et al., 1985; Krespan et al., 1984; Vicini et al., 1986), indicating the existence of endogenous positive modulators targeting BZD-site in the nervous system. Several candidates of endozepines have been suggested including oleamides (Cravatt et al., 1995), naturally occurring BZDs (Rothstein et al., 1992), and the protein diazepam-binding inhibitor (Costa and Guidotti, 1991). Oleamides is a fatty acid derivative isolated from sleep-deprived animals and has hypnotic effect (Cravatt et al., 1995). However, later studies have shown that this effect depends on the presence of $GABA_AR$ β3 subunit (Laposky et al., 2001), therefore it is unlikely for oleamide to bind to the same site as BZDs. A number of naturally occurring BZDs have been isolated from the brain in several studies (De Blas and Sangameswaran, 1986; Medina et al., 1988; Rothstein et al., 1992; Sangameswaran and De Blas, 1985). However, it is difficult to differentiate endogenous BZDs from the possible contamination from exogenous BZDs, precluding a definitive conclusion from these findings. Naturally occurring BZDs also exist in many plants, plant products and soil (Unseld et al., 1990; 1989; Wildmann et al., 1987; 1988). Thus, dietary sources may also contribute to the accumulation of BZD-like compounds in animal brain.

Diazepam-binding inhibitor (DBI) is a 10kDa protein that is highly conserved across species (Gray et al., 1986; Guidotti et al., 1983; Lihrmann et al., 1994; Mocchetti et al., 1986; Owens et al., 1989). It is also known as acyl-CoA binding protein, a cytosolic protein participating in fatty acid metabolism (Knudsen, 1991; Mogensen et al., 1987). In the brain, astrocytes are the main source of DBI (Christian and Huguenard, 2013a) and it is secreted through an unconventional pathway under various conditions (Loomis et al., 2010; Masmoudi et al., 2003; Qian et al., 2008; Tokay et al., 2008), supporting its role as an extracellular modulator of the $GABA_AR$. Application of exogenous DBI to cultured neurons reveals a negative allosteric modulation effect (Bormann, 1991; Costa and Guidotti, 1991). This DBI-mediated inhibition of GABA-signal is likely contributes to the neuronal proliferation in the subventricular zone (Alfonso et al., 2012). Paradoxically, a recent study has shown a positive modulation effect of DBI in the thalamic reticular nucleus (nRT). Deletion of the DBI gene in animals abolishes an endogenous BZD-site dependent potentiation of $GABA_AR$ current in nRT (Christian et al., 2013). Since DBI has a variety of cleavage products (Alho et al., 1991; Ferrero et al., 1986; Slobodyansky et al., 1992) and $GABA_ARs$ comprise different subunits in different brain regions, future research is needed to elucidate if the opposing actions of DBI is determined by the specific DBI peptide fragments or $GABA_ARs$ subunit composition. One possible way to do this is to use sniffer patch techniques. In a recent study, people developed a biosensor made from out-side-out membrane patches obtained from the ventrobasal nucleus (VB) and detected DBI-induced potentiation of $GABA_ARs$ only in nRT but not in the VB (Christian and Huguenard, 2013b). This result indicates that the regional specific expression and cleavage of DBI may be more important in determining its modulation effect.

1.2.2.5 Glutamate

Glutamate is the major excitatory neurotransmitter in the CNS. Interestingly, a previous research has reported that glutamate reversibly potentiates $GABA_AR$-mediated responses in acutely dissociated hippocampal neurons (Stelzer and Wong, 1989). This potentiation effect is associated with a change not in $GABA_AR$ reversal potential but in the slope of GABA I/V relationship, indicating that glutamate does not change the chloride membrane gradient but just affects the channel conductance of the $GABA_AR$. Several glutamate analogs, such as quisqualate, kainate and NMDA also elicited the similar potentiation effects, illustrating a possible common binding site on or closely association with the $GABA_AR$ for these agents (Stelzer and Wong, 1989). Recently, our lab has found that glutamate exerts an allosteric modulation effect on the glycine receptor, which is also a member of Cys-loop superfamily of the ligand-gated ion channels (Liu et al., 2010). Considering the structural similarity of the $GABA_AR$ and the glycine receptor, the glutamate-induced potentiation on the $GABA_AR$ may also acts through a similar mechanism involving allosteric interaction with the receptor.

It is worth mentioning that, glutamate is the agonist of several glutamate receptors such as NMDA receptors (NMDARs), AMPA receptors (AMPAR) and metabolic glutamate receptors (mGluRs). Glutamate can also affect the $GABA_AR$ functions through several indirect pathways. For example, it has been found that moderate NMDAR activation promotes $GABA_AR$ membrane insertion and facilitates inhibitory transmission. This long-term potentiation of $GABA_AR$-mediated inhibition (iLTP) is the result of CaMKII-dependent phosphorylation of the S383 residue on $GABA_AR$ β3 subunit and subsequent stabilization of synaptic $GABA_ARs$ (Marsden et al., 2010; Petrini et al., 2014). However, activation of the NMDAR by stronger glutamate

stimulation leads to a long-term depression of the $GABA_AR$-mediated inhibition (iLTD) in hippocampal neurons in a calcium dependent manner (Chen and Wong, 1995). Detailed characterization shows that this effect is due to both comprised receptor sensitivity to GABA and the enhanced dispersion of $GABA_ARs$ through calcineurin-induced dephosphorylation of the $GABA_AR$ γ2 subunit at the residue S327 (Bannai et al., 2009; Lüscher et al., 2011; Muir et al., 2010; Niwa et al., 2012). Although spatially distant from $GABA_ARs$, the synaptic NMDARs rather than the extrasynaptic NMDARs are suggested to be responsible for the suppression effect (Chisari et al., 2012). On the contrary, activation of the mGluR1 by ambient glutamate leads to the calcium release from IP_3Rs and stabilizes the $GABA_AR$ synaptic cluster through a PKC-dependent pathway in hippocampal neurons (Bannai et al., 2015). Thus, the different sources of calcium define the opposing mechanisms in controlling $GABA_AR$ functions. In immature neurons, hypoxia-induced seizure can be blocked by the antagonist of AMPAR but not NMDAR (Jensen et al., 1995; Koh and Jensen, 2001). Research has found that the activation of calcium-permeable AMPAR under hypoxia condition reduces the $GABA_AR$-mediated inhibition in hippocampal CA1 pyramidal neurons by calcineurin-induced dephosphorylation of the $GABA_AR$ β2/3 subunits (Sanchez et al., 2005). Notably, all these indirect pathways involve a time delay between glutamate stimulation and $GABA_AR$ potentiation/inhibition, ranging from several minutes to hours (Bannai et al., 2015; Chen and Wong, 1995; Marsden et al., 2010; Sanchez et al., 2005). Thus, the aforementioned allosteric modulation of $GABA_ARs$ by glutamate may serve as a rapid homeostatic regulatory mechanism in maintaining the excitation/inhibition balance in the CNS.

Type	Name	Modulation Role	Binding Site	Critical Binding Residues
Exogenous allosteric modulators	Benzodiazepines	Positive modulators (e.g. diazepam and alprazolam)	α+/γ- interface of the extracellular domain	α1H101, α2H101, α3H126, α5H105, γ2A79, γ2T81
		Negative modulators (e.g. Ro15-4513 and β-carbolines)		
		Benzodiazepine antagonist (e.g. RO 15-1788)		
	Barbiturates	Positive modulator (at low concentration) or agonist (at high concentration)	α+/β− and γ+/β− interfaces of the transmembrane domain	β1T262, β1S290, β2G219, β3N265, β3F289, β3M227, α1A291, α1Y294, γ2S301, α1S270, γ2S280
	General Anesthetics	Positive modulators	Solvent accessible pockets in the transmembrane domain	For propofol (α-/β+ interface): β3N265, β3H267, β1M286, β2G219, β2N265, β2Y444, β2Q185, β3Y143, β3F221, β3Q224, and β3T266

Type	Name	Modulation Role	Binding Site	Critical Binding Residues
Exogenous allosteric modulators	Alcohol	Positive modulator	Transmembrane domain	α2S270, α2A291, β1S265, β1M286, α2L232
	Picrotoxin	Open channel blocker	Transmembrane domain	Residues at 2' - 6' rings of the TM2 domain
	Penicillin-G	Open channel blocker	Transmembrane domain	Residues at 2', 6' and 9' rings of the TM2 domain
Endogenous allosteric modulators	Neurosteroid	Positive modulators (at low concentration) or agonist (at high concentration) (e.g. 3α,5α-THPROG and 3α,5α-THDOC)	Transmembrane domains	α1Q241, α1S240, α1N407, α1Y410, α1T236, β2Y284
		Negative modulators (e.g. PS and 3β-OH steroids)	Transmembrane domains	α1V256, β2A252

Type	Name	Modulation Role	Binding Site	Critical Binding Residues
Endogenous allosteric modulators	Zinc	Negative modulator	High-affinity binding site: TM2 domain Low-affinity binding site: α+/β- interface	High-affinity binding site: β3H267, β3E270 Low-affinity binding site: α1E137, α1H141, β3E182
	Copper	Negative modulator	conformationally linked with zinc binding site	Unknown
	2-AG	Positive modulator	Transmembrane domain	β_2V436, β_2F439
	Endozepine	Positive modulators	Same as benzodiazepine	Same as benzodiazepine
	Glutamate	Positive modulator	Unknown	Unknown

Table 1.1 Summary of the $GABA_AR$ allosteric modulators

1.3 Neurotransmitter glutamate

1.3.1 Glutamate synthesis

Glutamate is the endogenous ligand of the neuronal glutamate receptors such as AMPA receptors, kainate receptors and NMDA receptors, which depolarizes the postsynaptic neuron and increase its probability of firing an action potential. The synthesis and recycling of glutamate in the CNS is mainly through the glutamate-glutamine cycle (Bak et al., 2006). Glutamine is the precursor of glutamate and it is transported into the neurons via glutamine transporters (Bhutia and Ganapathy, 2015). Intracellular glutamine is converted into glutamate by mitochondrial enzyme phosphate activated glutaminase (PAG) (Rowley et al., 2012). It has been proposed that the glutamate synthesis from glutamine is a highly compartmentalized event that happens mainly in neuron due to the higher expression level of PAG in neuron than that in astrocyte (Hogstad et al., 1988; Kaneko et al., 1987; Zaganas et al., 2001). In neuron, the cytoplasmic glutamate concentration can reach up to 10-15mM (Attwell et al., 1993), which largely facilitates the fast packing of glutamate into the synaptic vesicles. In contrast, the cytoplasmic glutamate concentration in astrocytes is much lower (0.1–5 mM) (Attwell et al., 1993), mainly due to the glutamate-glutamine conversion in the astrocyte.

Several participants in the aforementioned pathways tightly control the rate of glutamate synthesis and may bear pathological and pharmacological significance (Bhutia and Ganapathy, 2015; Pochini et al., 2014). For example, human endogenous retroviruses (HERVs) are found to interact with glutamine transporters ASCT1/2 and reduce the amino acid intake, which may contribute to the pathogenesis of multiple sclerosis (Antony et al., 2011). Another family of glutamine transporters, SLC38 is under the regulation of PKA and PKC, which has an impact on

synaptic plasticity (Nissen-Meyer and Chaudhry, 2013; Ogura et al., 2007). PAG, the enzyme responsible for glutamate synthesis, can be pharmacologically inhibited by the compound 6-diazo-5-oxo-l-norleucine (DON) and exhibit anticonvulsive effect. The glutamate synthesis from glutamine is also coupled with other metabolic pathways, such as malate-aspartate shuttle (MAS, glutamate + oxaloacetate -> α-ketoglutarate + aspartate in mitochondria and the converse process in the cytoplasm) (Mangia et al., 2012). It has been found that under enhanced neuronal activities, there is an increase in glutamate concentration but a decrease in aspartate concentration in the brain (Dienel et al., 2002; Lin et al., 2012; Mangia et al., 2007), indicating more glutamate being shifted out of the MAS to increase the neuronal glutamate availability.

1.3.2 Glutamate release

1.3.2.1 Presynaptic release

The majority of the neurotransmitter glutamate is released from presynaptic sites. The cytoplasmic glutamate is packed into the presynaptic vesicles by vesicle glutamate transporters (VGLUT) (Hackett and Ueda, 2015; Thompson et al., 2005). Glutamate concentration can reach up to 100 mM inside the synaptic vesicles (Attwell et al., 1993). The neurotransmitter vesicles are enriched at the presynaptic sites by synapsin-mediated tethering to the actin cytoskeleton. In response to the elevated calcium concentration, the neurotransmitter vesicles move from the reserved vesicle pool through PKA and CaMKII mediated pathways and are docked and primed for release at the presynaptic membrane by the SNARE complex. Upon the arrival of action potential, the calcium influx into the presynaptic site through voltage gated calcium channels triggers the vesicle fusion and release of glutamate (Henley et al., 2014; Munson, 2015; Rizo and Rosenmund, 2008; Rowley et al., 2012).

Several types of presynaptic receptors play roles in regulation of presynaptic glutamate release (Engelman and MacDermott, 2004). For example, some of the GluN2B subunit containing (and lately found GluN3A subunit containing) NMDARs are shown to have presynaptic localization (Brasier and Feldman, 2008; Corlew et al., 2008; Larsen et al., 2011). Since the activation of NMDAR requires both glutamate and depolarization dependent removal of magnesium blockade, presynaptic NMDAR is considered as a coincidence detector (Bardoni et al., 2004; Bender et al., 2006; Sjöström et al., 2003). It has been shown that activation of presynaptic NMDAR enhances the spontaneous and evoked neurotransmitter release in different brain regions (Berretta and Jones, 1996; Brasier and Feldman, 2008; Casado et al., 2000; Mameli et al., 2005; Martin et al., 1991; McGuinness et al., 2010; Yang et al., 2006). The mechanism behind this phenomenon has been long-time proposed as the NMDAR-mediated increase of presynaptic calcium concentration (Berretta and Jones, 1996; Buchanan et al., 2012; Cochilla and Alford, 1999; Woodhall et al., 2001). However, recent evidence suggests a second calcium-independent but PKC-dependent mechanism may exist (Kunz et al., 2013).

Notably, presynaptically located $GABA_AR$ is also found to affect the glutamate release, although in a much more complex manner. In the spinal cord, the activation of presynaptic ρ2 containing $GABA_AR$ induces an inhibition of glutamate release and exerts anti-nociceptive effect (Kullmann et al., 2005; Rudomin and Schmidt, 1999; Tadavarty et al., 2015). However, in other brain regions, such as the cerebellum, hippocampus, amygdala and cerebral cortex, tonic activation of presynaptic $GABA_AR$ by ambient GABA produces a depolarization of presynaptic terminals and subsequently increases the probability of glutamate release (Alle and Geiger, 2007;

Awatramani et al., 2005; Nakamura et al., 2007; Stell et al., 2007; Szabadics et al., 2006; Woodruff et al., 2006). It has been shown that some axons lack the expression of KCC2 (Gulyás et al., 2001; Jarolimek et al., 1999; Ruiz et al., 2003), thus the excitation property of presynaptic $GABA_AR$ is probably a result of high local chloride concentrations at the presynaptic terminals (Khirug et al., 2008; Price and Trussell, 2006; Szabadics et al., 2006). Although it is known that presynaptic $GABA_AR$-mediated release facilitation may involve in mossy fiber LTP (Ruiz et al., 2010) and sound localization circuit (Weisz et al., 2016), the physiological significance of this phenomenon is still under investigation.

1.3.2.2 Astrocyte-mediated release

It has been confirmed that, astrocyte can also be an important source of glutamate in the CNS under certain physiological or pathological conditions, such as stroke or Alzheimer's disease (Rossi et al., 2000; Soria et al., 2014; Talantova et al., 2013). Several mechanisms have been proposed for the astrocyte-mediated glutamate release. First, astrocyte may utilize a similar releasing machinery as neurons and release glutamate through calcium-dependent exocytosis (Martineau, 2013). Studies have shown VGLUT1 containing small vesicles in astrocytes which can be released in a calcium dependent manner (Bezzi et al., 2004; Bowser and Khakh, 2007; Liu et al., 2011; Marchaland et al., 2008). A second mechanism may involve reversal of glutamate uptake by astrocytic glutamate transporters. Normally, the astrocytic glutamate transporters are responsible for cleaning-up the glutamate from the synaptic cleft to quickly terminate the neurotransmitter action (see below). However, under pathological conditions, such as ischemia, the changing of membrane ion gradient (e.g. high extracellular potassium) may reverse the function of astrocytic glutamate transporters and induce subsequent glutamate release

from astrocyte (Li et al., 1999; Rossi et al., 2000; Szatkowski et al., 1990). Interestingly, a recent study shows that blockade of glutamate transporters cannot prevent glutamate accumulation and ischemia-induced neuronal damage during oxygen and glucose deprivation (OGD) treatment. In contrast, they found that elevated activation of cystine/glutamate antiporter is responsible for those effects (Soria et al., 2014). Other proposed mechanisms include ATP-induced glutamate release through $P2X_7$ receptors (Duan et al., 2003; Fellin et al., 2006), activation of volume-sensitive organic osmolyte–anion channel (VRAC) during cell swelling (Hyzinski-García et al., 2014; Takano et al., 2005) and glutamate diffusion through unpaired connexons hemichannels (Ye et al., 2003). Thus, the astrocyte-mediated glutamate release is a complicated event and whether those mechanisms operate at the same time or separately at different conditions is still unclear.

1.3.2.3 Glutamate/GABA co-release

It has been found that vesicle glutamate transporter VGLUTs are expressed in neurons releasing other types of neurotransmitters, such as acetylcholine, serotonin and GABA (Amilhon et al., 2010; Gras et al., 2008; Nelson et al., 2014; Zander et al., 2010). The co-expression of VGLUT and VGAT (vesicle GABA transporter) has been observed in many brain regions including the cerebellum, hippocampus, cortex, habenular nucleus and auditory system (Herzog et al., 2004; Noh et al., 2010; Root et al., 2014; Zander et al., 2010). It is shown that the VGLUT does not only load glutamate into the same vesicle with GABA but it also facilitates the GABA vesicle packaging (Weston et al., 2011; Zander et al., 2010). Upon action potential, both glutamate and GABA can be released from the same vesicle and activate both inhibitory and excitatory receptors (Beltrán and Gutiérrez, 2012; Münster-Wandowski et al., 2013; Zimmermann et al.,

2015). It is not clear if GABAergic synapse also contains AMPARs, which mediate the excitatory postsynaptic responses (Rao et al., 2000; Zimmermann et al., 2015). However, there is evidence that co-released glutamate may activate the presynaptic mGluRs and NMDARs through a spillover manner (Gillespie et al., 2005; Noh et al., 2010; Somogyi et al., 2003; Stensrud et al., 2015). This co-release event is proposed to be involved in the topographic specification of inhibitory auditory pathway (Noh et al., 2010) and modulation of lateral habenula output (Root et al., 2014; Shabel et al., 2014).

1.3.2.4 Dendritic glutamate release

Unlike the VGLUT1 and VGLUT2, which are expressed almost exclusively on the presynaptic terminals, VGLUT3 can also be found in postsynaptic dendrites and cell bodies (Fremeau et al., 2002; 2004), suggesting the existence of vesicular glutamate release at the dendrites. Dendritic glutamate release has been documented in several brain regions such as the olfactory bulb, neocortex, midbrain and cerebellum. In accessory olfactory bulb, the mitral cell tufts connect with the periglomerular (PG) and granule cells via special dendrodendritic synapses (Egger and Urban, 2006). It has been found that back-propagating action potential evokes dendritic glutamate release in a calcium dependent manner and subsequently activates the PG and granule cells (Chen et al., 2000b; Isaacson, 2001; Isaacson and Strowbridge, 1998; Xiong and Chen, 2002). Glutamate receptors (e.g. AMPAR, NMDAR) on mitral cell can also be activated by dendritic released glutamate from itself or nearby cells through a spillover mechanism (Isaacson, 1999; Nicoll and Jahr, 1982; Pimentel and Margrie, 2008; Salin et al., 2001). It is interesting that subthreshold depolarization of mitral cell dendrites also triggers the glutamate release and its effect can be enhanced by activation of mGluRs, indicating the cells with weak sensory inputs

can also participate in the signal integration and autocrine regulation may exist in this process (Castro and Urban, 2009).

In the layer 2/3 neocortex near the inhibitory synapses between pyramidal cells and fast-spiking non-accommodating (FSN) interneurons, back-propagating action potentials in pyramidal cells also induce calcium transient and subsequent dendritic glutamate release. This event is followed by a suppression of FSN-mediated inhibition via an mGluR dependent pathway (Zilberter, 2000; Zilberter et al., 2005). Similarly, in the avian midbrain, depolarization of the postsynaptic neurons decreases the frequency of presynaptic spontaneous release through retrograde activation of presynaptic NMDAR. The retrograde messenger, most possibly glutamate, is released from postsynaptic dendrites via calcium-induced vesicle fusion (Penzo and Peña, 2011). The dendritic glutamate release from cerebellar Purkinje cells also plays a critical role in modulating its inhibitory synaptic plasticity. It will be discussed in detail in the next section.

Studies on dendritic vesicular release have confirmed the involvement of calcium signaling and the reliance on SNARE complex, which is also present in the traditional presynaptic release (Bergquist and Nissbrandt, 2003; Bergquist et al., 2002; Fortin et al., 2006; Higley and Sabatini, 2008; Ludwig et al., 2002). However, specific types of SNARE machinery, such as syntaxin 4 and SNAP-23, have been assigned to the dendritic secretion (Kennedy et al., 2010; Suh et al., 2010). Furthermore, unlike that of presynaptic neurotransmitter release, the existence of active zone for the dendritic secretion is still under debate (Kennedy and Ehlers, 2011; Matsui and Jahr, 2006; Tobin and Ludwig, 2007). There is also evidence that the temporal control of dendritic release is not as tight as that of traditional presynaptic release (Ludwig and Leng, 2006; Michael

et al., 2006; Pang and Südhof, 2010). This can be explained by the slow kinetics of dendritic calcium signal, which is probably due to the diffused calcium efflux from the intracellular stores (Ludwig, 2007; Ludwig et al., 2002). It has also been shown that dendritic secretion machinery is more sensitive to calcium, thus even a small elevation of dendritic calcium concentration (50-200 nM, like that from activation of NMDAR) is able to induce the vesicular release (Chen and Rice, 2001; Isaacson and Strowbridge, 1998; Kaiser et al., 2004; Zilberter et al., 1999). The protracted and ectopic release of dendritic vesicles makes it an unique event that may bear different physiological significance from the traditional presynaptic release.

1.3.3 Glutamate reuptake

1.3.3.1 Overview of glutamate reuptake and recycle

Once released, glutamate is rapidly removed from the synaptic cleft by astrocytic high affinity glutamate transporters, namely excitatory amino acid transporters (EAATs). EAAT1 and EAAT2 are the major types of glutamate transporters in astrocytes (Danbolt, 2001). The fast cleanup of glutamate by astrocyte ensures the extracellular glutamate is at a relatively low concentration (a few μM) (Anderson and Swanson, 2000; Moussawi et al., 2011). The tight control of glutamate concentration is critical for avoiding excitotoxicity (Danbolt, 2001). Inside astrocytes, glutamate is converted to glutamine by the enzyme glutamine synthetase (GS) before releasing out of the cell, thus avoiding the secondary activation of glutamate receptors (Uwechue et al., 2012). The synthesis of glutamine from glutamate happens exclusively in astrocytes as a result of the astrocyte-specific expression of GS (Martinez-Hernandez et al., 1977). Following the release, the extracellular glutamine is transported back into presynaptic neurons through glutamine

transporters. And then the glutamate-glutamine cycle can start over again to replenish the neuronal glutamate pool (Bak et al., 2006).

It is also well known that glutamate can be taken up directly by presynaptic neurons through neuronal expressed EAATs (Danbolt, 2001; Drejer et al., 1982; Russo et al., 2013). Blockade of the neuronal glutamate uptake reduces the presynaptic glutamate pool even when there is adequate supply of glutamine (Waagepetersen et al., 2005). The presence of EAATs in endothelial cells suggests another minor pathway for glutamate removal: the efflux of glutamate across the blood–brain-barrier into the blood stream (Cohen-Kashi-Malina et al., 2012; Hawkins, 2009; Smith, 2000). The glia cells lining the blood–brain-barrier actively facilitate this efflux process and maintain the proper direction of glutamate transportation (Cohen-Kashi-Malina et al., 2012; Helms et al., 2012).

1.3.3.2 Excitatory amino acid transporters

As summarized above, the excitatory amino acid transporters (EAATs) play an essential role in glutamate reuptake. EAATs utilize the gradient of Na^+ and K^+ ions to drive the transportation of glutamate (Anderson and Swanson, 2000). Each glutamate molecule is taken up by EAATs with the co-transport of 3 Na^+ and 1 H^+ and counter-transport of 1 K^+ (Danbolt, 2001; Vandenberg and Ryan, 2013). Till now, 5 subtypes of EAATs have been discovered, within which, EAAT2 is the major subtype and is responsible for 90% of glutamate uptake in the CNS (Jensen et al., 2015). EAAT1 and EAAT2 are predominantly expressed in astrocytes while EAAT3-5 is exclusively found in neurons (Danbolt, 2001). The astrocytic EAATs are enriched near the

synapse to ensure fast reuptake of glutamate and termination of neurotransmission (Chaudhry et al., 1995; Minelli et al., 2001). Notably, EAATs are also anion permeable (Fairman et al., 1995; Schneider et al., 2014; Wadiche et al., 1995). Depending on their different glutamate transportation efficacy and anion conductance, EAAT1-3 predominantly serves as glutamate transporters while EAAT4-5 mainly as anion channels (Mim et al., 2005; Schneider et al., 2014).

Dysfunction or down-regulation of EAATs has been implicated in the development of a variety of psychiatric disorders (e.g. depression (Hashimoto, 2009), schizophrenia (Purdon et al., 2008) and drug addiction (Shen et al., 2014)), neurodegenerative diseases (e.g. Alzheimer's disease (Li et al., 1997; Walton and Dodd, 2007) and Huntington's disease (Reynolds et al., 2008; Taylor-Robinson et al., 1996)) and neurological disorders (e.g. epilepsy (Tanaka et al., 1997) and hepatic encephalopathy (Cittolin-Santos et al., 2016)). Thus, EAATs may be a potential target for developing novel therapeutics. For example, transcriptional or translational increase of the EAAT2 produces neuroprotection effects in mice models of ischemia, amyotrophic lateral sclerosis (ALS), epilepsy and durg addiction (Kim et al., 2011; Kong et al., 2014; Rao et al., 2015).

1.3.3.3 Extracellular glutamate concentration under physiological and pathological conditions

Excess extracellular glutamate results in over activation of glutamate receptors and excitotoxicity (Danbolt, 2001). Thus, it is important to determine the extracellular glutamate concentration under physiological and pathological conditions. It is known that the physiological glutamate concentration in plasma is around 150 μM and in cerebrospinal fluid is around 10 μM (Danbolt,

2001; Featherstone and Shippy, 2008; Rahman et al., 2005). However, measuring the extracellular glutamate concentration in the CNS is much more difficult. Many techniques, such as microdialysis, biosensor voltammetry and electrophysiology, have been exploited to estimate the glutamate level in the extracellular space in the brain (Chefer et al., 2009; Herman and Jahr, 2007; Rahman et al., 2005). Depending on their experimental conditions, the resting glutamate concentration is estimated at a range from 0.02 to 30 μM (Cavelier and Attwell, 2005; Herman and Jahr, 2007; Jacobson et al., 1985; Miele et al., 1996; Oldenziel et al., 2004; Rahman et al., 2005). Following an action potential, the glutamate concentration in synaptic cleft rises up to more than 1 mM, but quickly comes back to the baseline level in less than 10 ms due to the rapid reuptake by glutamate transporters (Clements, 1996; Clements et al., 1992; Diamond and Jahr, 1997). The diffusion coefficient of glutamate is estimated to be around 0.3 $\mu m^2\ ms^{-1}$, but the diffusion is largely dependent on the physical barrier of surrounding glia cells and the distribution of glutamate transporters (Budisantoso et al., 2013; Kessler, 2013). It is worth mentioning that, due to the technique limitations, the estimated glutamate concentration may not fully reflect the real glutamate concentration in physiological conditions. For example, all of these techniques are invasive which may cause non-physiological elevations of glutamate due to tissue damage (Moussawi et al., 2011).

Notably, many studies have shown that glutamate can escape from the synapse where it is released and activates receptors in neighboring synapses or at extrasynaptic sites. This phenomenon, namely glutamate spillover, has been found in many brain regions under physiological conditions (Chalifoux and Carter, 2011; Diamond, 2002; Drew et al., 2008; Isaacson, 1999; Kullmann and Asztely, 1998; Okubo and Iino, 2011; Szapiro and Barbour, 2007;

Zhang and Sulzer, 2003). For example, communication between climbing fibers and molecular layer interneurons are exclusively through glutamate spillover in the cerebellum, (Szapiro and Barbour, 2007). Also, intensive stimulation of parallel fibers evokes AMPAR- and NMDAR-mediated EPSCs in stellate cells without any direct synaptic connections (Carter and Regehr, 2000). Activation of extrasynaptic NMDARs by glutamate spillover also contributes to the initiation of NMDAR-mediated spikes, an important calcium signal for neuronal plasticity (Chalifoux and Carter, 2011). These findings challenge the traditional concept of point-to-point signal transmission between synapses.

Elevated glutamate level in the CNS has been observed under many pathological conditions. Microdialysis measurement shows that during epilepsy, there is a 3-6 folds increase of brain glutamate concentration in mice models and conscious human patients (During and Spencer, 1993; Medina-Ceja et al., 2000; 2015). Similarly, at the centre of the ischemic lesion, the glutamate level can reach up to 80 times of normal concentration (Hillered et al., 1989; Uchiyama-Tsuyuki et al., 1994). In the penumbral region, the raise of glutamate concentration is to a lesser degree but still 25-folds of the baseline level (Takagi et al., 1993). During spread depression, the extracellular glutamate concentration is more than 90 μM higher than that of baseline (Zhou et al., 2013). The traumatic brain injury also initiates an 4-13 folds increase of extracellular glutamate concentration (Faden et al., 1989; Katayama et al., 1990; Nilsson et al., 1990). And a 2-fold increase of tonic glutamate level can still be observed 2 days after injury (Hinzman et al., 2010). Although controversial, excessive extracellular glutamate may also contribute to the pathogenesis of many other neurological disorders, such as multiple sclerosis, Parkinson's disease, Huntington's disease and drug addiction (Iwasaki et al., 1992; Lee et al.,

2013; Pitt et al., 2000; Shen et al., 2014; Tisell et al., 2013). The pathological accumulation of glutamate at the extracellular space may due to several reasons, such as massive glutamate release after neuronal injury (Katayama et al., 1990; Nishizawa, 2001; Xu et al., 2004), inflammation-induced glutamate release from glia cells (Vesce et al., 2007) and the compromised glutamate reuptake efficacy as I have discussed in the previous section.

1.3.4 Glutamate-binding site on glutamate receptors

Glutamate is the endogenous ligand of several ionotropic and metabotropic receptors in the CNS. This group of receptors is termed the glutamate receptors. Till now, 3 types of ionotropic receptors (AMPA receptor, NMDA receptor and kainate receptor) and a group of metabotropic glutamate receptors have been discovered in the mammalian CNS. Identifying the agonist (i.e. glutamate) binding sites is critical for understanding the receptor properties and developing novel therapeutics targeting those receptors.

1.3.4.1 AMPA receptor

The AMPA receptors (AMPARs) have 4 subunits families, namely GluA1, GluA2, GluA3 and GluA4. Most AMPARs are heterotetramers with two GluA2 subunits and another two subunits from other families, although homomeric receptors (e.g. homozygous GluA1) also exist (Greger et al., 2007; Sukumaran et al., 2012). Each subunit contains one agonist-binding site, making a total of four binding sites for glutamate (Armstrong et al., 1998). Occupying two of them by agonists is the minimum requirement for channel opening (Rosenmund et al., 1998). The binding pocket is formed by the N-terminal tails and the extracellular loop between the 3rd and 4th transmembrane domains (Armstrong et al., 1998). Based on the crystal structure and homology

models, several studies have identified the critical residues for agonist binding, including Y450, P478, T480, R485, S654, T655 and E705 on GluA2 subunit and their corresponding residues on other subunits (Armstrong and Gouaux, 2000; Armstrong et al., 1998; Bjerrum et al., 2003; Gill et al., 2008; Mankiewicz et al., 2007; Pentikäinen et al., 2003). All of these residues are conserved among all four subunit families. The agonist docking shows that Y450, T480, R485 and S654 form interaction with the α-carboxyl group of glutamate molecules while T655 is with γ-carboxyl groups. T480, P478 and E705 attract the α-amino group of glutamate molecules (Armstrong and Gouaux, 2000; Armstrong et al., 1998; Mankiewicz et al., 2007).

1.3.4.2 NMDA receptor

The NMDA receptor (NMDAR) is a heterotetramer comprising of two GluN1 and two GluN2 subunits. The extracellular domain of each subunit contains two globular structures: an amino terminal domain (ATD) contributes to channel modulation and a ligand-binding domain responsible for agonists (e.g. glutamate and NMDA) and co-agonist (e.g. glycine and D-serine). The glutamate binds to the ligand-binding domain of the GluN2 subunits (Furukawa et al., 2005; Karakas and Furukawa, 2014; Lee et al., 2014). Using mutagenesis techniques, the critical residues for glutamate binding has been found in previous studies, including H466, S492, T494, R499, F637, S670, T671 and D712 on GluN2A subunits and E387, F390, K459, H460, R493, V660, S664 and V709 on GluN2B (Chen et al., 2005a; Laube et al., 1997; Ren et al., 2007). The α-carboxyl group of glutamate molecule is anchored by the positively charged R499 on GluN2A and R493 on GluN2B. The α-amino group interacts with the negatively charged D712 on GluN2A and E387 on GluN2B. The γ-carboxyl group is predicted to interact with either charged

receptor surface or hydroxyl groups from serine and threonine residues (Chen et al., 2005a; Laube et al., 1997).

1.3.4.3 Kainate receptor

Similarly as the AMPAR and NMDAR, the kainate receptor (KAR) is also tetramer comprised of four subunits from five different families, namely GluK1-5. GluK1-3 can form either homomeric or heteromeric receptors while GluK4 and GluK5 can only assemble functional receptors with one of the GluK1-3 subunits (Kumar et al., 2011). The secondary structure of KAR and AMPAR are highly conserved. Thus the ligand-binding site of KAR is comparable to that of AMPAR (Traynelis et al., 2010). Crystallography studies have shown that the critical residues for glutamate binding on KAR include Y474, P501, T503, R508, S674, T675 and E723 on GluK1 subunit and Y457, P485, A487, R492, A658, T659 and E707 on GluK2 subunit (Mayer, 2005; Mayer et al., 2006). The electrostatic interactions are formed between glutamate molecules with the charged residues, such as R492(508) and E707(723).The presence of threonine and serine residues on GluK1 instead of alanine on GluK2 explains why GluK1 has higher binding affinity to glutamate (Lerma, 2003; Mayer, 2005).

1.3.4.4 Metabotropic glutamate receptor

Metabotropic glutamate receptors (mGluRs) are members of G-protein-coupled receptors, which modulate neuronal functions through intracellular signaling pathways. There are three groups of mGluRs with a total of eight receptor subtypes (Niswender and Conn, 2010). The dimerization of the receptors is required for glutamate-induced activation (Moustaine et al., 2012). The large extracellular domain of mGluRs is termed the Venus flytrap domain (VFD). Each VFD contains

two lobes and the glutamate-binding site is located at the cleft between them (Pin et al., 2003). Residues involve in glutamate binding includes Y74, R78, S164, S165, S186, T188, D208, Y236, E292, G293, D318, R323, K409 on mGluR1 and R64, R68, S151, A172, T174, Y222, S278, D301, K389 on mGluR3 (Acher and Bertrand, 2005; Tsuchiya et al., 2002). Using mGluR1 as a model, five residues (S186, T188, D208, Y236 and D318) are found to be critical for binding of the α-amino group and two basic residues (R78 and K409) are vital for binding of the γ-carboxyl group. S165 and T188 also involve in binding of the α-carboxyl group of the glutamate molecules (Wellendorph and Bräuner-Osborne, 2009).

1.3.4.5 Presence of charged residues in glutamate binding sites

The glutamate binding sites on glutamate receptors share several common characteristics. One outstanding feature of glutamate binding sites is the presence of the charged residues, which is crucial for securing the glutamate through electrostatic interactions (Table 1.2). The positively charged residues, such as arginine, histidine and lysine are responsible for anchoring the two negatively charged carboxyl groups of glutamate. And the positively charged amino group on glutamate requires the presence of at least one negatively charged residue, such as aspartic acid or glutamate to tightly bind to the pocket (Wellendorph and Bräuner-Osborne, 2009). Mutating those charged residues in the binding pocket leads to a significant increase of glutamate EC_{50} (Chen et al., 2005a). Other types of residues, such as hydroxyl containing residues (serine and threonine) and aromatic residues (tyrosine and phenylalanine), are also often involved in the glutamate binding. However, their modes of interactions with glutamate are complicated since both their backbones and side chains may contribute to the interactions (Armstrong et al., 1998; Chen et al., 2005a; Mayer, 2005; Tsuchiya et al., 2002).

Receptors	Critical residues for glutamate binding
AMPAR	GluA2: Y450, P478, T480, R485, S654, T655 and E705
NMDAR	GluN2A: H466, S492, T494, R499, F637, S670, T671 and D712 GluN2B: E387, F390, K459, H460, R493, V660, S664 and V709
KAR	GluK1: Y474, P501, T503, R508, S674, T675, E723 GluK2: Y457, P485, A487, R492, A658, T659, E707
mGluR	mGluR1: Y74, R78, S164, S165, S186, T188, D208, Y236, E292, G293, D318, R323, K409 mGluR3: R64, R68, S151, A172, T174, Y222, S278, D301, K389

Table 1.2 Glutamate binding residues on glutamate receptors.

Each binding site contains at least one positive charged residue (R, H or K, underlined) to interact with the negative charged carboxyl group of glutamate molecule and one negative charged residues (E or D, double underlined) to interact with the positively charged amino group of glutamate molecule.

1.4 Plasticity at inhibitory synapses in Purkinje cells

1.4.1 Overview of cerebellar Purkinje cells

1.4.1.1 Purkinje cell anatomy

Cerebellar Purkinje cells were first described in 1837 and named after their discoverer, Czech anatomist Jan Evangelista Purkyně. Their unique morphology makes them among the most distinguishable neurons in the brain. The characteristics of their structure are their large, spherical cell body and the highly branched dendritic arbors (Shepherd, 2004). In the cerebellum, Purkinje cell bodies are packed into a one-cell-thick layer, namely Purkinje layer, in the middle region of the cerebellar cortex. The dendrites of Purkinje cells project towards the outer layer (molecular layer) of the cerebellar cortex, and are flattened in a parasagittal plane perpendicular to the cerebellar folds (Kaneko et al., 2011; Pfaff, 2012). Purkinje cells are GABAergic neurons. Their axons grow into the deep cerebellar nuclei and exert inhibitory effect on the target cells (Obata and Takeda, 1969). This Purkinje cell-mediated inhibitory projection is the sole output of all cerebellar cortex signals (Purves et al., 2001; Shepherd, 2004). In this regard, Purkinje cells play a dominant role in cerebellar circuits.

1.4.1.2 Neuronal connections of Purkinje cells

The dendritic branches of each Purkinje cell are covered with around 200,000 dendritic spines, which receive two types of excitatory inputs from either the parallel fibers or the climbing fibers (Purves et al., 2001; Shepherd, 2004). The parallel fibers are the axons of granule cells, which receive afferent from mossy fibers, the major signal inputs of the cerebellum. The parallel fibers run vertically through the flattened dendritic arbor of Purkinje cells and form 80-100 synapses with Purkinje cell dendrites (Shepherd, 2004). Each Purkinje cell can receive input from more

than 100,000 parallel fibers, but the effect from each parallel fiber is very weak. It requires many parallel fibers to fire spontaneously in order to fire a single action potential in the Purkinje cell (simple spike), which may contribute to the precise timing of motor coordination (Heck and Sultan, 2002). The climbing fibers arise from the inferior olivary nucleus in the brainstem. The climbing fibers 'climb' up the dendritic tree of Purkinje cells and form around 300 synapses at the cell bodies and proximal dendrites regions (Shepherd, 2004). Each Purkinje cell only receives input from one climbing fiber. The excitatory inputs from climbing fibers are very strong, each of which can induce multiple action potentials in Purkinje cells (complex spikes) (Ohtsuki et al., 2009). The function of this strong input is still under debate, although most of the studies believe that it represents the error signals in motor performance and regulates the Purkinje cells' excitability by inducing long-term depression of the parallel fiber-Purkinje cell synapses (Apps and Garwicz, 2005; Coesmans et al., 2004; Ito, 2008).

Purkinje cell activity is also refined by the inhibitory inputs from two types of interneurons, namely basket cells and stellate cells (Ito, 1987; 2008). Those interneurons are located at the molecular layer and their dendrites lie in a plane parallel to that of the Purkinje cells dendrites (Shepherd, 2004). Basket cells are excited by parallel fibers and send powerful inhibitory projection to the axon initial segment of the Purkinje cells (Konnerth et al., 1990; Southan and Robertson, 1998). The feature of the basket cell-Purkinje cell connection is presence of the large-amplitude miniature IPSCs, which is due to the spontaneous multivesicular release from the ryanodine-sensitive calcium stores at the basket cell presynaptic terminals (Conti et al., 2004; Llano et al., 2000). On the contrary, although also receive afferent from the parallel fiber, the stellate cells send inhibitory projection onto Purkinje cell distal dendrites, which results in

smaller IPSCs (Hirano et al., 2002; Shepherd, 2004). Stellate cells are believed to protect the Purkinje cell from the over-excitation by parallel fibers (Hirano et al., 2002; Marr, 1969). It has also been found that inhibitory signals from stellate cells can prevent the Purkinje cell long-term depression induced by simultaneously activation of parallel fibers and climbing fibers (Ekerot and Kano, 1985; Kleim et al., 1997) (Figure 1.3).

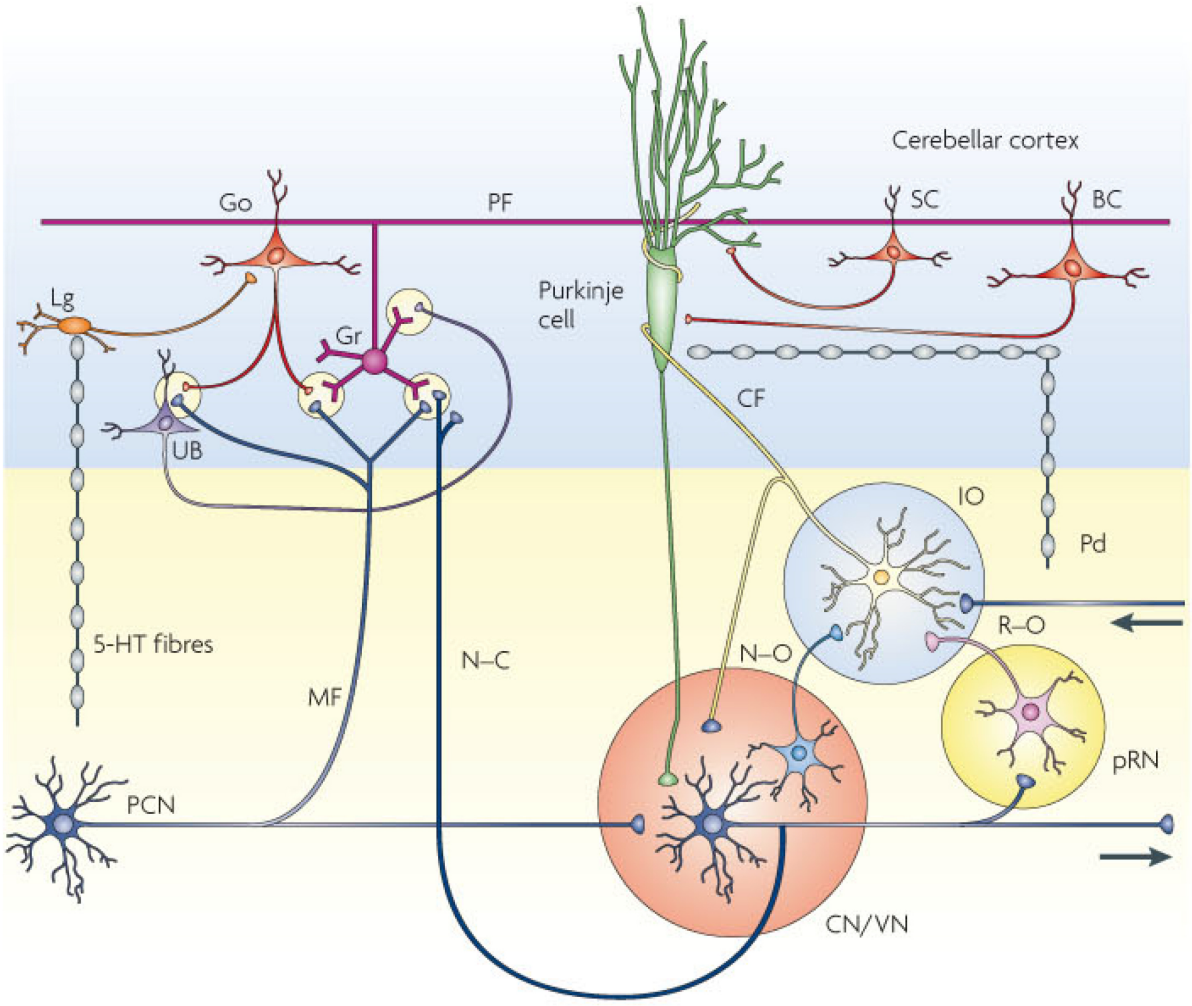

Figure 1.3 Neuronal connections of Purkinje cells

Purkinje cells play dominant roles in the cerebellar circuits. It received excitation inputs from the climbing fibers (CF) and parallel fibers (PF); and generates outputs to the cerebellar nuclei/vestibular nuclei (CN/VN). The inhibitory inputs in PCs are from stellate cells (SC) and basket cells (BC), which refine the information processing. Abbreviations: PF, parallel fiber; CF, climbing fiber; SC, stellate cell; BC, basket cell; CN/VN, cerebellar nuclei/vestibular nuclei; Go, Golgi cell; Gr, granule cell; IO, inferior olive; Lg, Lugaro cell; MF, mossy fiber; N–C, nucleo–cortical projection; N–O, nucleo–olivary projection; PCN, precerebellar nucleus; Pd, peptidergic fiber; pRN, parvocellular red nucleus; R–O, rubro– olivary projection; UB, unipolar brush cell; 5-HT, 5-hydroxytryptamine (serotonin). Reprinted with permission from Ito, M. Control of mental activities by internal models in the cerebellum. *Nat Rev Neurosci* 9.4, 304–313 (2008).

1.4.1.3 Cerebellum and Purkinje cell function

The cerebellum is the brain region responsible for motor coordination. It participates in precise and effective execution of purposeful movements as well as the presence of appropriate posture (Brooks, 1984; Strata, 2009). Cerebellar dysfunction results in abnormal movements, such as ataxia and dysmetria (Manto and Marmolino, 2009; Schmahmann, 2004). It is also involved in some cognitive activities such as attention and language, and in regulating fear and pleasure responses (Gillig and Sanders, 2010; Schmahmann and Caplan, 2006; Strick et al., 2009; Wolf et al., 2009). It has been shown that cerebellar diseases can cause potential cognitive deficits or even personality changes (Hoche et al., 2015; Rapoport et al., 2000; Timmann et al., 2010).

As the sole output of the cerebellum, the Purkinje cell is the heart of cerebellar signal processing. The lack of feedback system makes the information passes through the Purkinje cell in a quick and precise manner (Eccles, 2013). The Purkinje cell also plays an important role in adaptive motor learning. Climbing fiber receives the motor error signals from the spinal cord and adjust the Purkinje cell activity accordingly by inducing long-term depression at the Parallel fiber-Purkinje cell synapse (Apps and Garwicz, 2005; Ito, 2008). Purkinje cells dysfunctions are observed in many motor-related neurological disorders, such as Huntington's disease, Parkinson’s disease and multiple sclerosis, and are associated with motor deficiency symptoms in these diseases (Giuliani et al., 2011; Kishore et al., 2014; Redondo et al., 2015; Ruegsegger et al., 2016; Tu et al., 1997). The link between Purkinje cell and autism is also heavily discussed recently, as studies found the Purkinje cell abnormality may contribute to many autistic

symptoms such as impairment of motor control, delays in language skills and social deficits (Fujita et al., 2012; Lotta et al., 2014; Piochon et al., 2014; Skefos et al., 2014).

1.4.2 Purkinje cell inhibitory plasticity

There are three major types of plasticity found at the interneuron-Purkinje cell synapse, depolarization-induced suppression of inhibition (DSI), rebound potentiation (RP), and depolarization-induced potentiation of inhibition (DPI). All of them can be induced by depolarization of postsynaptic Purkinje cells, but different downstream signaling pathways are involved in each type of plasticity (Figure 1.4).

1.4.2.1 Depolarization-induced suppression of inhibition

Depolarization-induced suppression of inhibition (DSI) in Purkinje cells was first observed in 1991. Researchers have found that postsynaptic depolarization of Purkinje cells induces calcium entry and subsequently suppresses the inhibitory GABAergic current recorded from Purkinje cells for a short period of time (~ 60s) (Llano et al., 1991). The studies of similar phenomenon in hippocampal CA1 pyramidal cells largely facilitate our understanding of the Purkinje cell DSI (Ohno-Shosaku et al., 1998; Pitler and Alger, 1992). Although originally thought as a presynaptic mGluR-mediated event (Morishita et al., 1998), the DSI in CA1 pyramidal cells is found to be through the endocannabinoids retrograde signaling pathway (Maejima et al., 2001b; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001; Wilson et al., 2001). Following these studies, researchers have tested the role of endocannabinoids and their receptors in Purkinje cell DSI and show that either CB1 receptor antagonist or knockout of CB1 receptors can eliminate DSI (Yoshida et al., 2002). Thus, it is proposed that the depolarization-induced intracellular

calcium elevation in postsynaptic Purkinje cells initiate the synthesis of endocannabinoids, which retrogradely activate the presynaptic CB1 receptors and reduce GABA release. Purkinje cell depolarization can be achieved by stimulation from either parallel fiber or climbing fiber. It is interesting that climbing fiber stimulation can suppress the GABA release from basket cells through a second pathway: activation of basket cell presynaptic AMPAR by glutamate-spillover from climbing fiber terminals (Rigby et al., 2015; Satake et al., 2004).

The similar effect, namely depolarization-induced suppression of excitation (DSE), has been observed at the excitatory synapses of the Purkinje cells (Kreitzer and Regehr, 2001a). Although controversy exists, studies have shown the involvement of endocannabinoids system in DSE, similarly as that of DSI (Kreitzer and Regehr, 2001b; Maejima et al., 2001a). DSI has also been discovered in many other brain regions, such as the basal ganglia, cortex, amygdala, and hypothalamus (Bodor et al., 2005; Jo et al., 2005; Katona et al., 2001; Mátyás et al., 2006). Recently, it has been shown that facial stimulation of anesthetized mice induced Purkinje cell DSI *in vivo*, indicating DSI may contribute to the conversion of the sensory inputs into the motor learning signals (Bing et al., 2015).

1.4.2.2 Depolarization-induced potentiation of inhibition

Similar to DSI, depolarization-induced potentiation of inhibition (DPI) is also a type of presynaptic plasticity. It has been found that, following the initial DSI, depolarization of Purkinje cells can also cause a long lasting potentiation of GABA transmission, represented by the increase of IPSC frequency, at the interneuron–Purkinje cell synapse through activation of presynaptic NMDARs (Duguid and Smart, 2004). It is known for a long time that activation of

presynaptic NMDAR enhances GABA release from basket and stellate cells (Banerjee et al., 2016; Glitsch and Marty, 1999; Glitsch, 2008), although some controversies exist (Pugh and Jahr, 2011). This study has further suggested that a calcium-dependent retrograde messenger, presumably glutamate, is involved in this process. The following study has confirmed the presence of glutamate release machinery at the postsynaptic site and its involvement in DPI (Crepel et al., 2011; Duguid et al., 2007). This type of dendritic glutamate release has also been observed in several other types of Purkinje cell plasticity, such as depolarization-induced slow current (DISC) and late phase of DSE (Crepel, 2007; 2009; Crepel et al., 2011; Duguid et al., 2007; Shin et al., 2008). Thus, dendritic glutamate release may play a unique role in regulation of Purkinje cell neurotransmission.

Notably, glutamate spillover from excitatory synapses may make additional contribution to NMDAR activation. Studies have found that either bust stimulation or stimulation with a pattern mimicking physiological activities can induce glutamate spillover from parallel fibers which activates stellate cell presynaptic NMDARs and subsequently leads to a lasting increase of presynaptic GABA release at the stellate cell-stellate cell synapse or the stellate cell autaptic synapse (Dubois et al., 2016; Lachamp et al., 2009; Liu and Lachamp, 2006). This type of activity-dependent glutamate spillover from parallel fibers has also been reported in several other studies before (Carter and Regehr, 2000; Clark and Cull-Candy, 2002). However, Purkinje cells are tightly wrapped by Bergmann glial cells, which express high densities of glutamate transporters. It has been found that inhibition of glutamate transporters on Bergmann cells significantly increases the mIPSC frequency, presumably through glutamate spillover-presynaptic NMDAR pathway (Huang and Bordey, 2004). However, whether the glutamate

spillover can reach the interneuron-Purkinje cell synapse under physiological conditions is still unknown.

1.4.2.3 Rebound potentiation

At the interneuron-Purkinje cell synapse, depolarization of Purkinje cell can affect not only the presynaptic GABA release efficacy (as in DSI and DPI), but also the postsynaptic responsiveness to GABA. The rebound potentiation (RP), characterized by a long-lasting (up to 75 min) increase of IPSC amplitude, can be induced by either climbing fiber stimulation or direct depolarization of Purkinje cells. A calcium-dependent pathway is suggested to be responsible for this up-regulation of postsynaptic GABA responsiveness (Kano et al., 1992). Mechanism studies has shown that calcium influx via voltage-gated calcium channel and subsequent activation of CaMKII are involved in this process (Kano et al., 1996). Interestingly, blocking PKA, the cAMP-dependent protein kinase, is also able to diminish RP (Kano and Konnerth, 1992). The interaction of CaMKII and PKA pathways in RP induction has been discussed in later studies, suggesting that PKA phosphorylates DARPP-32 and release CaMKII from the inhibition of protein phosphatase 1 (PP-1). In contrast, activation of metabotropic GABA receptor ($GABA_BR$) can suppress RP by inhibiting PKA activation (Kawaguchi and Hirano, 2000; 2002). Following researches have revealed the role of calcineurin and DPE1 (a Ca^{2+}/calmodulin-dependent phosphodiesterase) in determining the thresholds and temporal integration of intracellular calcium signaling in RP induction (Kawaguchi et al., 2011; Kitagawa et al., 2009). The CaMKII autophosphorylation and CaMKII-mediated inhibition of PDE1 may also be involved in RP, further complicated the signaling cascade for RP induction (Kawaguchi et al., 2011; Kitagawa et al., 2009).

CaMKII is the central part of the RP signaling cascade. It is well known that CaMKII can directly phosphorylate the β and γ2 subunits of the $GABA_AR$ and enhance the amplitude of GABA-induced current (Houston et al., 2007; 2009; McDonald and Moss, 1994). Thus, the endogenous $GABA_AR$, which contains α1β2/3γ2 subunits, in Purkinje cells may also be susceptible to CaMKII-induced phosphorylation (Laurie et al., 1992a; Pirker et al., 2000). A recent study has shown that RP relies on the phosphorylation of $GABA_AR$ β2 subunits and subsequent increase of $GABA_AR$ surface expression (He et al., 2015). The CaMKII-induced conformational change of $GABA_AR$ associated protein (GABARAP) is also vital for RP induction (Kawaguchi and Hirano, 2007). Since anchoring of the $GABA_AR$ on the cytoskeleton protein tubulin is mediated by the structural altered GABARAP, the activation of CaMKII may induce RP by facilitating the receptor trafficking and surface expression of $GABA_ARs$ (Chen and Olsen, 2007; Kanematsu et al., 2007; Leil et al., 2004).

RP has been proposed as a contributor to motor learning for a long time (Hirano and Kawaguchi, 2014; Kano et al., 1992; Kawaguchi and Hirano, 2002). To test this hypothesis, a transgenic mouse strain was developed recently with Purkinje cell specific expression of a peptide that blocked the binding between the $GABA_AR$ and GABARAP. As expected, the RP could no longer be induced in this mice strain. Vestibulo-ocular reflex (VOR) was tested and the RP-deficient mice showed normal baseline VOR dynamics, but impaired VOR adaptation during training. However, the adaptation of optokinetic response, another type of eye reflex is intact in the RP-deficient mice (Tanaka et al., 2013). These results suggest a physiological role of RP as it contributes to some but not all types of motor learning.

It is worth noting that, after depolarization of Purkinje cells, there is a transient potentiation of $GABA_AR$ function (up to 10 min) that is independent with CaMKII and PKA pathways. This component of RP, termed as the early phase of RP, is not well studied. One cue comes from a study showing that blockade of postsynaptic vesicular transportation by N-ethylmaleimide (NEM), guanosine 5-[β-thio]diphosphate (GDP-β-S) or botulinum neurotoxin light-chain B (BoNT-B) can abolish both the late phase of RP by interruption of $GABA_AR$ trafficking, but also the early phase of RP (He et al., 2015), suggesting a role of postsynaptic vesicle in the transient augment of the $GABA_AR$ function. Previous studies on DPI have already revealed that, postsynaptic vesicles are also involved in the dendritic glutamate release at the interneuron-Purkinje cell synapses (Crepel et al., 2011; Duguid and Smart, 2004; Duguid et al., 2007). Since glutamate has been previously indicated as a positive allosteric modulator of the $GABA_AR$ (Stelzer and Wong, 1989), it is possible that depolarization-induced dendritic glutamate release is one of the mechanisms underlying the early phase of rebound potentiation.

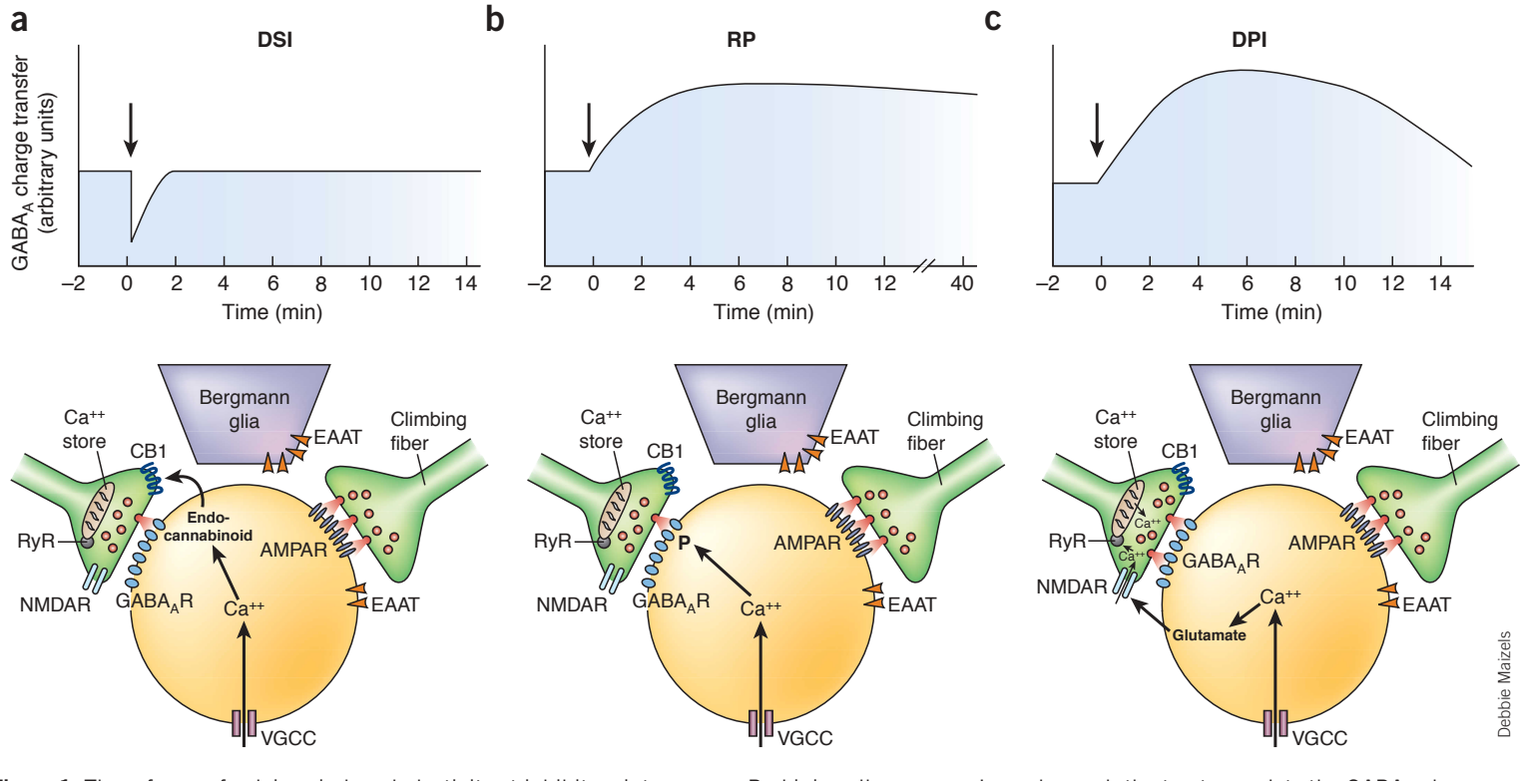

Figure 1.4 Inhibitory plasticity of Purkinje cells

a) Depolarization induced suppression of inhibition (DSI) is mediated by calcium-dependent endocannabinoids release and retrograde activation of CB1 receptors that reduces presynaptic GABA release. **b)** Rebound potentiation (RP) is through calcium activated kinase cascades that increase $GABA_AR$ surface expression and enhance GABA responsiveness. **c)** Depolarization induced potentiation of inhibition (DSI) is induced by dendritic glutamate release and retrograde activation of presynaptic NMDAR that increases GABA release probability. Reprinted with permission from Tzingounis, A. V. & Nicoll, R. A. Presynaptic NMDA receptors get into the act. *Nat Neurosci.* 7, 419-420 (2004).

1.5 Rationale, hypothesis and objectives

Stelzer and Wong (1989) have shown that glutamate and several glutamate analogs can reversibly potentiate the GABA-induced chloride current in acute isolated hippocampal neurons (Stelzer and Wong, 1989). However, whether it is through direct interaction between glutamate and the $GABA_AR$, or through activation of certain glutamate receptors and heterosynaptic regulation of $GABA_ARs$ is not clear. Recently, our lab found that glutamate allosterically potentiate GlyRs-mediated current in both neurons and HEK293 cells expressing recombinant GlyRs (Liu et al., 2010). Taking the structural homology between GlyRs and $GABA_ARs$ into consideration, we hypothesize that **the excitatory neurotransmitter glutamate may serve as a positive allosteric modulator of the inhibitory $GABA_AR$**. The main objective of this dissertation is to test this hypothesis by pursuing the following specific aims:

1. **Investigate the phenomenon and determine the underlying mechanism of the glutamate-induced potentiation of $GABA_AR$ function.** As discussed above, we consider that **glutamate-induced potentiation of $GABA_ARs$ is also through allosteric interaction between glutamate and $GABA_ARs$.** Following Stelzer and Wong's work, in Chapter 3, we confirmed their discovery using cultured hippocampal neurons. With recombinant $GABA_ARs$ expressed in HEK293 cells, we further illustrated that glutamate-induced potentiation effect is through the ligand binding to the $GABA_AR$ but not a glutamate receptor/protein. A binding assay with [^{3}H]-glutamate was conducted to demonstrate the physical binding of glutamate on the $GABA_AR$.

2. **Identify and characterize the glutamate-binding site on the $GABA_AR$.** The binding site of an allosteric modulator determines its unique property. Thus, it is important to identify and characterize the glutamate-binding site on $GABA_ARs$. Analysis of previously identified $GABA_AR$ allosteric binding site reveals the subunit interface to be a critical region for ligand binding for modulation (Chiara et al., 2013; Maldifassi et al., 2016; Sigel, 2002; Wallner et al., 2014). In this region, small molecules (i.e. glutamate) which bind in between two subunits are more likely to cause conformation changes, leading to functional alteration and modulation effects of the receptor. Small molecules binding to surface sites on individual subunits do not usually cause modulation effects because they are less likely to cause the necessary conformational changes for the modulation to occur. (Hibbs and Gouaux, 2011). Thus, we hypothesize that **the glutamate-binding site is located at the subunit interface of the $GABA_AR$**. In Chapter 4, with the help of systematic mutagenesis analysis, we identified this glutamate-binding site at α+/β- interface of the $GABA_AR$. Further characterization showed that both spatial and electrostatic accessibilities to the pocket are critical for glutamate binding. This binding site is conserved among all α and β subunit families, suggesting glutamate as the modulator of most, if not all $GABA_ARs$.

3. **Investigate the potential of the glutamate-binding site as a novel target for developing $GABA_AR$-based therapeutics**. As the principal inhibitory receptors in the CNS, $GABA_AR$ is an important target for therapeutics development. Benzodiazepine is the most widely used $GABA_AR$ positive allosteric modulator, demonstrating strong potency in treating neurological diseases, such as epilepsy. However, the effect of

benzodiazepine depends on the presence of γ subunit, which limits its application (Sigel, 2002). Moreover, long-term exposure to benzodiazepine may cause drug resistance as a result of subunit switch (Deeb et al., 2012; Vinkers and Olivier, 2012). In contrast, drug that binds to α+/β- interface has broader effect on $GABA_AR$, as 95% of $GABA_ARs$ contain α and β subunits and may be suitable for long-term treatment (Mohler, 2006; Ramerstorfer et al., 2011). Since the glutamate binding pocket is also located at the α+/β- interface, we hypothesize that **this glutamate binding site can be a more preferable therapeutic target for developing broad-spectrum $GABA_AR$ modulators**. In Chapter 5, using *in silicon* and electrophysiological screening, we identified ampicillin and BRC640 as novel positive modulators of the $GABA_AR$ targeting the glutamate-binding site. As expected, they enhanced both the phasic and tonic inhibition mediated by $GABA_AR$, and thus served as candidate drugs for future optimization.

4. **Elucidate the physiological role of glutamate/$GABA_AR$ crosstalk in Purkinje cell inhibitory plasticity.** Depolarization of Purkinje cells leads to a calcium-dependent potentiation of postsynaptic $GABA_ARs$ functions (rebound potentiation, RP), reflected by the increased amplitude of IPSCs (Kano et al., 1992). The late phase of RP was found through the CaMKII and PKA pathways (Kawaguchi and Hirano, 2000; 2002), but the mechanism of the early phase is unknown. Meanwhile, PC excitation results in another type of inhibitory plasticity called depolarization-induced potentiation of inhibition (DPI), which is characterized by the increased frequency of IPSCs (Duguid and Smart, 2004). The mechanism of DPI involves calcium-induced dendritic glutamate release at the inhibitory postsynaptic site and retrograde activation of presynaptic NMDARs to enhance

GABA release probability (Crepel et al., 2011; Duguid and Smart, 2004; Duguid et al., 2007). Considering the spatial proximity between the dendritic glutamate releasing site and the $GABA_AR$ in Purkinje cells, it is highly possible that the glutamate can directly interact with postsynaptic $GABA_ARs$ and potentiate their function. Therefore, we hypothesize that **following depolarization of PCs, dendritically released glutamate may serve as an autocrine factor that potentiates the nearby $GABA_ARs$, and this mechanism may fully or partially contribute to the early phase of RP.** Model of our hypothesis is illustrated in Figure 1.5. In Chapter 6, we tested this hypothesis using the compound we identified in Chapter 5 to determine the presence of glutamate/glutamate-like compound induced potentiation on Purkinje cell expressed $GABA_ARs$. Then, we conducted an occlusion experiment demonstrating that occupying the glutamate-binding site before Purkinje cell depolarization would prevent the induction of early phase of RP. These results indicate the glutamate-$GABA_AR$ crosstalk may play a unique role in Purkinje cell inhibitory plasticity.

Overall, the study in this dissertation revealed that glutamate, a classic excitatory neurotransmitter, enhanced the function of $GABA_AR$, the principle inhibitory receptor in the brain via a mechanism of allosteric modulation. The discovery of the glutamate-binding site on the $GABA_AR$ blurs the traditional distinction between excitatory and inhibitory neurotransmitters. Moreover, the unique location of the glutamate-binding site makes it a great target for future drug development. Finally, our study on the Purkinje cell inhibitory plasticity demonstrated one of the possible physiological roles that the glutamate/$GABA_AR$ interaction may play and emphasized its significance in regulating neuronal excitability. Taken together, this study largely

enhanced our understanding on the endogenous modulation of the $GABA_AR$ and revealed a novel form of excitation/inhibition crosstalk, which may lead to the redefinition of the concept of a classic excitatory or inhibitory neurotransmitter.

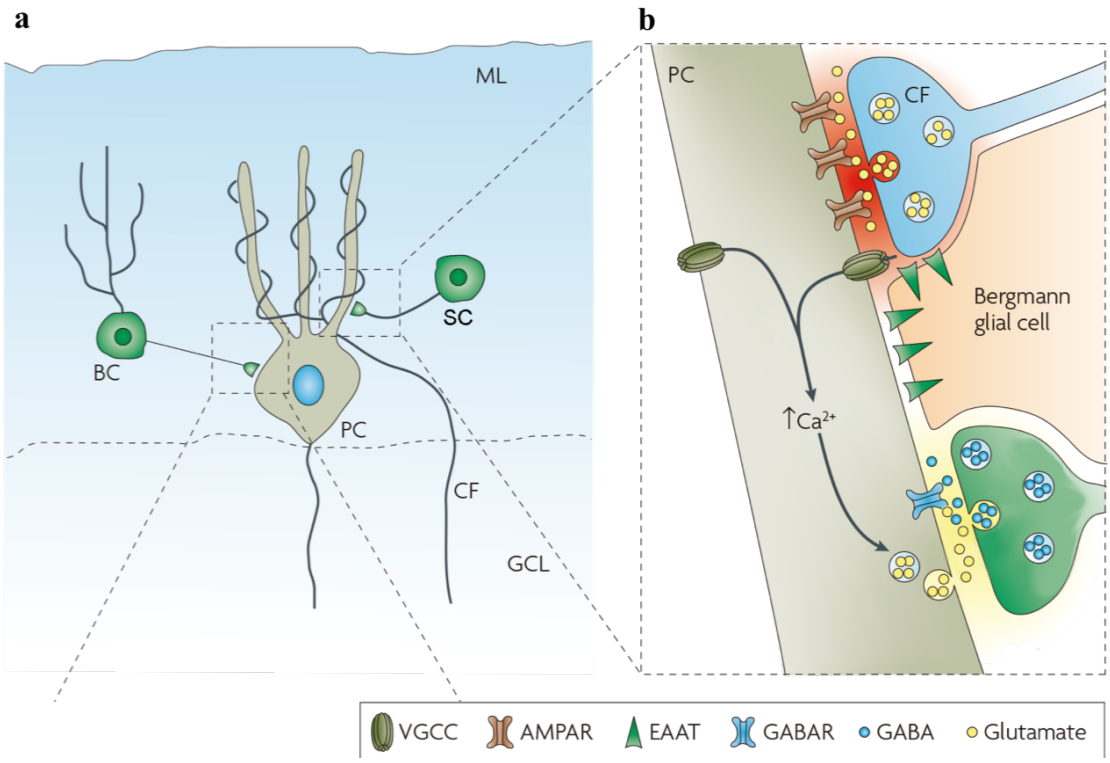

Figure 1.5 Model of the dendritic glutamate release and autocrine potentiation of $GABA_AR$s on Purkinje cells

a) Purkinje cells receive inhibitory inputs from basket cell (BC) and stellate cell (SC) as well as excitatory inputs from climbing fiber (CF) and parallel fiber (not shown here). **b)** Depolarization of Purkinje cell by climbing fiber or direct current injection causes elevated intracellular calcium level and induces dendritic glutamate release near inhibitory synapse of Purkinje cells. Glutamate diffuses to postsynaptic $GABA_AR$ and produces autocrine potentiation of PC inhibitory synapse. Modified with permission from Pinheiro, P. S. & Mulle, C. Presynaptic glutamate receptors: physiological functions and mechanisms of action. *Nat Neurosci.* 9.6, 423-436 (2008).

Chapter 2: Methods and material

2.1 Neuronal culture

Cultured hippocampal and cerebellar neurons were prepared from the brains of D18-19 fetal Wister rats. Tissues were digested with a 0.05% trypsin-EDTA solution (Invitrogen) for 15-30 min at 37 °C, and then triturated with the 100-300 µl pipette tips to ensure a single cell suspension. Next, the cell suspension was centrifuged at 2500 ×g for 50 s and the cell pellets were resuspended in Neurobasal media containing 0.5mM GlutaMAX-1 and 2% B27 supplement. Cells were seeded on poly-D-lysine-coated 24-well coverslips at a density of 1.5×10^5 cells/well for hippocampal neurons and 4×10^5 cells/well for cerebellar neurons. Cultures were maintained in a humidified incubator with 5% CO_2 at 37 °C. Media swap was performed every 4 days thereafter. Hippocampal neurons were used for electrophysiological recordings 10-14 days after plating. Cerebellar neurons were used 7-9 days after plating.

2.2 HEK293 cell culture and transfection

HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were grown to 70-80% confluence and transiently transfected by using Lipofectamine 2000 with 1:0.5-1 plasmid/lipid ratio. Cells were transfected with a combination of pcDNA3-CMV expression vectors, each of which expressed one of the rat recombinant $GABA_AR$ α, β or γ2 subunits. The transfection ratio with α/β/γ2 and α/β plasmids was 2:2:1 and 1:1, respectively. pcDNA3-GFP was co-transfected with $GABA_AR$ subunits in order to facilitate the visualization of the transfected cells during electrophysiological experiments. Cells were re-plated on poly-D-lysine-coated glass coverslips in 24-well plates after 24 hours transfection and were cultured for an additional 24 hours before whole-cell patch-clamp recordings.

2.3 Site-directed mutagenesis

The site-directed mutagenesis of α1 or β2 subunits were performed using the QuikChange method (Stratagene). All mutant clones were confirmed by DNA sequencing. Wild-type or mutant subunits were transfected in HEK293 cells and subjected to electrophysiology examinations.

2.4 Electrophysiology

Whole cell patch-clamp 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 for HEK293 cells and cultured hippocampal neurons, and -70mV for cultured cerebellar neurons. Signals were filtered at 2 kHz, digitized at 10 kHz (Digidata 1322A). Recording pipettes (3-5 MΩ) were filled with the intracellular solution containing (mM): CsCl 140, HEPES 10, Mg-ATP 4, QX-314 5, pH 7.20; osmolarity, 290-295 mOsm. BAPTA (10 mM) was added in the intracellular solution for HEK293 cell and hippocampal neuron recording and BAPTA (0.1mM) was used for Purkinje cell recording to minimize the interruption of intracellular calcium signaling. The extracellular solution contains (mM): NaCl 140, KCl 5.4, HEPES 10, $MgCl_2$ 1.0, $CaCl_2$ 1.3, glucose 20, pH 7.4; osmolarity, 310-315 mOsm. NMDA current was recorded in Mg^{2+} free extracellular solution.

GABA induced currents were evoked by applying GABA through perfusion fast-step system (Warner Instruments) using a two-square barrel glass tubing. For recordings of mIPSCs and GABA evoked postsynaptic currents in cultured neurons, CNQX (10 μM) and TTX (0.5 μM)

were added in the extracellular solution to minimize the activation of ionotropic glutamate receptors and voltage-gated sodium channels, respectively. For sIPSC recording, only TTX (0.5 μM) was added. Stimulation protocol for RP and DPI induction followed the work of Duguid & Smart (Duguid and Smart, 2004). The membrane potential of Purkinje cells was stepped from -70 mV to 0 mV for 5 s. All experiments were performed at room temperature. The evoked current was analyzed using Clampfit 10. mIPSC and sIPSC were analyzed using MiniAnalysis 6.0.

2.5 [^{3}H]-glutamate binding assay

For membrane preparation, transfected HEK293 cells were washed twice with cold PBS and harvested by scraping into 5 ml cold PBS. Cells were then centrifuged at 1200 x g for 12 min at 4°C and medium was removed. The washing procedure was repeated once by resuspending the cell pellet into 5 ml cold PBS and centrifuging again. Then the cell pellet was re-suspended into 1ml of 50 mM Tris-HCl buffer (pH 7.4, with protease inhibitor) and homogenized using syringes with 18-G, 21-G and 23-G needles.

To separate the membrane, the homogenate was centrifuged for 20 min at 20,000 x g at 4°C. Then the pellet was re-suspended into 1ml of 50 mM Tris-HCl buffer, pH 7.4 and centrifuged again. After repeating this procedure for one more time, the pellet was re-suspended into 1ml of 50 mM Tris-HCl buffer, pH 7.4 and the protein concentration was measured.

To conduct the binding assay, 100 μg membrane was incubated with 40 nM [^{3}H]-Glutamate in a total volume of 0.5 ml in 50 mM Tris-HCl buffer, pH 7.4 for 1h on ice. For competition assay,

0.4 mM non-labeled glutamate was added with 40 nM [^{3}H]-Glutamate in a total volume of 0.5 ml. Then the reaction was terminated by quickly filtering the solution on Whatman filter paper and washing by 3.5 ml Tris-HCl buffer. Radioactivity was measured in a Beckman liquid scintillation counter.

2.6 Data analysis

Values are expressed as mean ± SEM (n = number of experiments). The two-tailed Student's t test was used for comparison of two groups. Comparison of three or more groups was done using one-way ANOVA test followed by Tukey's post hoc analysis. P values less than 0.05 were considered statistically significant. In figures, * represents $P<0.05$, ** represents $P<0.01$. Concentration–response curves were created by fitting data to Hill equation: $I = Imax/[1+(EC_{50}/[A])^n]$, where I is the current, Imax is the maximum current, [A] is a given concentration of agonist, n is the Hill coefficient

2.7 Materials

Neurobasal Media, B27 supplement, GlutaMAX-I supplement, fetal bovine serum (FBS), DMEM, Trypsin-EDTA and Lipofectamine 2000 were purchased from Thermo Fisher Scientific (Waltham, MA, US). N-methyl-D-aspartate (NMDA), D-2-amino-5-phosphonovaleric acid (APV), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), kinate acid (KA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), TBOA, MK-801, TTX, Mg-ATP 4, QX-314 and CsCl were purchased from Tocris (Ellisville, Missouri, US). L-Glutamic acid, [^{3}H]-Glutamate, gamma-Aminobutyric acid (GABA), ampicillin sodium salt (Amp), Hepes, Glucose and poly-D-lysine hydrobromide were purchased from Sigma-Aldrich (St. Louis, MO, US). Bicuculline

methobromide was purchased from Alexis Biochemicals (Farmingdale, NY, US). The drugs subjected to screening was purchased from Vitas-M lab (BRC366, BRC204, BRC021, BRC888), 3B SCIENTIFIC CORP (BRC487), nci/ABI Chem (BRC334, BRC562, BRC640, BRC213, BRC071, BRC421, BRC997, BRC183, BRC073) and AKOS (BRC648, BRC649, BRC603, BRC588, BRC419)

Chapter 3: Glutamate as a positive allosteric modulator of the $GABA_AR$

3.1 Introduction

Chemical neurotransmission is the major form of neuronal communication in the CNS. Signaling molecules, i.e. neurotransmitters, are released by the presynaptic neurons, diffuse across synaptic cleft, bind to and activate the receptors on the postsynaptic neurons. Neurotransmissions are classified as excitatory or inhibitory according to their effects on postsynaptic neurons. Excitatory neurotransmission is mediated by excitatory neurotransmitter glutamate. It activates cation-selective ionotropic receptors, such as AMPARs and NMDARs, to depolarize the target cell and to increase the probability of firing an action potential (Traynelis et al., 2010). By contrast, inhibitory neurotransmission involves releasing of inhibitory neurotransmitters, GABA or glycine, which bind to $GABA_ARs$ or glycine receptors respectively. Activation of these inhibitory receptors leads to chloride influx, which hyperpolarizes the cell and reduces neuronal firing (Farrant and Nusser, 2005; Fatima-Shad and Barry, 1993).

The balance between excitatory and inhibitory neurotransmissions (E/I balance) is essential for maintaining normal brain functions. Processing of neural information is thought to occur by integration of excitatory and inhibitory neuronal inputs (Heiss et al., 2008; Shew et al., 2011; Yizhar et al., 2011). Also, epilepsy, insomnia and many psychiatric and neurodegenerative diseases have been linked to unbalanced synaptic excitation and inhibition (Cirelli and Tononi, 2015; Dudek, 2009; Gogolla et al., 2009; Hynd et al., 2004; Yates, 2011). Several models of the reciprocal interactions between excitatory and inhibitory systems have been proposed, ranging from network-level, cellular-level to receptor-level. Network-level interactions include those feedback or recurrent inhibitory circuits between excitatory and inhibitory elements in a neuronal

network (Windhorst, 1996). Cellular-level regulation is through intracellular signaling pathways, such as G-protein signaling triggered by metabotropic excitatory amino acid receptors that regulates inhibitory synaptic transmission (Errington et al., 2011; Hanson and Smith, 1999) and NMDAR initiated signaling pathway that modulates the $GABA_AR$ functions (Marsden et al., 2007; Muir et al., 2010; Potapenko et al., 2013). However, the excitation/inhibition crosstalk at the receptor level is not as well documented. This type of E/I crosstalk is achieved through direct neurotransmitter-receptor interaction. One well-studied example is the glycine/NMDAR interaction, in which glycine, an inhibitory neurotransmitter, allosterically acts on NMDAR and modulates its function (Lerma et al., 1990; Papouin et al., 2012).

In 1989, Stelzer and Wong reported that glutamate, the principal excitatory neurotransmitter, enhanced inhibitory $GABA_AR$-mediated current in acute dissociated hippocampal pyramidal cells (Stelzer and Wong, 1989). They also found that applying several glutamate analogs, such as APV, NMDA, KA, to hippocampal neurons also mimicked the effect of glutamate on GABA-induced current, which suggested these agents might exert the potentiation effect by acting at a common binding site on the $GABA_AR$. This study provided us with another strong evidence on the receptor-level E/I regulations. However, the detailed mechanism behind this phenomenon is still unclear. Recently, our lab has found that glutamate also elicits a potentiation effect on the glycine receptors, another type of inhibitory receptor (Liu et al., 2010). This potentiation effect is independent of the activation of any known glutamate receptors and is very likely caused by direct allosteric interaction between glutamate and the glycine receptor. Considering the structural similarity between the $GABA_AR$ and the glycine receptor (Connolly and Wafford, 2004), we hypothesize that glutamate may also potentiate the $GABA_AR$ function through a

similar allosteric interaction, which can be a novel model for fast excitatory/inhibitory neurotransmission crosstalk.

3.2 Results

3.2.1 Glutamate analog, APV, potentiates GABA-induced current in cultured hippocampal neurons

Following Stelzer and Wong's work, we first confirmed the existence of glutamate/glutamate analog-induced potentiation effect in hippocampal neurons. Since Stezler and Wong have shown that the NMDA receptor antagonist APV could mimic glutamate in potentiating $GABA_AR$ functions (Stelzer and Wong, 1989), we used APV instead of glutamate in this experiment to avoid the activation of any known native ionotropic glutamate receptors. Whole-cell voltage clamp recording was conducted on Day 10-14 culture hippocampal neurons with a pipette filled with a Cl^- based intracellular solution and at a holding membrane potential of -60mV. 0.5 μM Tetrodotoxin (TTX) and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were added into the extracellular solutions (ECS) to block both action potentials and AMPA/kainate receptor mediated current. Fast perfusion of GABA (0.5 μM) evoked an inward current (Figure 3.1a). Co-application of APV with GABA reversibly and significantly potentiated the GABA-induced currents (157.7 ± 9.1% of the control, $P < 0.01$, $n = 9$; Figure 3.1b). The APV-potentiated currents were completely blocked by applying 10μM Bicuculline, a $GABA_AR$ antagonist, suggesting there is no activation of other ionotropic receptors except for $GABA_ARs$ (Figure 3.1a). Through systematic concentration-response analysis, we confirmed that the APV-induced potentiation of GABA currents was concentration-dependent with an EC_{50} of 150 ± 27 μM (Figure 3.1c).

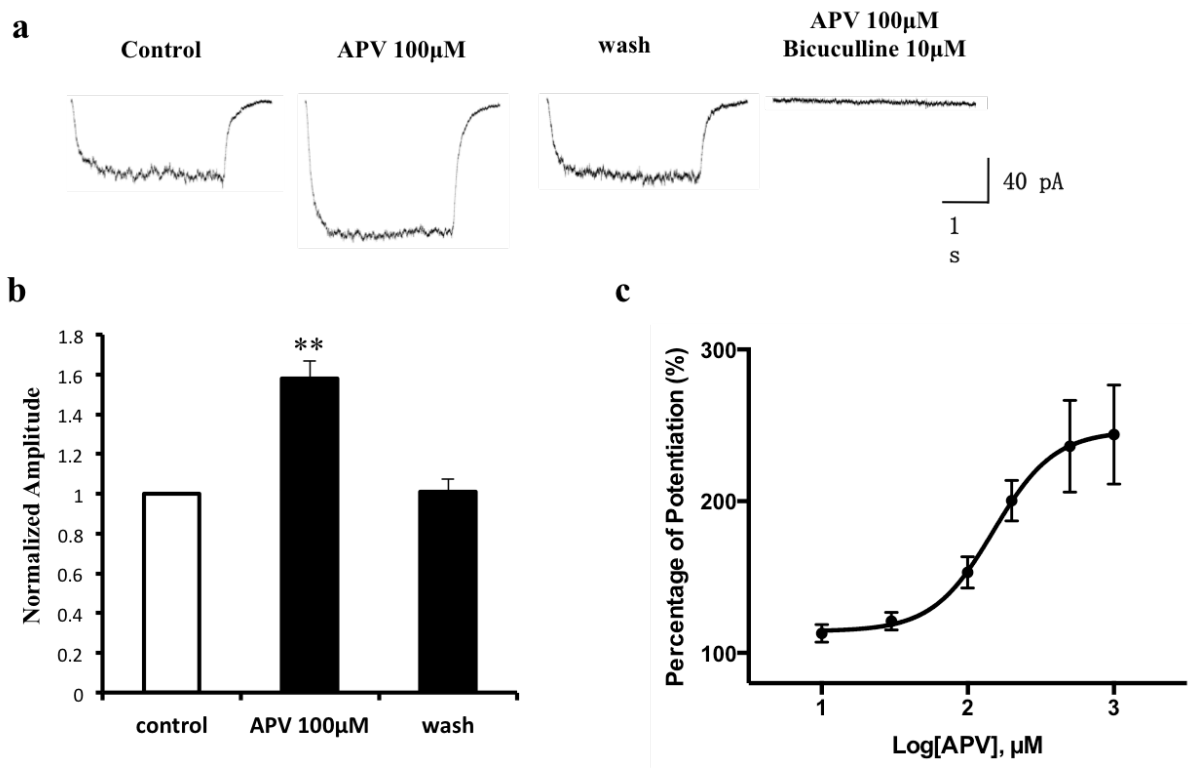

Figure 3.1 APV enhanced GABA-induced current in cultured hippocampal neurons

a) Representative traces showing GABA (0.5 μM) evoked an inward current in cultured hippocampal neurons. APV (100μM) reversibly potentiated the GABA-induced current. Bicuculine (10 μM) completely blocked the APV-enhanced current. **b)** Quantified results from 9 neurons. APV (100 μM) significantly potentiated GABA (0.5 μM)-induced current in hippocampal neurons (157.7 ± 9.1% of the control, $P < 0.01$, one-way ANOVA test). **c)** Concentration-response relationship of APV-induced potentiation on GABA currents in hippocampal neurons (n=6).

Since APV is a NMDAR antagonist, it was possible that the observed effect was elicited by NMDAR-related pathway but not the direct interaction between APV and $GABA_A$Rs. To exclude the potential involvement of NMDARs, we used dizocilpine (also known as MK801), an uncompetitive NMDAR open-channel blocker to block NMDARs before applying APV. MK801 has previously been found having no effect on $GABA_A$R function (Dr. Dongchuan Wu, unpublished data). Cultured hippocampal neurons were first incubated with MK801 (10 μM) and NMDA (50 μM) for 3 min in Mg^{2+}-free extracellular solution. This led to a complete and long lasting blockage of NMDAR even after wash-out of MK801 and NMDA (Figure 3.2a). Under this condition, application of APV (200μM) still produced a significant increase of GABA current amplitude (162.1 ± 15.3% of the control, $P<0.05$, n=4; Figure 3.2b). This result strongly indicated that NMDAR was not responsible for the APV-induced potentiation effect on $GABA_A$R.

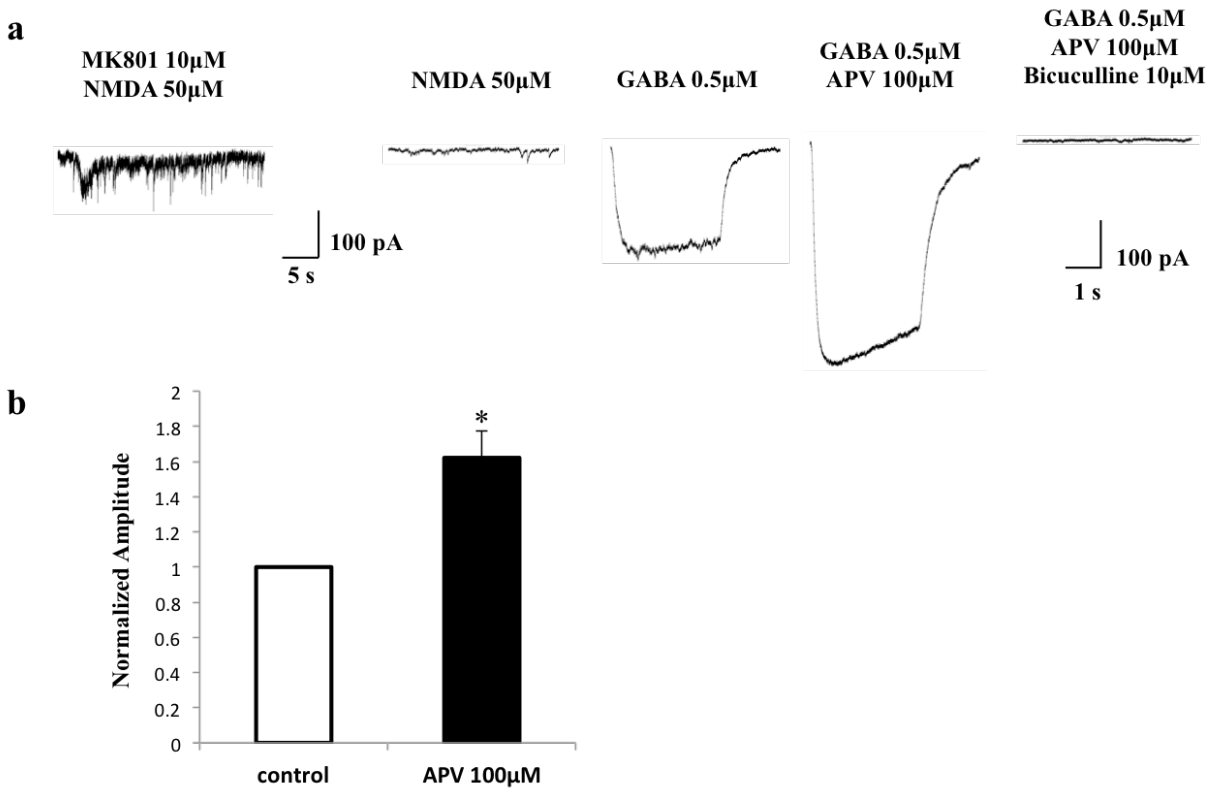

Figure 3.2 APV-induced potentiation of GABA current is not through NMDAR-related pathway

a) Representative traces showing blockage of NMDAR by co-application of MK801 (10μM) with NMDA (50μM) (left) for 3 min. After wash-out MK801 and NMDA, fast perfusion NMDA (50 μM) still failed to evoke any current (middle). However, application of APV (100 μM) still produced a significant potentiation of GABA-induced current, which can be completely blocked by bicuculline (10 μM) (right). **b)** Quantified results from 4 neurons. APV significantly increased GABA current amplitude (162.1 ± 15.3% of the control, $P<0.05$, Student's *t*-test) even with the blockage of NMDARs.

3.2.2 APV potentiates $GABA_AR$-mediated phasic and tonic inhibition in cultured hippocampal neurons

$GABA_ARs$ mediate both phasic and tonic inhibition in the CNS. The former one represents the synaptic $GABA_AR$-mediated fast inhibition and the latter one represents the chronic inhibition caused by ambient GABA acting on extrasynaptic $GABA_ARs$ (Farrant and Nusser, 2005). To test APV effect on phasic inhibition, miniature inhibitory postsynaptic currents (mIPSC) was recorded in cultured hippocampal neurons with ECS containing TTX (0.5 μM) and CNQX (10 μM). Bath application of APV (200 μM) produced a reversible and significant increase in mIPSC amplitude (control: 29.0±2.9pA vs APV: 36.6±3.6pA, $P<0.01$; n=7; Figure 3.3b) but not frequency (control: 0.89±0.27Hz vs APV: 0.90±0.29Hz, $P>0.05$, n=7; Figure 3.3c), indicating that APV regulated postsynaptic $GABA_AR$ function but did not affect presynaptic GABA release.

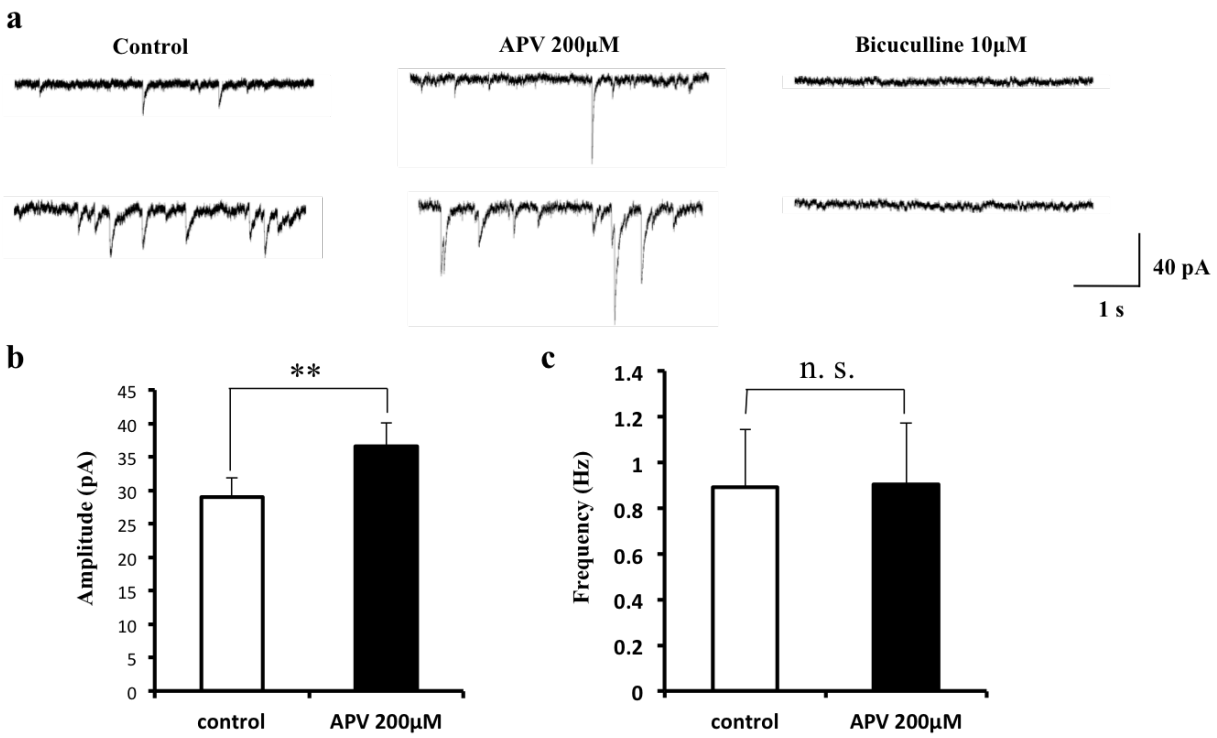

Figure 3.3 APV potentiates $GABA_AR$-mediated phasic inhibition

a) Representative trace of mIPSCs before and after APV (200 µM) application. The mIPSC can be blocked by 10 µM bicuculline. **b)** Quantified results from 7 neurons. APV (200 µM) significantly increased the mIPSC amplitude (control: 29.0±2.9pA vs APV: 36.6±3.6pA, P<0.01, Student's *t*-test) **c)** APV (200 µM) induced no significant change in mIPSC frequency (control: 0.89±0.27Hz vs APV: 0.90±0.29Hz, P>0.05, n=7, Student's *t*-test).

Tonic inhibition can be measure by the shift of baseline holding currents upon blockage of $GABA_A$Rs. With whole-cell recording of cultured hippocampal neurons in ECS containing TTX (0.5 μM) and CNQX (10 μM), application of bicuculline (10 μM) led to a tonic current represented by an upward shift of the baseline holding current (9.67±1.12 pA, n=7, Figure 3.4). Incubating cells in APV (200 μM) significantly increased the amplitude of tonic currents (17.28±2.80 pA, n=7, $P<0.05$ compared with control; Figure 3.4). Since phasic and tonic inhibitions are mediated by spatially and pharmacologically distinct $GABA_A$Rs, our results suggested that the glutamate/glutamate analogs-induced potentiation might be a universal mechanism in regulating most, if not all native $GABA_A$Rs.

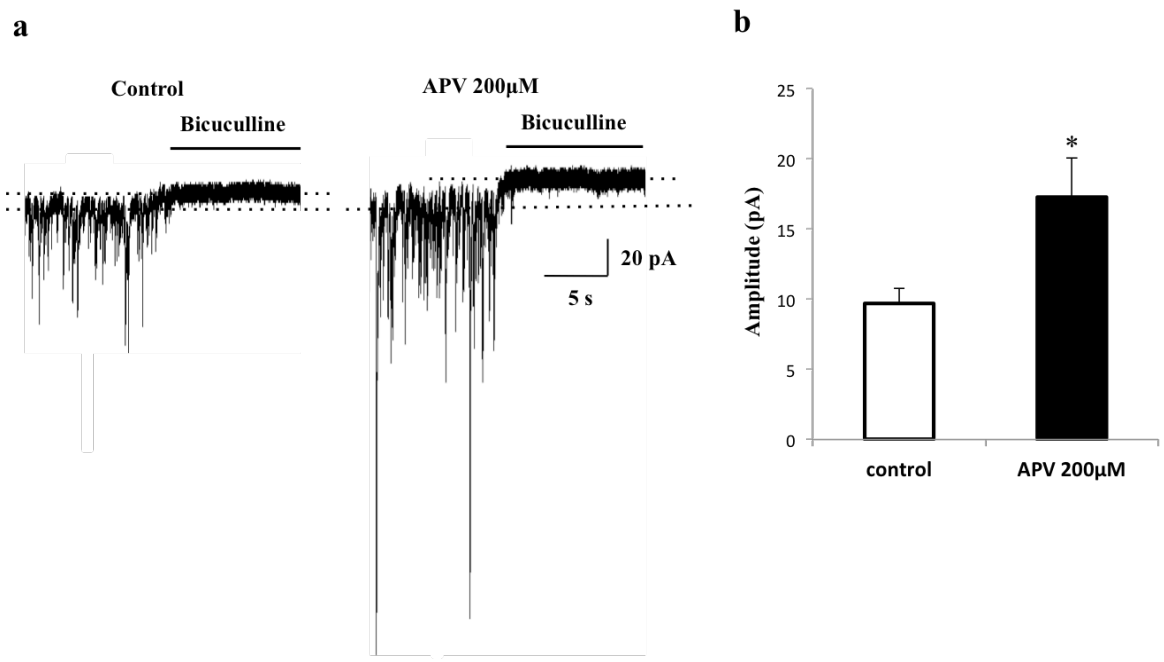

Figure 3.4 APV potentiates $GABA_AR$-mediated tonic inhibition

a) Representative trace of tonic GABA current measured as the holding baseline shift after blocking the persistently activated $GABA_ARs$. APV (200 μM) application increased the amplitude of tonic current. **b)** Quantified results from 7 neurons. APV (200 μM) significantly increased the amplitude of tonic GABA current (control: 9.67±1.12 pA vs APV: 17.28±2.80 pA, $P<0.05$, Student's *t*-test).

3.2.3 Glutamate and its analogs potentiate the function of recombinant $GABA_A$Rs in HEK293 cells

To further differentiate the effect elicited by direct interaction of glutamate or its analogs with $GABA_A$Rs from those caused by other indirect mechanisms such as activation of unknown glutamate receptors, we tested the glutamate effect on recombinant $GABA_A$Rs expressed in HEK293 cells. Plasmids expressing $GABA_A$R α1 and β2 subunits were co-transfected into HEK 293 cells to fulfill the subunit minimum requirement for $GABA_A$R membrane expression (Malherbe et al., 1990; Sigel et al., 1990). Under whole-cell patch recording configuration, glutamate (1 mM) itself could not induce any noticeable current on the transfected HEK 293 cells (Figure 3.5a), confirming there was no ionotropic glutamate receptors expressed in HEK 293 cells. However, it significantly potentiated GABA (1 μM) induced inward current (331.2 ± 44.5%, n=6, $p<0.01$; Figure 3.5b). The potentiated current was completely blocked by $GABA_A$R antagonist, bicuculline (100 μM) (Figure 3.5a), which confirmed the currents were gabaergic. Systematic concentration-response analysis showed that, glutamate concentration-dependently enhanced the GABA currents with an EC_{50} close to 180 μM and a lowest effective concentration (20% potentiation) around 30 μM (Figure 3.5c). Applying a fixed concentration of glutamate at 100 μM led to a leftward shift of the GABA concentration-response curve and decreased GABA EC_{50} from 13.19±1.08 μM to 5.46±1.10 μM (Figure 3.5d). However, there was no significant change in either Hill coefficient (1.28±0.12 and 1.42±0.18 in the absence and presence of 100 μM glutamate, respectively), or the maximum GABA responses. These results suggested that glutamate increased GABA binding affinity on $GABA_A$R but not the channel conductance. This property of glutamate is very similar to that of benzodiazepine, a classic $GABA_A$R positive allosteric modulator.

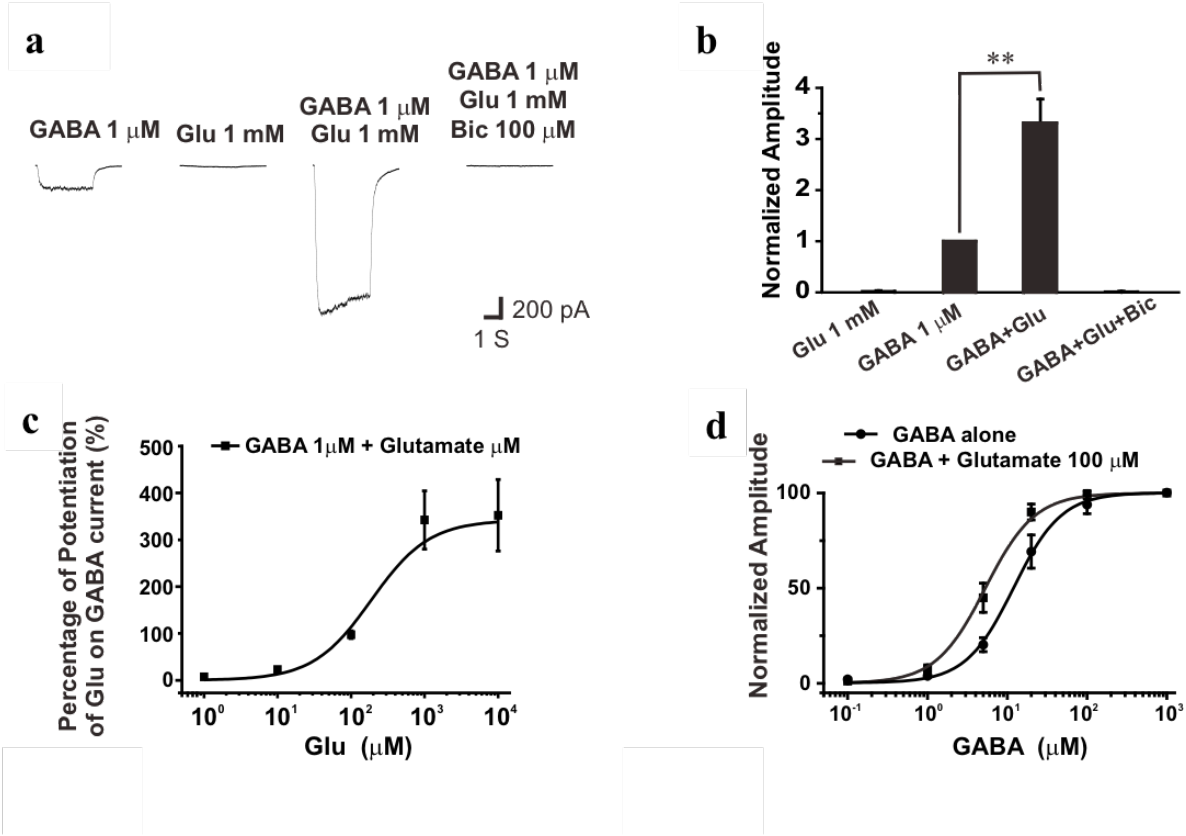

Figure 3.5 Glutamate potentiates the function of recombinant $GABA_A$Rs in HEK293 cells

a) Representative traces showing glutamate (Glu, 1 mM) itself did not evoke any noticeable current but it potentiated the GABA-induced current that can be fully blocked by bicuculline (Bic, 100 μM). **b)** Quantified results from 6 cells, glutamate (1 mM) significantly potentiated the amplitude of GABA (1 μM) evoked current (331.2 ± 44.5%, n=6, $p<0.01$, Student's *t*-test). c) Concentration-response curve of glutamate potentiation effect on GABA (1 μM) evoked current. d) Glutamate (100 μM) left shifted the GABA concentration-response curve.

Stezler and Wong have shown that glutamate analogs mimicked glutamate, potentiating $GABA_AR$ functions in hippocampal neurons (Stelzer and Wong, 1989). Here we tested if the similar effects existed in HEK293 cells transiently expressing recombinant $GABA_ARs$. As shown in Figure 3.6, various ionotropic glutamate receptor agonists, including AMPA (100 μM; an agonist for AMPA type glutamate receptor), kainic acid (100 μM; an agonist for both kainate and AMPA glutamate receptors), and NMDA (100 μM; an agonist for NMDA receptor) all enhanced GABA-mediated currents in HEK 293 cells expressing α1β2 $GABA_ARs$ (AMPA, 339.2±38.0% of currents induced by GABA alone, $p=0.0007$; kainic acid, 388.2±61.0%, $p<0.01$; and NMDA, 267.6±20.5%, $p<0.01$; compared with GABA currents in the absence of an agonist; Figure 3.6a). Similarly, the competitive antagonists of glutamate receptors, including APV (100 μM, a competitive antagonist for NMDARs) and TBOA (100 μM, a competitive antagonist for glutamate transporters) also greatly potentiated GABA currents (APV, 254.9±33.0%, $p<0.01$; TBOA, 202.8±18.3%, $p<0.01$). In contrary, the non-competitive NMDA receptor antagonist MK-801, which does not share the structural features and binding site with glutamate, showed no effect on α1β2 containing $GABA_ARs$ expressed in HEK 293 cells (96.9±5.8%, $p>0.05$).

Since majority of endogenous $GABA_ARs$ contain one copy of γ subunits (Mohler, 2006), we also tested glutamate and its analogs' effects on γ containing $GABA_ARs$ using heterologous expression system. We transiently transfected HEK 293 cells with plasmids expressing $GABA_AR$ α1, β2 and γ2 subunits. Similarly as what we have seen with α1β2 containing $GABA_ARs$, glutamate and glutamate analogs also significantly potentiate HEK 293 currents mediated by α1β2γ2 containing $GABA_ARs$ (Figure 3.6b; AMPA: 165.8±6.2%, $p<0.01$; kainic acid: 188.7±5.5%, $p<0.01$; NMDA: 171.7±5.4%, $p<0.01$; APV: 173.3±3.7%, $p<0.01$; glutamate:

169.3±6.9%, $p<0.01$; and TBOA: 159.9±7.8%, $p<0.01$). Interestingly, the potentiation effects observed with α1β2γ2 $GABA_A$Rs appears to be much smaller than that of α1β2 $GABA_A$Rs (Figure 3.6), suggesting that incorporation of a γ subunit may compromise the glutamate/glutamate analogs induced potentiation.

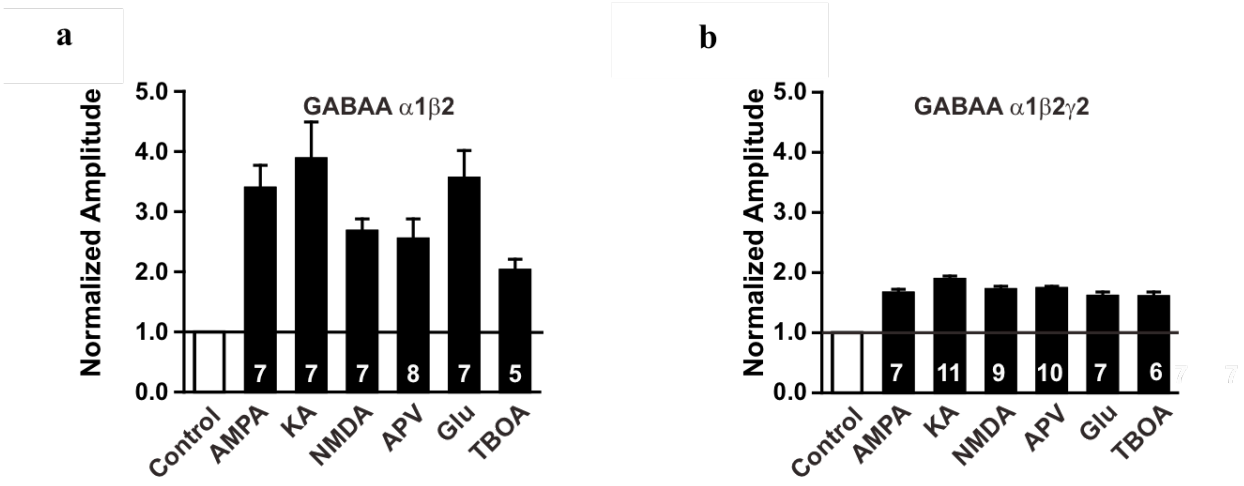

Figure 3.6 Glutamate analogs potentiate the GABA-induced current in HEK293 cells

a) Glutamate analogs (100 μM) potentiate the GABA (1 μM) evoked current in HEK193 cells expressing $GABA_AR$ containing α1β2 subunits (AMPA, 339.2±38.0%; kainic acid, 388.2±61.0%; NMDA, 267.6±20.5%; APV, 254.9±33.0%; TBOA, 202.8±18.3%; compared with GABA currents in the absence of a glutamate analog). **b)** Glutamate analogs (100 μM) potentiate the GABA (1 μM) evoked current in HEK193 cells expressing $GABA_AR$ containing α1β2γ2 subunits (AMPA: 165.8±6.2%; kainic acid: 188.7±5.5%; NMDA: 171.7±5.4%; APV: 173.3±3.7%; glutamate: 169.3±6.9%; and TBOA: 159.9±7.8%; compared with GABA currents in the absence of a glutamate analog). (Numbers in each bars in a and b indicate the number of independent recording in each groups)

3.2.4 Glutamate physically binds to the $GABA_AR$

Our previous results from neuron and HEK 293 cells suggested that glutamate and its analogs potentiate GABA current most likely through a direct interaction with $GABA_ARs$. To further investigate the mechanism of this interaction, we performed [^{3}H]-glutamate binding assays to test if glutamate physically binds to $GABA_ARs$. Plasma membranes were isolated from HEK 293 cells transfected with α1 and β2 subunits of $GABA_ARs$. Using plasma membranes from non-transfected HEK 293 cells as the control, we found that [^{3}H]-glutamate (40 nM) showed significant binding affinity to membranes from $GABA_ARs$ overexpressed cells (938.2±82.7 CPM vs control: 204.0±20.6 CPM, $P<0.05$, Figure 3.7a). This type of specific binding was concentration-dependent (Figure 3.7b) and could be efficiently competed by increasing concentration of non-radio-labeled (cold) glutamate (Figure 3.7c). High concentration (0.4 mM) of cold glutamate and APV reduced the binding activity in the $\alpha1\beta2$ expressing cells to a level similar to the nonspecific background activity observed in the non-transfected cells (cold glutamate: 262.2±47.8 CPM, n=6, $P>0.05$; APV: 240.8±36.8 CPM, n=6, $P>0.05$, compared with non-transfected control group; Figure 3.7a). In contrast, GABA (40mM) failed to alter the [^{3}H]-glutamate binding activity (939.2±88.8 CPM, n=6, $P>0.05$ compared with [^{3}H]-glutamate only group; Figure 3.7a). These results indicate that glutamate and its analog APV can directly and specifically bind to the $GABA_AR$ through the same binding sites on the receptors, and that the glutamate-binding site does not overlap with the known GABA-binding site, strongly suggesting glutamate as a positive allosteric modulator of $GABA_ARs$.

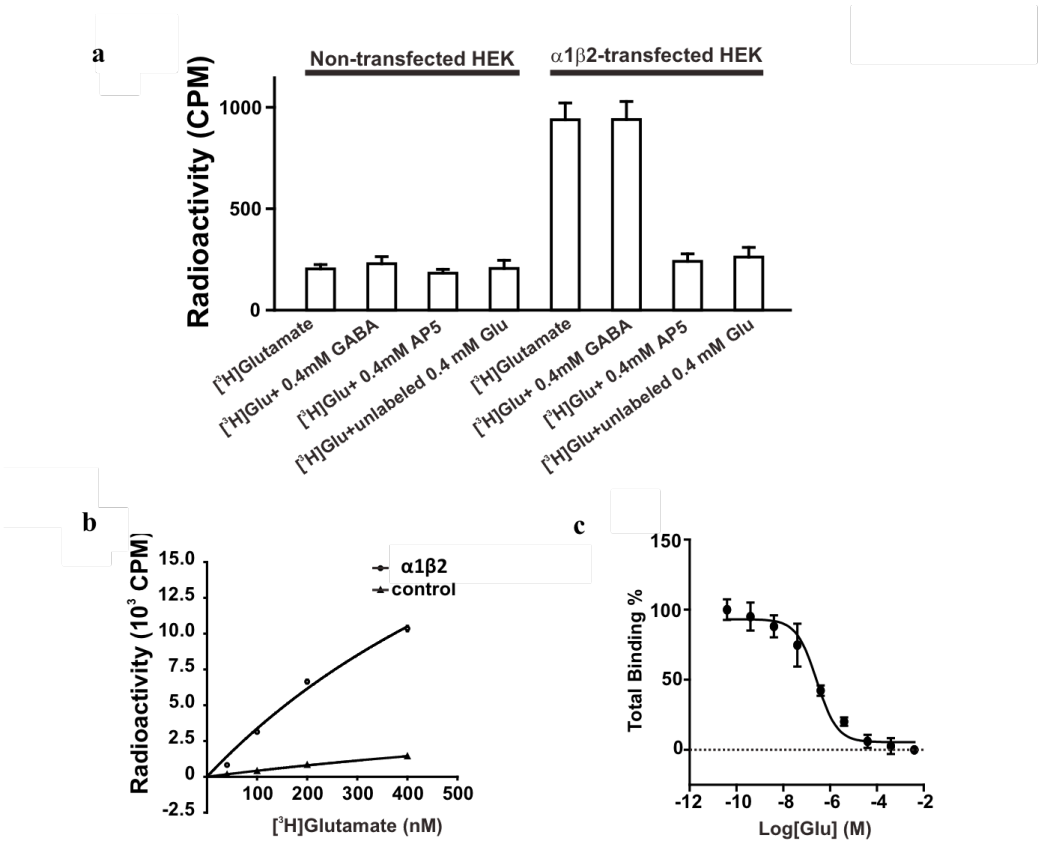

Figure 3.7 Glutamate physically binds to the $GABA_AR$ at an allosteric binding site

a) [^{3}H]-glutamate binding assays showed [^{3}H]-glutamate (40 nM) specifically bound to the plasma membranes of HEK transiently transfected with α1β2 $GABA_ARs$ (control: 204.0±20.6 CPM vs $GABA_AR$ transfected membrane: 938.2±82.7 CPM, $P<0.05$, n=6, Student's *t*-test), but not that of none-transfected HEK controls; and the specific [^{3}H]-glutamate binding was competitively blocked by a high concentration (0.4 mM) of non-radio-labeled glutamate or APV (cold glutamate: 262.2±47.8 CPM, n=6, $P>0.05$ compared with non-transfected control group; APV: 240.8±36.8 CPM, n=6, $P>0.05$ compared non-transfected control group), but not affected by a high concentration (0.4 mM) of non-radio-labeled GABA (939.2±88.8 CPM, n=6, $P>0.05$ compared with [^{3}H]-glutamate only group). **b)** Concentration dependent binding of [^{3}H]-glutamate on plasma membrane containing $GABA_ARs$. **c)** Non-radio-labeled glutamate competitively inhibited the [^{3}H]-glutamate (40 nM) binding (n=4).

3.3 Discussion

Excitation/inhibition crosstalk at the receptor level contributes to the fast reciprocal regulation of E/I balance in the CNS. Following Stezler and Wong's work, we investigated a novel type of E/I crosstalk where excitatory neurotransmitter glutamate and its analogs exert positive modulation effects on $GABA_A$Rs. This glutamate-induced potentiation occurs with both phasic and tonic inhibition, indicating such a positive modulation by excitatory transmitter glutamate may exist in various $GABA_A$R subfamilies, highlighting the significance of the modulation in both synaptic and extrasynaptic GABA events.

Glutamate (and its analogs) potentiated GABA currents without inducing any detectible currents on its own in HEK293 cells transiently transfected with α1β2 or α1β2γ2 compositions of $GABA_A$Rs. Given that HEK 293 cells express naturally very low levels of endogenous glutamate receptors, our results are consistent with a mode of allosteric modulation by direct binding of glutamate ligands to the $GABA_A$R. The observation that glutamate potentiation can be mimicked by most glutamate receptor ligands suggests that the glutamate-binding site on $GABA_A$Rs is pharmacologically less stringent compared to other glutamate binding sites on iontropic glutamate receptors.

Using a combination of [^{3}H]-glutamate binding assays, we confirmed the physical binding of glutamate to $GABA_A$Rs. APV, but not GABA, was able to compete [^{3}H]-glutamate binding, demonstrating that the glutamate binding site presents a novel site that does not overlap with known GABA agonist binding sites. Taking all the results together, we provide strong evidence that glutamate may serve as a novel positive allosteric modulator of $GABA_A$R. Along with the

recently reported glutamate modulation of glycine receptor (Liu et al., 2010), our study suggests that this type of reciprocal regulation may be a common phenomenon among inhibitory transmitter receptor-gated chloride channels. This previously unrecognized novel crosstalk blurs the traditional distinction between excitatory and inhibitory transmitters

It is important to note that the EC_{50} of glutamate modulation we observed is at high μM ranges, which is much higher than basal levels of extracellular glutamate (Herman and Jahr, 2007; Jacobson et al., 1985). However, extracellular glutamate concentrations can be increased under certain physiological and pathological conditions. Thus, the higher EC_{50} may have physiological and/or pathological significances. For instance, under basal conditions, due to the presence of parasynaptic glial and/or neuronal glutamate transporters, presynaptically released glutamate cannot spill-over to adjacent GABAergic synapses at concentrations required for the glutamate allosteric potentiation of $GABA_AR$s. Thus, the higher EC_{50} would ensure that glutamate only functions as an excitatory transmitter at glutamatergic synapses under most of these physiological conditions. However, during intensive neuronal activities or under certain pathological conditions, the extracellular glutamate concentration may be reached to the level close or even above the EC_{50}, thereby engaging the glutamate allosteric potentiation of the adjacent $GABA_AR$s. For example, glutamate concentrations measured from brain slices can reach above 90 μM during spreading depression (Zhou et al., 2013), a pathophysiological condition related to stroke or migraine with aura. As another important source, astrocytes can release glutamate through opening of two-pore-domain potassium channels TREK-1 upon Gi pathway activation by protease-activated receptor 1 (Woo et al., 2012). The peak concentration of glutamate released by this pathway can reach as high as 100 μM (Woo et al., 2012). The

astrocyte-originated release possibly provides one of the major sources of ambient glutamate, which may affect tonic GABAergic inhibition. Furthermore, studies have revealed that glutamate could be co-released with GABA at GABAergic synapses in the auditory pathway during development (Gillespie et al., 2005; Noh et al., 2010). Purkinje cell dendrites can also release glutamate during excitation in close proximity with the $GABA_A$Rs (Duguid and Smart, 2004). Under these conditions, glutamate may allosterically potentiate adjacent synaptic and/or extrasynaptic $GABA_A$Rs, thus homeostatically increasing $GABA_A$R inhibition and counteracting overexcitation.

Chapter 4: Identification and characterization of glutamate-binding site on the $GABA_AR$

4.1 Introduction

Our previous results have demonstrated that glutamate positively modulated GABA-mediated currents by physically acting at allosteric sites in both recombinant and endogenous $GABA_ARs$. Next, we explored the amino acid residues critical for the glutamate-binding site on the $GABA_AR$. $GABA_AR$ is a pentameric receptor and the majority of $GABA_ARs$ contain 2 α subunits, 2 β subunits and 1 γ subunits. Since the glutamate modulation can be observed in the recombinant $GABA_AR$ containing α1β2 subunits, and its efficacy is reduced by the introduction of a γ subunit (Figure 3.6), we predict that the glutamate-binding site(s) is likely located in α and/or β subunits. We also reasoned that a modulation site of a small molecule would be located in an interface region of the two subunits due to its ability to cause the necessary conformational changes, leading a functional alteration of the receptor. (Hibbs and Gouaux, 2011). As glutamate cannot compete with GABA at the GABA agonist binding sites (Figure 3.7) which are located at the β+/α- interface (Lummis, 2009), we further speculated that the glutamate binding may occur somewhere around the α+/β- interface.

Lacking the proper crystal structure of $GABA_ARs$, our lab generated a computer-based homology model of the most common native $GABA_AR$ which has a subunit composition of two α1, two β2 and one γ2 subunits based on the crystal structures of the glutamate-gated chloride channel (Hibbs and Gouaux, 2011). This homology model is reconfirmed by the recently resolved β3 homomeric $GABA_AR$ crystal structure (Miller and Aricescu, 2014), Based on our

previous assumption, our lab conducted an *in-silicon* molecular docking to search for the putative glutamate-binding pockets in the $GABA_AR$, with a particular focus on the extracellular α+/β- interface region.

After docking glutamate to this region, we found that there were several potential binding pockets that could potentially interact and accommodate a glutamate molecule at the α+/β- interface (Figure 4.1b). To further increase the predicting accuracy and thereby decrease the number of possibilities, we also tried to incorporate another glutamate-like ligand TBOA into the modelling. TBOA is at least one hydrophobic benzyl group larger than glutamate itself (Figure 4.1a). Since TBOA mimics glutamate, potentiating $GABA_AR$-mediated responses (Figure 3.6), we predict that the glutamate-binding sites/pockets should also be able to accommodate the larger sized TBOA and, as such, TBOA docking analysis should help us to exclude these predicted glutamate-binding pockets that are too small to accommodate TBOA. As we expected, this led us to focus our efforts on two potential binding pockets located at the α+/β- interface, one under the loop-C at a site homologous to the α-/β+ GABA binding site (P1) and the other just below the loop-C site (P2), as respectively indicated in Figure 4.1c.

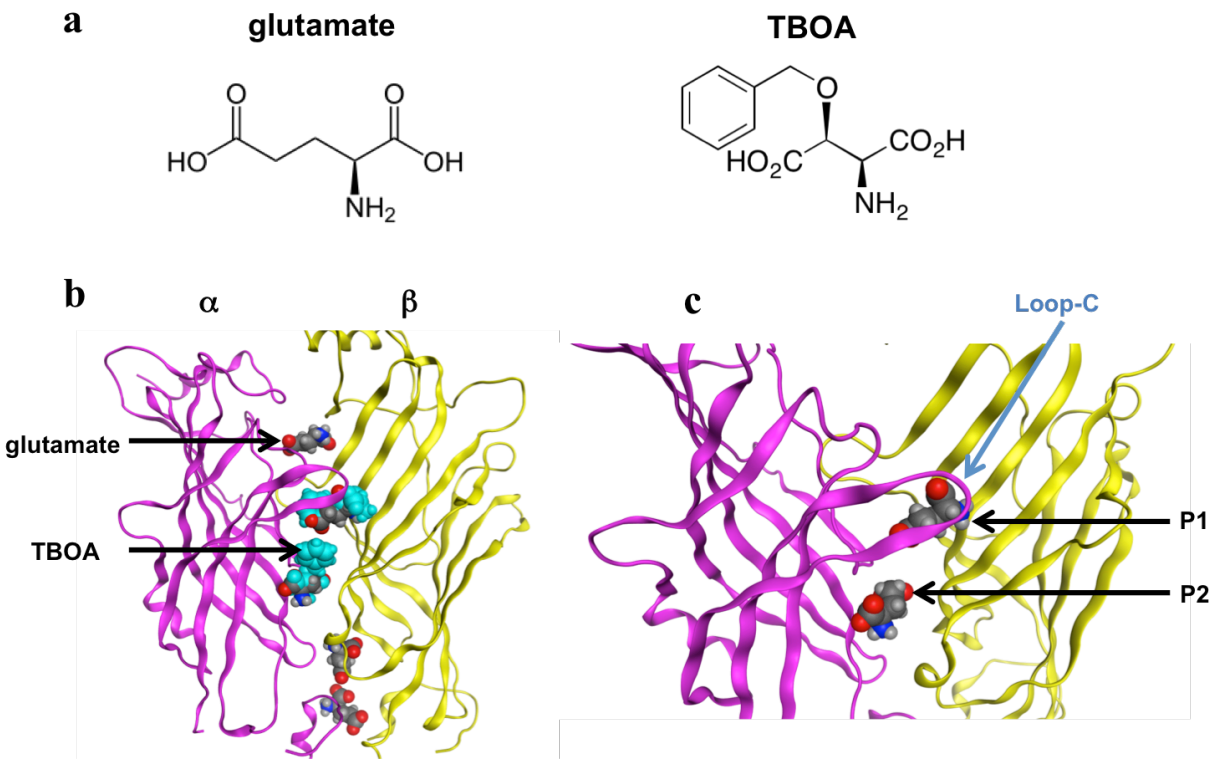

Figure 4.1 *in-silicon* molecular docking and the putative glutamate-binding pockets

a) Structures of glutamate and TBOA. **b)** Potential binding pockets found by glutamate and TBOA docking at the α+/β- interface. The grey molecule represents glutamate, and blue molecule represents TBOA. There are two pockets that can accommodate both glutamate and TBOA, which are identified as our candidate pockets. **c)** A closer view on the two candidate binding pockets (P1 and P2) at the α+/β- interface. P1 is under loop-C around the site homologous to the GABA-binding site and P2 is just below P1.

4.2 Results

4.2.1 The glutamate-binding pocket is located at the α+/β- interface of the $GABA_A$Rs

Based on the docking results, we performed a systemic mutational analysis of critical amino acids around these two putative binding pockets followed by followed by electrophysiological characterizations of their impacts on glutamate potentiation in HEK cells expressing wild or mutated α1β2γ2 $GABA_A$Rs (Table 4.1). As summarized in Table 4.1, we found either that mutations of amino acids surrounds the putative loop-C pocket (P1, Figure 4.1c) had no obvious effect on glutamate-induced potentiation or the mutation itself produced a significant reduction in GABA's ability to evoke $GABA_A$R-gated currents. The data clearly suggested that the loop-C pocket is not the binding site by which glutamate produces allosteric potentiation of $GABA_A$R function. In great contrast, mutation any of the five amino acids surrounding the putative glutamate-binding pocket just below the loop-C site (P2, Figure 4.1c) was able to significantly reduce the glutamate-induced potentiation of $GABA_A$R function (Table 4.1). The mutations of the 4 charged residues (α1K104D, α1E137G, α1K155D and β2E181G) showed larger impacts on glutamate-induced potentiation than that of the mutation of the neutral residue (β2I180A) (Table 4.1), indicting stronger interactions between glutamate, a charged molecule, with those charged residues in the binding pocket due to electrostatic attraction. Therefore, we identified those four charged residues (α1K104, α1E137, α1K155 and β2E181) as the critical residues for glutamate binding.

Pocket	Mutation	Glutamate (100 μM)-induced Potentiation (% of wild type)*	GABA EC_{50} (μM)	n
	α1β2γ2 WT	100.00%	10.4±1.1	6
P1	α1S158Aβ2γ2	106.9±39.8%	6.4±1.1	3
	α1Y159Aβ2γ2	90.8±26.7%	7.9±1.1	3
	α1S205Aβ2γ2	113.1±33.0%	10.2±1.0	3
	α1T206Aβ2γ2	129.3±61.7%	8.6±1.3	3
	α1E208Aβ2γ2	90.9±28.7%	9.4±1.3	3
	α1β2G126Aγ2	Not tested**	152.7±1.1	3
	α1β2Q63Aγ2	Not tested**	163.4±1.2	3
	α1F99Aβ2γ2	96.4±23.0%	8.9±1.3	3
	α1β2V177Aγ2	89.5±28.3%	8.7±1.1	3
	α1β2Y61Aγ2	Not tested**	176.5±1.2	3
P2	α1K104Dβ2γ2	8.0±10.7%	11.0±1.1	6
	α1E137Gβ2γ2	2.1±7.7%	11.9±1.0	6
	α1K155Dβ2γ2	9.0±10.0%	8.5±1.1	6
	α1β2E181Gγ2	1.6±11.6%	10.0±1.1	6
	α1β2 I180Aγ2	18.8±12.9%	8.0±1.2	6

Table 4.1 Summary of systematic mutagenesis analysis

* Glutamate-induced potentiation effect was tested with 1 μ M GABA induced currents.

** Potentiation effect was not tested because no obvious current could be evoked by 1 μ M GABA.

As shown in Figure 4.2a, mutating any one of the four critical residues (α1K104D, α1E137G, α1K155D, β2E181G) essentially eliminated the potentiation of GABA currents by glutamate at both low (100 μM) and high (1 mM) concentrations. Importantly, neither of these mutations affected the ability of GABA to activate $GABA_AR$, inducing inward currents (Figure 4.2b). Computer modeling confirmed the involvement of those four residues in glutamate binding (Figure 4.2e). The positive charged residues (α1K104D and α1K155D) were responsible for interacting with the carboxyl (COO^-) group of glutamate and the negative charged residues (α1E137G and β2E181G) were responsible for interacting with the amino (NH_3^+) group of glutamate. These results demonstrate that these residues are critically required for glutamate-induced potentiation of $GABA_ARs$, and thereby provide a strong support for the modeling predicted glutamate-binding pocket below the loop-C site being the glutamate-binding pocket.

As the putative binding pocket is located at the α+/β-, not involving any amino acid residue from a γ subunit, we then performed further mutational analysis in HEK cells expressing α1β2 $GABA_ARs$. It is interesting that in comparison with HEK cells expressing αβγ $GABA_ARs$, an individual mutation of any one of these glutamate-binding pocket forming amino acid residues produced a much weaker effect on glutamate induced potentiation (Figure 4.2c), in comparison with the same individual mutation in α1β2γ2 receptor (Figure 4.2a). Single mutation could only produce either partial inhibition (α1K104D and β2E181G) or no effect (α1E137G and α1K155D) on 100 μM glutamate-induced potentiation (α1K104D, 42.0 ± 6.9 % of the wild type receptor, $p<0.05$; βE181G, 51.2 ± 9.4 %, $p<0.05$; Figure 4.2c, Left). As for the potentiation induced by saturated concentration of glutamate (1 mM), only β2E181G successfully produced significant reduction (Figure 4.2c, Right; β2E181G, 50.6 ± 8.4 %, $P<0.05$), indicating βE181G may play a

more significant role in glutamate binding. However, a combination of simultaneously mutating two of these four amino acids provided a complete elimination of glutamate potentiation (Figure 4.2d). Substituted α1E137G and β2E181G on different subunits dramatically reduced the sensitivity to glutamate at both 100 μM (9.2 ± 3.0 % of control, $P<0.05$) and 1 mM (16.2 ± 1.3 % of control, $P<0.05$; Figure 4.2d). Co-expression of α1K104D and β2E181G also strongly decreased the sensitivity to glutamate at both 100 μM (6.6 ± 6.1 % of control, $P<0.05$) and 1 mM (27.4 ± 12.6 % of control, $P<0.05$). The double mutation K104D and E137G of α1 subunit, which respectively impairs the interaction with COO^- and $NH3^+$ groups of glutamate, decreased receptor sensitivity to 100 μM (15.1 ± 6.9 % of control, $P<0.05$) and 1 mM glutamate (34.4 ± 10.8 % of control, $P<0.05$).

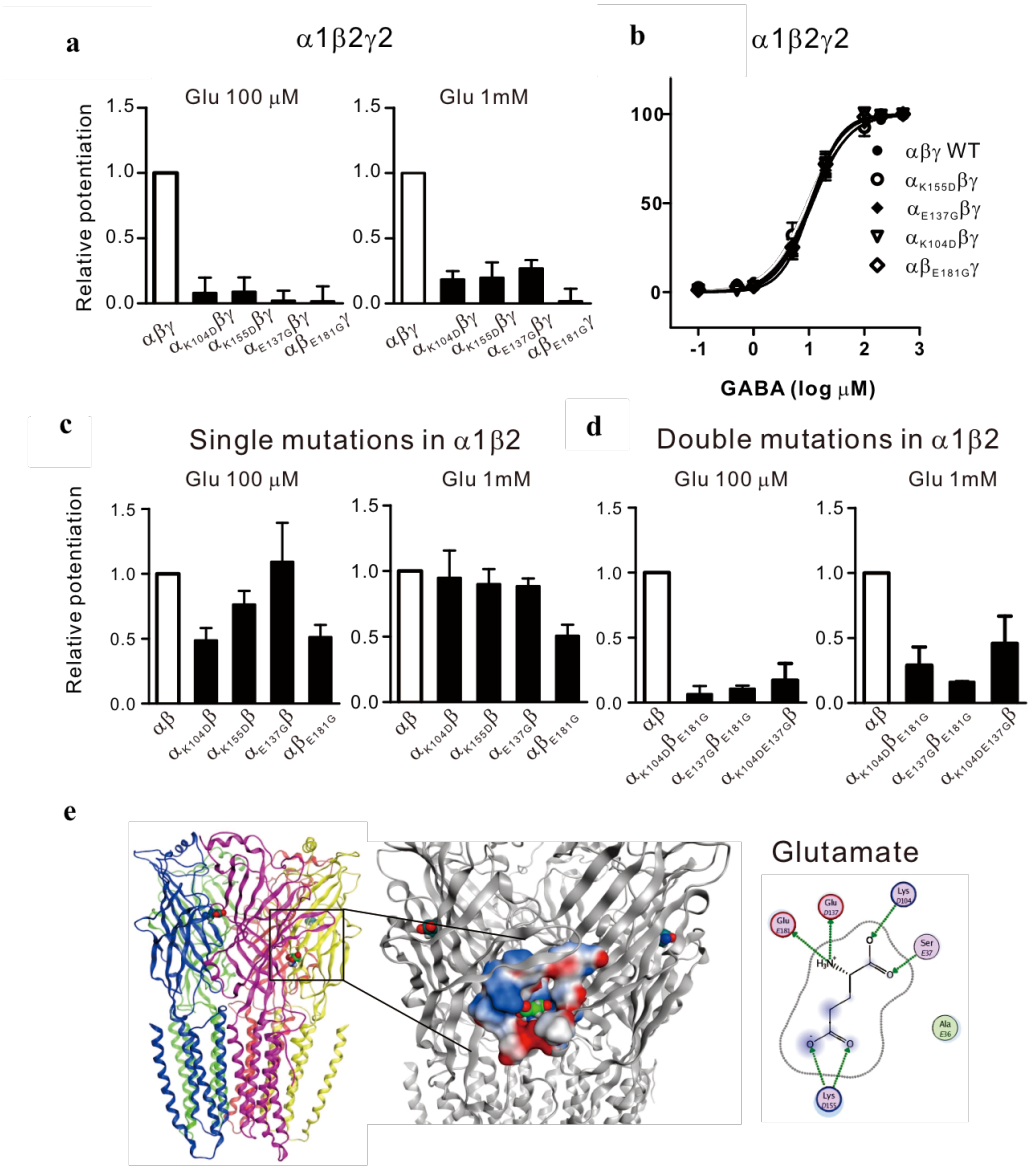

Figure 4.2 Identification of glutamate-binding site in the α+/β- interface of the $GABA_AR$

Mutational characterization of the putative glutamate-binding pocket in HEK cells transiently expressing recombinant $GABA_ARs$. Relative potentiation was obtained by normalizing potentiation observed in the mutated receptor to that in the respective wild type receptor. **a)** In cells expressing α1β2γ2 $GABA_ARs$, mutation of any of these putative pocket-forming residues impairs of glutamate-induced potentiation of GABA (1μM) currents (Glu 100 μM: αK104Dβγ: 7.9 ± 11.7 % of control, n=5, P<0.01; αK155Dβγ: 9.0 ± 10.9 % of control, n=5, P<0.01;

αE137Gβγ: 2.1 ± 7.6 % of control, n=6, P<0.01; αβE181Gγ: 1.6 ±11.6 % of control, n=6, P<0.01; Glu 1mM: αK104Dβγ: 18.6 ± 6.3 % of control, n=5, P<0.01; αK155Dβγ: 19.8 ± 11.6 % of control, n=5, P<0.01; αE137Gβγ: 26.9 ± 6.4 % of control, n=6, P<0.01; αβE181Gγ: 1.9 ±9.6 % of control, n=6, P<0.01, Student's *t*-test). **b)** Those four mutations did not affect the GABA activation of the α1β2γ2 receptor (n=6). **c)** In cells expressing α1β2 $GABA_A$Rs, any single mutation appears less effective (Glu 100 μM: αK104Dβ: 48.8 ± 9.5 % of control, n=6, P<0.05; αK155Dβ: 76.3 ± 10.5 % of control, n=6, P>0.05; αE137Gβ: 109.1 ± 30.2 % of control, n=5, P>0.05; αβE181G: 51.2 ±9.5 % of control, n=7, P<0.05; Glu 1mM: αK104Dβ: 94.7 ± 20.7 % of control, n=5, P>0.05; αK155Dβ: 90.1 ± 11.3 % of control, n=5, P>0.05; αE137Gβ: 88.4 ± 5.9 % of control, n=5, P>0.05; αβE181G: 50.6 ±8.4 % of control, n=6, P<0.05, Student's *t*-test). **d)** Elimination of the potentiation requires a combination of any of two residues being simultaneously mutated (Glu 100 μM: αK104DβE181Gγ: 6.6 ± 6.1 % of control, n=5, P<0.01; αE137GβE181Gγ: 10.7 ± 2.3 % of control, n=5, P<0.01; αK104DE137Gβγ: 17.3 ±12.8 % of control, n=5, P<0.01; Glu 1mM: αK104DβE181Gγ: 27.4 ± 12.6 % of control, n=5, P<0.05; αE137GβE181Gγ: 16.3 ± 6.7 % of control, n=5, P<0.05; αK104DE137Gβγ: 45.9 ±20.9 % of control, n=5, P<0.05, Student's *t*-test) . **e)** The overall structures of the glutamate bound α1β2γ2 $GABA_A$Rs are shown on the Left panels. The subunits were coloured individually. The boxed regions are further enlarged in the middle panels, highlighting the identified glutamate-binding pocket in the α+/β- interface. The pocket-forming amino acid residues (particularly, β2E181, α1K104, α1K155 and α1E137) and their interactions with glutamate are illustrated in the panels on the right.

4.2.2 Spatial and electrostatic accessibility of glutamate to the binding pocket is crucial for the potentiation effects

To further determine the relative significance between the electrostatic and the side chain steric arrangements of these critical amino acid residues in their interaction with glutamate, we next substituted the aforementioned critical residues with amino acids with various sizes or charges and studied the subsequent effects on the glutamate-induced potentiation. We found that substitution of α1K104, α1E137, α1K155, or β2E181 with a non-charged, large side-chain residue tryptophan, which perturbed the spatial and electrostatic accessibility of glutamate to the binding pocket, greatly reduced glutamate potentiation of the GABA currents in HEK cells expressing α1β2γ2 $GABA_A$Rs (Figure 4.3a). In contrast, substitution of any of α1E137, α1K155, or β2E181 with a similarly charged amino acid (aspartic acid for α1E137 and β2E181; arginine for α1K155) failed to significantly affect glutamate-induced potentiation (Figure 4.3b). However, substitution of α1K104 with arginine exhibited reduced potentiation by glutamate, possibly due to the larger side chain volume of arginine in comparison with lysine. Therefore, both spatial and electrostatic accessibility of glutamate to the binding pocket is crucial for the induction of the potentiation effects. Collectively, these data confirmed that α1K104, α1E137, α1K155, and β2E181 play a critical role in forming the glutamate-binding pocket at the α+/β- interface of $GABA_A$Rs, likely through their direct electrostatic interactions with either COO^- or NH_3^+ groups of glutamate.

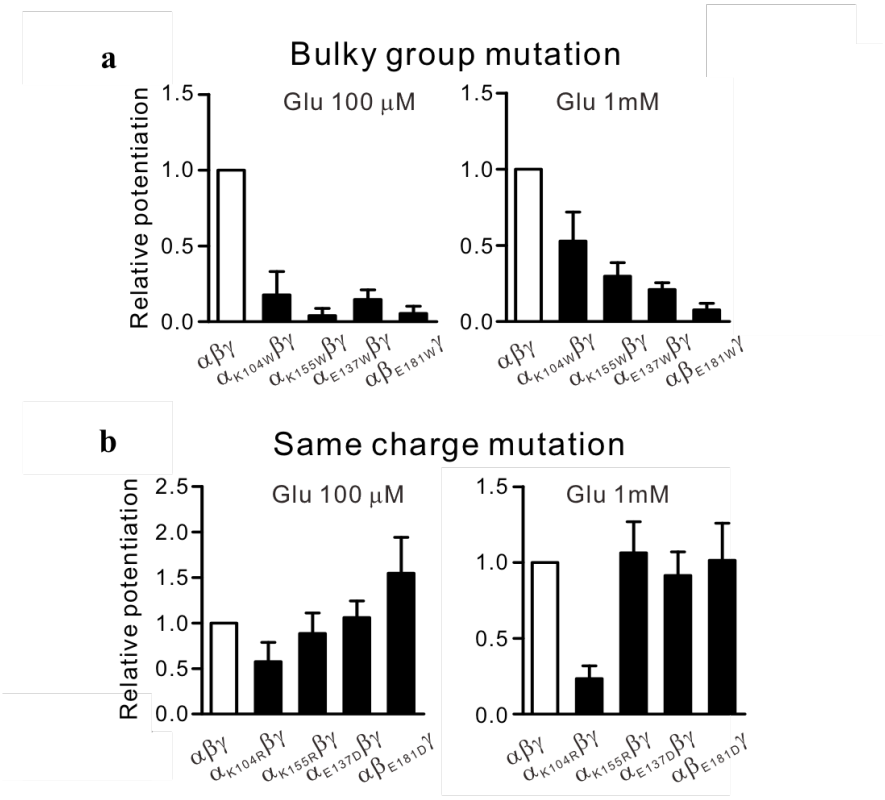

Figure 4.3 Characterization of the $GABA_AR$ glutamate-binding site in HEK cells

a) Substitution of the residue α1K104, α1K155, α1E137, or β2E181 with non-charged, bulky tryptophan residue impaired glutamate-mediated potentiation (Glu 100 μM: αK104Wβγ: 17.8 ± 15.3 % of control, n=5, P<0.01; αK155Wβγ: 4.0 ± 4.7 % of control, n=6, P<0.01; αE137Wβγ: 14.7 ± 6.3 % of control, n=6, P<0.01; αβE181Wγ: 5.4 ±4.8 % of control, n=6, P<0.01; Glu 1mM: αK104Wβγ: 50.8 ± 17.2 % of control, n=5, P<0.05; αK155Wβγ: 29.8 ± 8.8 % of control, n=6, P<0.01; αE137Wβγ: 21.1 ± 4.4 % of control, n=6, P<0.01; αβE181Wγ: 7.6 ±4.3 % of control, n=6, P<0.01, Student's *t*-test). **b)** Mutation of the residue α1K104, α1K155, α1E137, or β2E181 into a different residue with the same charge had little effect on the potentiation, except for the α1K104R mutation possibly due to the it large side chain (Glu 100 μM: αK104Rβγ: 57.5 ± 21.1 % of control, n=6, P>0.05; αK155Rβγ: 88.4 ± 22.5 % of control, n=6, P>0.05; αE137Dβγ: 105.9 ± 18.3 % of control, n=6, P>0.05; αβE181Dγ: 154.7 ±39.5 % of control, n=6, P>0.05; Glu 1mM: αK104Rβγ: 23.4 ± 8.4 % of control, n=6, P<0.01; αK155Rβγ: 106.4 ± 20.4 % of control, n=6, P>0.05; αE137Dβγ: 91.5 ± 15.4 % of control, n=6, P>0.05; αβE181Dγ: 101.5 ±24.5 % of control, n=6, P>0.05, Student's *t*-test).

4.2.3 Critical residues of the glutamate-binding site are conserved among their respective subfamilies

After confirming the glutamate-binding site in α1β2γ2 containing $GABA_A$Rs, we next used sequence alignment and mutational analysis to determine if this newly identified glutamate-binding pocket, and hence glutamate modulation are conserved among all α and β containing $GABA_A$Rs. Alignment of the sequences of α1 to α6 showed that the critical residues K104, E137, and K155 in α1 are also conserved among all other α subunits (Figure 4.4a). We next used α2 as an example to test the functional conservation of glutamate-induced potentiation of GABA currents. As shown in Figure 4.4b, similar to that observed in α1 containing $GABA_A$Rs (Figure 3.5), glutamate was also capable of potentiating GABA currents in HEK cells expressing α2/β2/γ2 $GABA_A$Rs, and as expected, individual mutation of those conserved critical residues of α2K104, α2E137, and α2K155 with either non-charged or oppositely charged residues also abolished glutamate-mediated potentiation of GABA currents in these cells (Figure 4.4b). Similarly, sequence alignment also showed that E181 in β2 was also conserved in other two β subunits; corresponding E182 in the β1 and β3 subunits (Figure 4.5a). In agreement with this sequence conservation, we found that glutamate could also potentiate GABA-induced currents in either α1/β1/γ2 or α1/β3/γ2-expressing HEK cells, and most importantly, mutation of E182 in either β1 or β3 prevented glutamate-induced potentiation of GABA-induced currents (Figure 4.5b), confirming the conservation of glutamate-binding site among different subfamilies of $GABA_A$Rs.

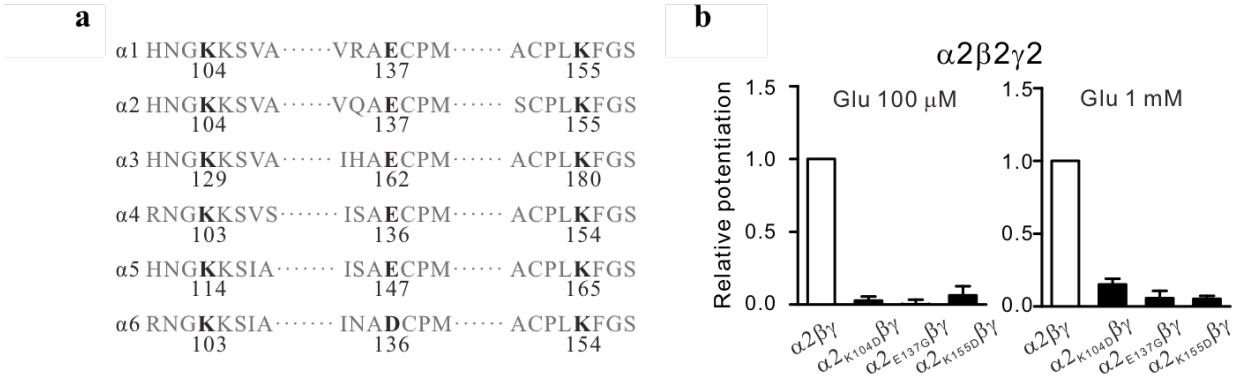

Figure 4.4 Conservation of the glutamate-binding site in the α subunit family

Residues critical for glutamate binding pocket in α1 are conserved among all α subunits. **a)** Sequence alignment showed that α1K104, α1K155, and α1E137 are conserved in all six α subunits. **b)** Mutating either of these conserved residues in α2 abolished the glutamate-mediated potentiation (Glu 100 μM: α2K104Dβγ: 2.7 ± 3.1% of control, n=6, P<0.01; αE137Gβγ: 0.3 ± 3.4% of control, n=6, P<0.01; α2K155Dβγ: 6.3 ± 6.8% of control, n=6, P<0.01; Glu 1mM: α2K104Dβγ: 15.1 ± 4.4% of control, n=6, P<0.01; αE137Gβγ: 5.7 ± 5.6 % of control, n=6, P<0.01; α2K155Dβγ: 2.7 ± 3.1% of control, n=6, P<0.01, Student's *t*-test).

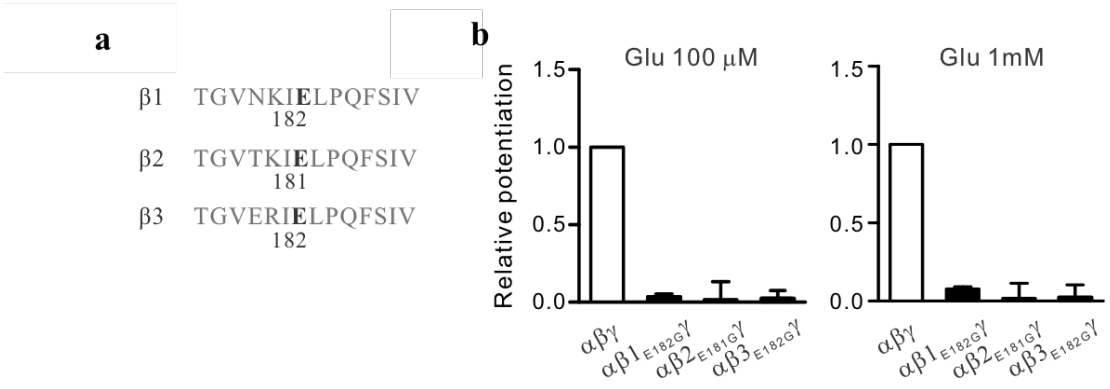

Figure 4.5 Conservation of the glutamate-binding site in the β subunit family

a) Sequence alignment showed that β2 E181 is conserved, corresponding E182 in β1 and β3 subunits. **b)** Mutating β1E182G, β2E181G, and β3E182G equally impaired glutamate-mediated potentiation in respective receptors (Glu 100 μM: αβ1E182Gγ: 2.4± 2.0% of control, n=6, P<0.01; αβ2E181Gγ: 1.6 ±11.6 % of control, n=6, P<0.01; αβ3E182Gγ: 2.3 ± 5.0% of control, n=6, P<0.01; Glu 1mM: αβ1E182Gγ: 7.6 ± 1.0% of control, n=6, P<0.01; αβ2E181Gγ: 1.9 ±9.6 % of control, n=6, P<0.01; αβ3E182Gγ: 5.2 ± 5.6% of control, n=6, P<0.01, Student's *t*-test).

4.2.4 Incorporation of γ subunit compromises glutamate allosteric potentiation of GABA$_A$Rs by reducing the number of glutamate-binding pockets

Previously, we have found that introducing a γ subunit compromised the efficacy of glutamate modulation (Figure 3.6). Given the glutamate-binding pocket is formed by critical residues of α and β subunits at the α+/β- interface, we hypothesize that the incorporation of γ subunit negative impacts glutamate potentiation by reducing the number of glutamate-binding pockets on GABA$_A$Rs. As shown in Figure 4.6a, for the GABA$_A$R containing only α and β subunits, there are two α+/β- interfaces, with potentially two glutamate-binding pockets (Figure 4.6a, Right). By contrast, for the GABA$_A$Rs containing α1β2γ2 subunits, there appears only one α+β- interface and hence only a single glutamate-binding pocket (Figure 4.6a, Left & Middle). We next, questioned why the corresponding residues in the γ subunit at α+/γ- or γ+/β- interfaces cannot form the glutamate-binding pocket by comparing the corresponding residues of γ2 with their counterparts in either α and β subunits.

Alignment of γ2 with β2 showed that, in the position corresponding to β2E181 at the β2-interface, the γ2 subunit contains a positively charged residue R197 (Figure 4.6b; Top panel on the left). As the negatively charged E181 is required for its interaction with the positively charged NH_3^+ group of glutamate for glutamate-binding to the receptor, the opposite charged γ2R197 will not be likely able to mimic β2E181 in forming the glutamate-binding pocket with these critical residues in α+ side of α1 subunit. If this reasoning is correct, we hypothesize that substitution of γ2R197 with the negatively charged amino acid glutamate, mimicking the critical E181 residue of β subunit at the α+/β- interface, would be able to generate a new site at the α+/mutated γ- interface (Figure 4.6b, Bottom panel on the left), and consequently restore the

reduced level of glutamate potentiation in αβγ receptors to that in αβ receptors. Indeed, as we expected, that co-expression of γ2R197E with wild type α1 and β2 subunits significantly increased glutamate-induced potentiation of GABA currents in α1β2γ2 containing $GABA_A$Rs at both glutamate concentrations of 100 μM (204.9 ± 48.6% of wild-type α1β2γ2, $P<0.05$) and 1 mM (225.7 ± 51.9% of wild-type α1β2γ2, $P<0.05$; Figure 4.6b, Right). The level of increased potentiation effect was comparable with that observed in α1β2 receptors (Glu 1μM: 92.7 ± 22.0% of wild-type α1β2, no significant difference; Glu 1mM: 82.4 ± 16.9% of wild-type α1β2, no significant difference, Figure 4.6b), suggesting that the single mutation on γ subunit created an additional glutamate-binding site at the α+/γ- interface.

Similarly, alignment of γ2 and α1 showed that γ2 contains a negatively charged residue (E168) at the position corresponding to the positively charged α1K155 that is required for forming glutamate-binding pocket along with β subunit in the α+/β- interface (Figure 4.6c left). Importantly, mutating this negative E168 residue into a positively charged lysine residue (γE168K) also significantly enhanced glutamate-mediated potentiation of GABA currents, comparing with wild-type α1β2γ2 $GABA_A$Rs (Glu 100μM: 169.4 ± 32.5% of wild-type α1β2γ2, $P<0.05$; Glu 1mM: 190.9 ± 39.2% of wild-type α1β2γ2, $P<0.05$; Figure 4.6c). However, the γE168K mutation failed to fully restore the compromised potentiation in γ2 containing $GABA_A$Rs (Glu 100μM: 76.9 ± 16.3% of wild-type α1β2, $P<0.05$; Glu 1mM: 45.4 ± 10.1% of wild-type α1β2, $P<0.05$; Figure 4.6c), indicating that the newly created binding site at the γ+/β- interface is not as potent as the original binding site at the α+/β- interface.

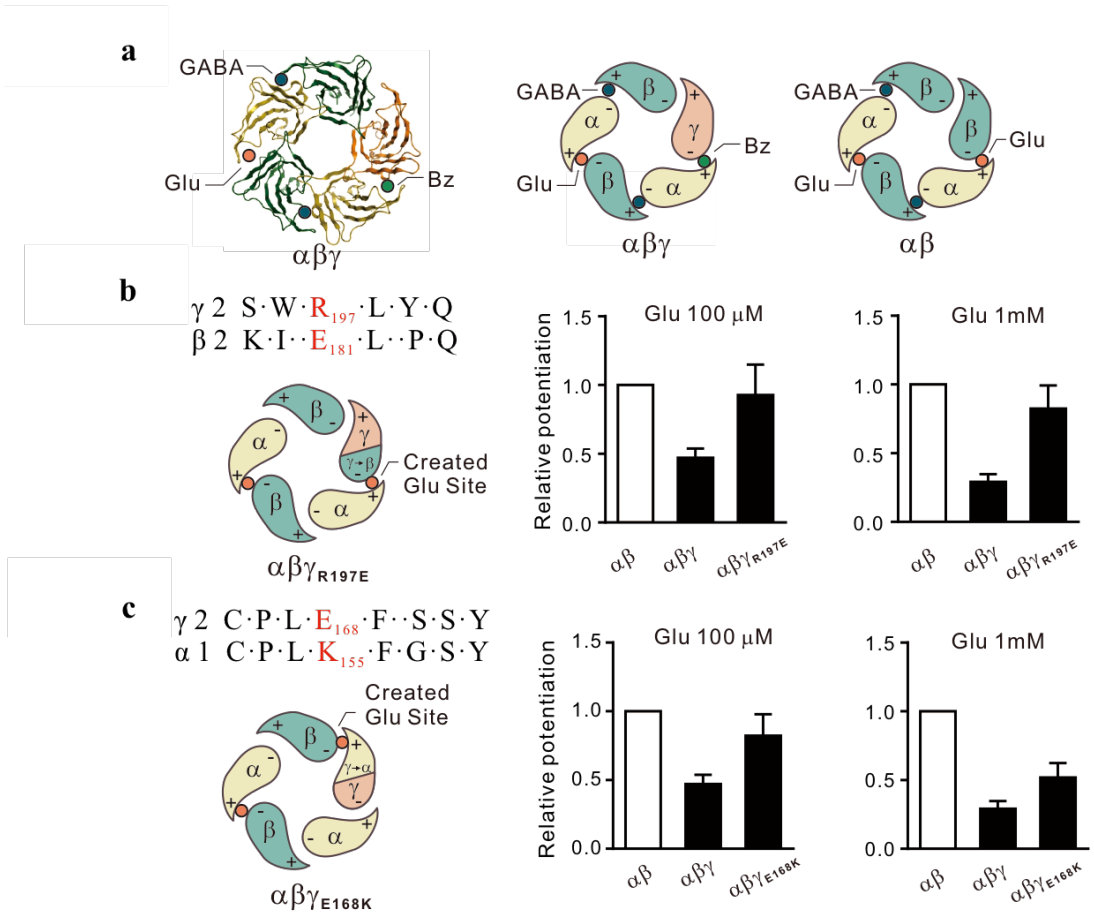

Figure 4.6 γ subunit reduces the glutamate potentiation by disrupting the binding pocket in the $GABA_AR$

a) Top-down structural views of pentameric $GABA_AR$ with α1β2γ2 (Left and Middle panels) and α1β2 (Right panel). GABA (Black dot), glutamate (Glu; Red dot) and benzodiazepine (Bz; Green dot) binding sites are respectively located at different interfaces in αβγ (middle) and αβ $GABA_ARs$ (right). The requirement of critical residues for the glutamate-binding pocket in the α+/β- interface predicts that the αβγ receptor contains only one of the functional glutamate binding sites (Left and Middle panels), whereas αβ receptor has two (Right panel). **b)** The sequence alignment indicates the substation of β2E181 at the corresponding position of γ2 subunit with an opposite charged residue of R197 (Left), preventing the formation of the second glutamate binding pocket; and reversing the charge with the γ2R197E mutation creates a new glutamate binding pocket at the α+/γ- interface, increasing the potentiation to the level comparable to that of α1β2 receptors (Right panel; n=6). **c)** Similarly, γ2E168K mutation mimics α1K155 creating a new glutamate binding pocket at the γ2+/β- interface (Left panel), and thereby increases the level of glutamate potentiation (Right panel; n=10).

In results shown in Figure 4.2a, we demonstrated that single mutation of these critical amino acid residues of either α1 or β2 in α1β2γ2 receptors is sufficient to eliminate glutamate-induced potentiation, likely due to the loss of the sole glutamate-binding pocket formed at the single α+/β- interface. If that is the case, we reason that by creating a new glutamate-binding pocket at β+/γ- with the γ2E168K mutation described above (Figure 4.7a), we should be able to restore the loss of glutamate sensitivity in these glutamate-binding deficit mutants. Indeed, we found that in HEK cells expressing α1K155D along with β2γ2, glutamate failed to increase GABA currents even at high glutamate concentration, but the glutamate potentiation was partially restored by mutating the wild type γ2 into γ2E168K (Glu 100μM: 4.2 ± 1.8% v.s. 71.8 ± 14.7% of wild-type α1β2γ2, in the absence or presence of γ2E168K mutation, respectively, $P<0.05$; Glu 1mM: 12.0 ± 4.6% v.s. 76.4 ± 10.5% of wild-type α1β2γ2, in the absence or presence of γ2E168K mutation, respectively, $P<0.05$; Figure 4.7a). Similarly, creating a new glutamate-binding pocket at α+/γ- with the γ2R197E mutation (Figure 4.7b) also rescued the loss glutamate potentiation due to the disrupting the single glutamate-binding pocket caused by β2E181G mutation (Glu 100μM: 3.2 ± 2.8% v.s. 76.6 ± 10.4% of wild-type α1β2γ2, in the absence or presence of γ2 R197E mutation, respectively, $P<0.05$; Glu 1mM: 4.3 ± 2.4% v.s. 84.5 ± 16.9% of wild-type α1β2γ2, in the absence or presence of γ2 R197E mutation, respectively, $P<0.05$; Figure 4.7B). The above results further emphasized the critical role of the four afore-identified residues in formation of the glutamate-binding pocket.

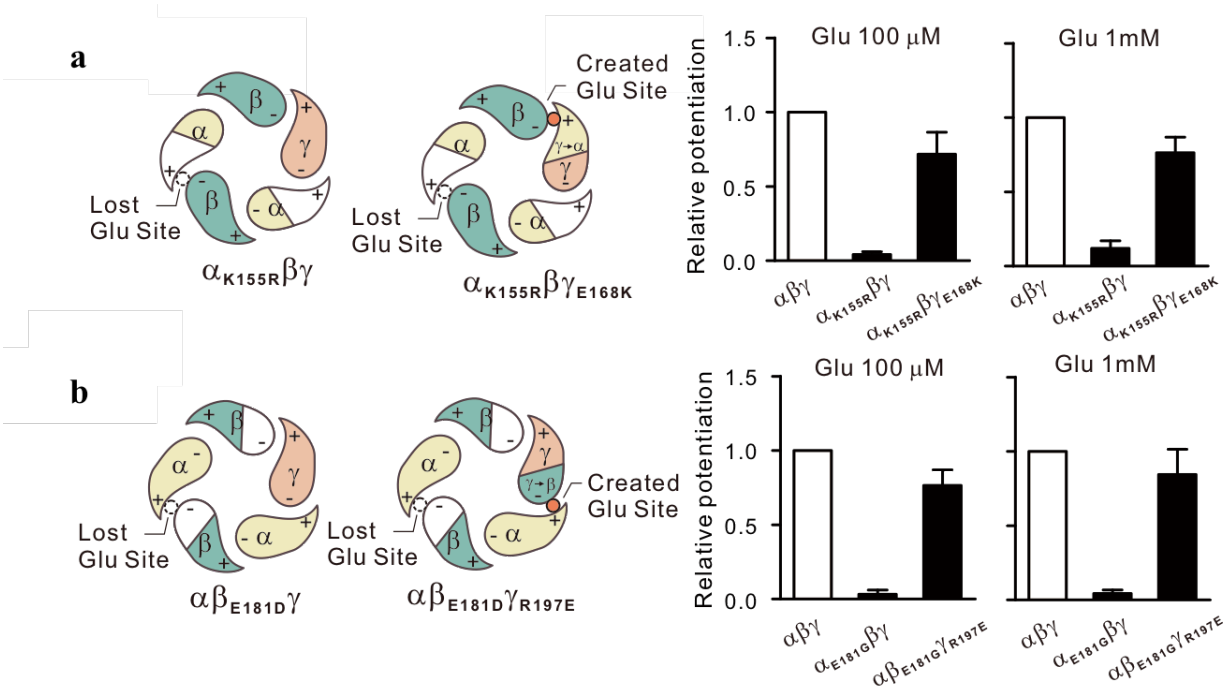

Figure 4.7 Creating an artificial glutamate-binding site at α+/γ- or γ+/β- interface rescues the potentiation deficit in α+/β- mutated $GABA_AR$

a) The newly created glutamate binding pocket at the γ+/β- interface by either γ2E168K (Left panels) is capable of rescuing glutamate potentiation deficit caused by disrupting the glutamate binding pocket in the α+/β- interface with α1K155D (Right panels; n=6). **b)** Similarly, creating a new glutamate binding pocket at the α1+/γ2- interface with γ2R197E mutation (Left panels) rescues glutamate potentiation deficit produced with β2E181G (Right panels; n=7).

4.3 Discussion

In Chapter 3, we have confirmed the direct binding of glutamate to $GABA_A$Rs at a novel site that does not overlap with known GABA agonist binding sites. Based on the result of computer-assisted *in silicon* docking screening, we did a systematic mutational analysis and positively identified the glutamate-binding pockets located in the α+/β- interface with four critical residues including α1K104, α1E137, α1K155 and β2E181 (Figure 4.2a). Notably, single mutation of those 4 critical residues produced larger negative impact on glutamate-induced potentiation of αβγ containing $GABA_A$Rs than that of αβ containing $GABA_A$Rs. More specifically, only single mutation of β2 at E181 (β2E181G) resulted in a significant reduction in the potentiation of GABA responses by both low (100μM) and high (1mM) concentration of glutamate. These results implied that compared with other critical residues, β2 E181 may be more critical for its interaction with NH_3^+ group of glutamate, and that the less impaired interaction between glutamate and the receptor by a single mutation at α1 K104, or E137, or K155 are possibly due to the partial compensation by the stronger binding of β2 E181.

Through double substitution experiment, we found that simultaneously mutating 2 critical residues that are located at different (α1β2) subunits or that interact with different functional groups ($NH3^+$/COO^-) of glutamate could dramatically reduce the $GABA_A$Rs' sensitivity to glutamate, confirming the involvement of those 4 critical residues in glutamate-binding site. The requirement of double mutation in α1β2, but only single mutation in α1β2γ2 receptors are in a good agreement with the electrophysiological results that showed glutamate produced a more pronounced potentiation in HEK cells expressing α1β2 $GABA_A$Rs (Figure 3.6a) when compared to GABAARs expressing α1β2γ2 receptors (Figure 3.6b), and provide further support for the

modeling predicted glutamate-binding pocket (P2) at the α+/β- interface: it is encompassed by 5 amino acids listed in Table 4.1, and particularly, the four charged residues (K104, K155, E137 on α1, and E181on β2 subunit), respectively interacting with COO^- and NH_3^+ groups of both TBOA and glutamate (Figure 4.1 and 4.2e).

It is interesting to note that these electrostatic interactions between charged amino acid residues and glutamate are common features among several glutamate-binding pockets recently identified on other glutamate-binding proteins/ion channels/receptors co-crystallization studies (Acher and Bertrand, 2005; Armstrong and Gouaux, 2000; Armstrong et al., 1998; Chen et al., 2005a; Laube et al., 1997; Mayer, 2005; Mayer et al., 2006; Tsuchiya et al., 2002; Wellendorph and Bräuner-Osborne, 2009). The common amino acid residues involved in interacting with glutamate within these glutamate-binding pockets are two positive charged residues of either arginine (R) or lysine (K) that form electrostatic interactions with the negatively charged termini carboxyl group (COO^-) of the glutamate, and two negative charged residues of either aspartic acid (D) or glutamate (E) that electrostatically interact with the positively charged amino group (NH_3^+) of glutamate. In this regard, the glutamate-binding pocket on $GABA_AR$ we identified here also shares the same characteristics, having the positive charged residues (α1K104 and α1K155) and negatively charged residues (α1E137 and β2E181) capable of directly binding to the opposite charged groups of glutamate (Figure 4.2e). These analyses provide additional support for the identified pocket as the glutamate-binding sites by which glutamate produces allosteric potentiation of $GABA_ARs$. It is also important to note that these critical residues α and β subunits are conserved among their respective subfamilies (Figure 4.4 & 4.5). Given, most native $GABA_ARs$ contain α and β subunits (Mohler, 2006), the glutamate-binding pocket likely exists

and functionally operates among most, if not all $GABA_AR$s, thereby having more profound and wild spread physiological, pathological and therapeutic significance.

Another notable feature of the $GABA_AR$ glutamate-binding pocket is that it is distinct from any other known ligand and modulatory sites on the receptor. First, it does not overlap with the GABA agonist binding sites previously identified at the β+/α- interfaces (Lummis, 2009), and this is fully supported by our results that even a high concentration of GABA failed to competitively replace glutamate binding in the [^{3}H]-glutamate binding assays (Figure 3.7) and that mutation of either these critical residues involved in glutamate binding did not effect GABA activation of $GABA_AR$s (Figure 4.2b). Moreover, this newly identified glutamate-binding pocket is also structurally and functionally distinct from the most well-characterized allosteric benzodiazepine modulatory site. Previous studies have pinned down the benzodiazepine binding site at the α+/γ- interface, requiring the presence of a γ subunit in the $GABA_AR$ (Figure 4.6a, Middle) (Benson et al., 1998; Sigel, 2002; Sigel and Steinmann, 2012; Wieland et al., 1992). However, the glutamate-binding site identified in the present study is located the α+/β- interface, not requiring the presence of a γ subunit (Figure 4.6a, Right). In fact, incorporation of a γ actually partially impairs the glutamate-induced potentiation. Our sequence alignment and mutational analysis confirm that the inability of γ subunit to support glutamate binding and modulation is primarily due the lack of certain amino acid residues required for the formation of the glutamate-binding pocket (Figure 4.6 b & c). Specifically, in comparison with γ2 with β2 and α1 subunits, γ2 has the oppositely charged amino acid residues E168 and R197 at corresponding positions of α1 K155 and β2 E181, respectively, and these make the γ subunit unable to form the glutamate-binding pocket at either the α1+/γ2- interface (Figure 4.6b) or γ2+/β2- interface

(Figure 4.6c). These results provide further strong support for our identification of the glutamate-binding pockets in the α+/β- interface of the $GABA_A$Rs. It is interesting to note that some benzodiazepine related chemicals such as CGS9895 can also positively modulate $GABA_A$R function via interacting with a putative binding pocket located adjacent to loop C in the α+/β- interface that is close to the predicted binding pocket P1 in the present study (Figure 4.1b) (Ramerstorfer et al., 2011; Sieghart et al., 2012). Since none of the residue mutations surrounding the loop-C region, that is critically required for CGS9895 (Sieghart et al., 2012), affected glutamate potentiation of $GABA_A$R function (Table 4.1), the CGS9895 binding pocket in the α+/β- interface is clearly distinct from the new glutamate-binding pocket identified in the present study.

It is worth mentioning that, the glutamate-binding pocket found here slightly overlaps with a previous known low-affinity zinc-binding pocket. The glutamate pocket is located above the zinc pocket but they share two critical residues, α1E137 and β2E181 (Hosie et al., 2003). This phenomenon is interesting because zinc is a negative allosteric modulator of the $GABA_A$R, which means pulling those two critical residues towards different directions by either zinc or glutamate can induce distinct effect on the $GABA_A$R. There is a possibility that glutamate exerts the potentiation by relieving the $GABA_A$R from zinc inhibition. However, it is unlikely since 90% of zinc inhibition is mainly through another binding located in the transmembrane domain (Horenstein and Akabas, 1998; Hosie et al., 2003; Wooltorton et al., 1997a).

Taken together, our results clearly demonstrate that glutamate exerts an allosteric potentiation of $GABA_AR$ function by direct binding to a novel glutamate-binding pocket formed by 4 charged amino acid residues of α and β subunits at the α+/β- interface of the $GABA_AR$.

Chapter 5: Development of novel positive allosteric $GABA_AR$ modulators targeting the glutamate-binding site

5.1 Introduction

$GABA_ARs$ are important targets for various therapeutic drugs. In particular, benzodiazepines have been one of the safest and most popular therapeutics for anxiety, sedation, and as anticonvulsants for the treatment of seizures (Shorter, 2005). Benzodiazepines, by binding to the benzodiazepine site located in the interface of γ and α subunits, allosterically modulate the function of γ-containing $GABA_ARs$ (Figure 4.6) (Sigel, 2002; Sigel and Steinmann, 2012). However a large portion of extrasynapatic $GABA_ARs$ do not contain the γ subunit, which makes them insensitive to benzodiazepines (Brickley and Mody, 2012). As such, benzodiazepines have limited effect on tonic GABA currents known to be critical for maintaining neuronal excitability (Brickley and Mody, 2012). Thus, it is imperative to develop new $GABA_AR$-based therapeutics that targets both synaptic and extrasynaptic $GABA_ARs$.

Since the novel glutamate-binding site we identified here only requires the α and β subunits (Figure 4.6), and would therefore be present on almost all native $GABA_ARs$, including those located extracellularly. To test if this site can be a therapeutic target for developing novel positive $GABA_AR$ modulators that can allosterically potentiate both synaptic and tonic GABA currents, Our lab employed the computer-assisted *in-silicon* molecular docking approach and performed a virtual high throughput chemical library screening. By using the same modeled receptor shown in Figure 4.2 we iteratively searched for small molecule compounds that can be docked into this newly identified glutamate binding pockets from the ZINC chemical library

(Irwin and Shoichet, 2005). This initial screening led to the identification of a group of glutamate-like molecules that can dock to the identified $GABA_AR$ glutamate binding pocket with high docking scores. We considered these compounds as the potential candidates acting at this glutamate-binding site.

5.2 Results

5.2.1 Screening of novel positive allosteric modulators of the $GABA_AR$

Based on the result of our initial *in-silicon* screening, we functionally characterized the identified compounds using recombinant $GABA_ARs$ in HEK 293 cells. 100 μM of each compound was bath applied to HEK 293 cells expressing α1β2γ2 containing $GABA_AR$ and their effects on GABA-induced current were tested using whole-cell patch clamp recording. As shown in Figure 5.1, among the 20 compounds we tested, 5 of them showed no effect on GABA current, 7 of them reduced GABA current and 8 of them showed potentiation of GABA current. Among those positive modulators of the $GABA_AR$, BRC640 exerted highest potentiation effect on GABA-induced current (Figure 5.1), suggesting it as the putative lead compound targeting the glutamate-binding site. Interestingly, ampicillin (Amp, CAS number 69-52-3), the widely used antibiotic, also met our initial selection criteria and bore a high docking score in our *in-silicon* screening. Electrophysiological test showed that ampicillin was capable to induce a significant potentiation of $GABA_AR$-mediated current in HEK 293 cells (Figure 5.1). Therefore, we classified ampicillin and BRC640 as tour candidate compounds and evaluated for their effects on the $GABA_AR$ in details.

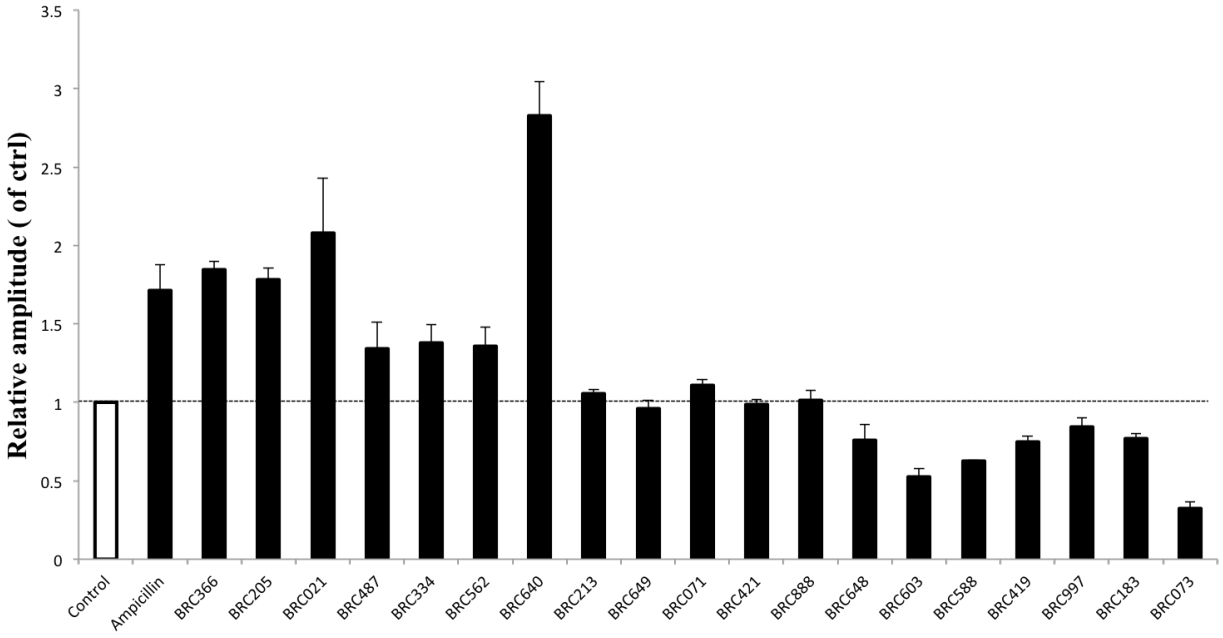

Figure 5.1 Screening of novel positive allosteric modulators of the $GABA_AR$

Results summary of the drug (100 μM) effect in HEK293 cells expressing α1β2γ2 $GABA_AR$. The relative amplitude compared with control were as below: Ampicillin: 171.3±16.6%; BRC366: 184.4±5.5%; BRC205: 178.1±7.3%; BRC021: 207.9±35.2%; BRC487: 134.1±16.9%; BRC334: 137.8±11.5%; BRC562: 135.6±12.5%; BRC640: 282.8±21.5%; BRC213: 105.5±2.9%; BRC649: 95.7±5.6%; BRC071: 110.8±3.5%; BRC421: 98.4±3.6%; BRC888: 101.4±6.0%; BRC648: 75.7±9.8%; BRC603: 52.6±5.3%; BRC588: 62.2±3.4%; BRC419: 74.6±3.6%; BRC997: 84.1±5.8%; BRC183: 77.0±2.9%; BRC073: 32.2±4.3%

5.2.2 Ampicillin positively regulates $GABA_A$Rs-mediated current by acting at the glutamate-binding site

Our initial screening identified ampicillin as a positive modulator of $GABA_A$Rs. As shown in Figure 5.2, in HEK 293 cells expressing α1β2γ2 $GABA_A$Rs, application of Amp (300 μM, a concentration to induce the approximate maximum response), like glutamate, produced no currents on its own, but reversibly potentiated currents induced with GABA (1 μM) (171.3±16.5% of control, $P<0.05$, n=15, Figure 5.2b, Left). This Amp-mediated potentiation effect is concentration-dependent with an EC_{50} of 6.06±1.24 μM (Figure 5.2c, Left). Moreover, 300 μM Amp shifted the GABA concentration-response curve towards left and deceased the EC_{50} of GABA from 4.78±1.12 to 1.97±1.23 μM (Figure 5.2c, Right). This reduction of EC_{50} was not associated with an obvious alteration in either the Hill coefficient (1.21±0.13 and 1.41±0.15 in the absence and presence of 300 μM Amp, respectively) or the maximum GABA responses, indicating that Amp may affect GABA binding affinity on $GABA_A$Rs. Notably, ampicillin also significantly potentiated the function of α1β2 containing $GABA_A$Rs (233.1±56.7% of control, $P<0.05$, n=6, Figure 5.2b, Right), indicating ampicillin very likely bound to the α+/β- interface, where glutamate-binding site was located.

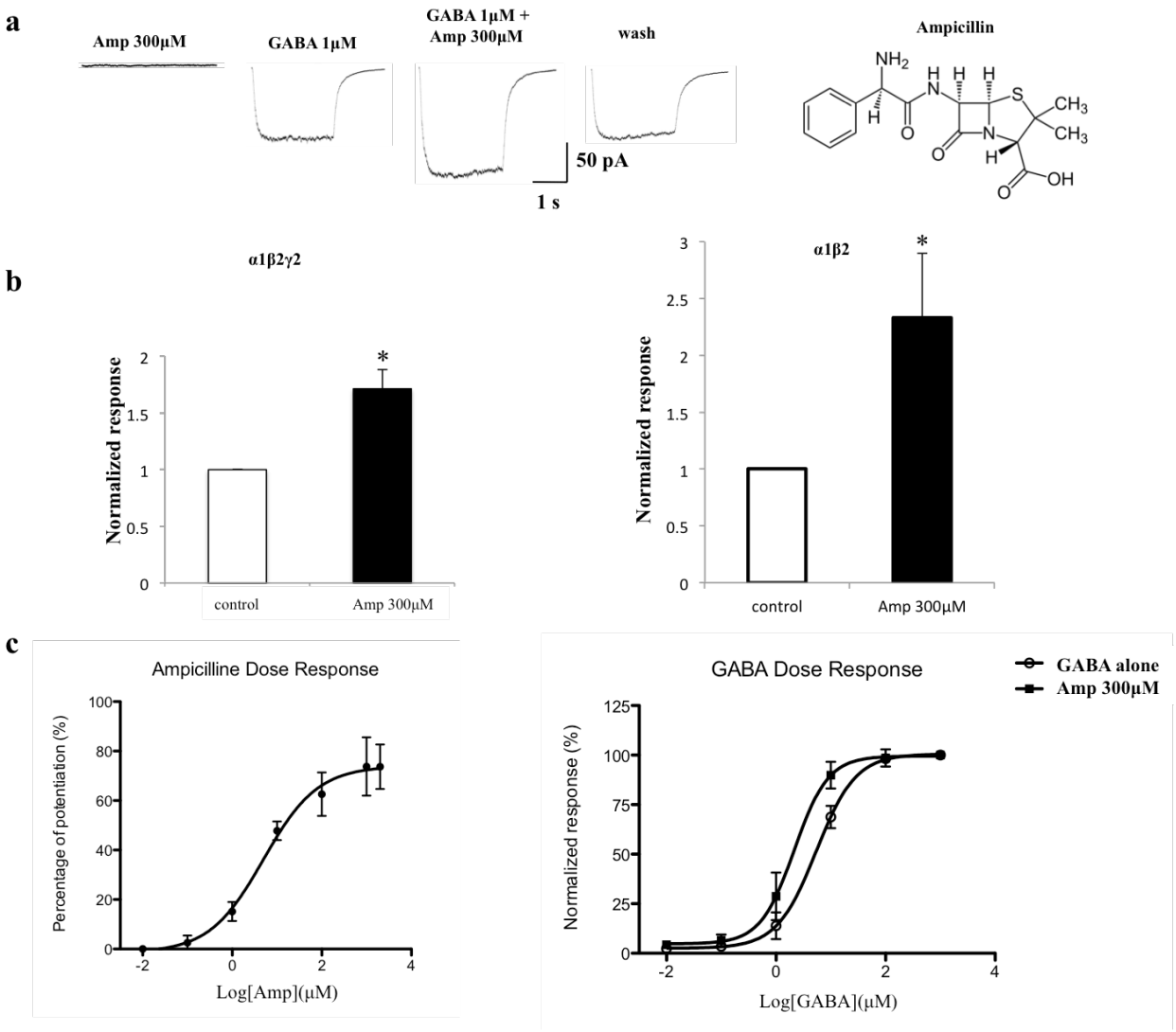

Figure 5.2 Ampicillin positively modulates the $GABA_AR$-mediated current in HEK293 cells

a) Representative traces showing ampicillin (Amp, 300 μM) alone did not induce any noticeable current but it reversibly enhanced the GABA (1 μM)-induced current in HEK293 cells expressing α1β2γ2 $GABA_ARs$ (Left panel). The right panel shows the structure of Amp. **b)** Quantified results showing Amp (300 μM) significantly potentiated the amplitude of GABA (1 μM) evoked current in HEK293 cells expressing either α1β2γ2 $GABA_ARs$ (171.3±16.5% of control, n=15, $p<0.05$, Student's *t*-test), or α1β2 $GABA_ARs$ (233.1±56.7% of control, n=6, $p<0.05$, Student's *t*-test). c) Concentration-response curve of Amp potentiation effect on GABA (1 μM) evoked current (Left panel, n=6). Amp (300 μM) left shifted the GABA concentration-response curve (Right panel, n=6).

Next, we tested if Amp was capable to elicit a similar potentiation effect on native $GABA_A$Rs. Whole-cell patch clamp recording was conducted in cultured hippocampal neurons. Fast perfusion of Amp (300 μM) did not produce any noticeable current in neurons (Figure 5.3a). However, co-application of Amp (300 μM) dramatically increased the amplitude of GABA (0.5 μM)-induced currents (158.0±5.6%, $P<0.05$, $n=9$; Figure 5.3b). Additional tests revealed that Amp had no effects on AMPA or NMDA glutamate receptor-mediated currents (Figure 5.3c). These results suggest that Amp mimics glutamate, being a specific agonist at the putative $GABA_A$R glutamate-binding site and allosterically potentiating $GABA_A$R function, but is not an agonist of any known ionotropic glutamate receptor.

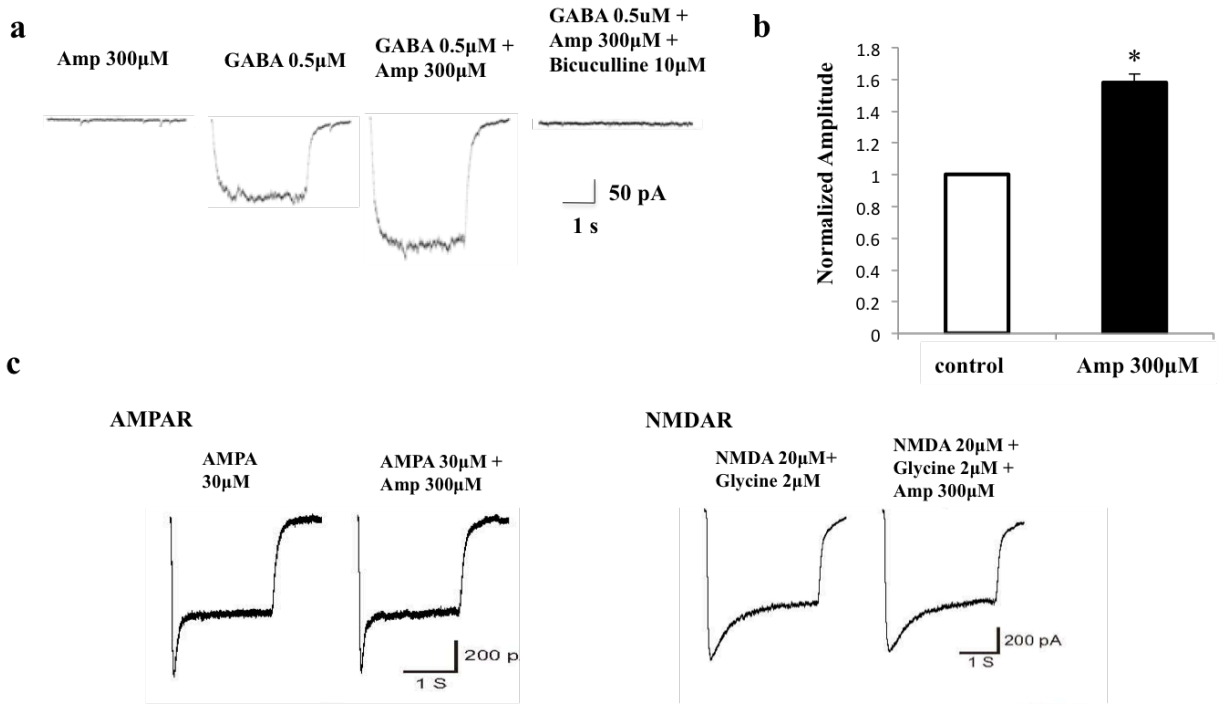

Figure 5.3 Ampicillin positively modulates the $GABA_AR$-mediated current in cultured hippocampal neurons

a) Representative traces showing Amp (300 μM) did not evoke any current in cultured hippocampal neuron. GABA (0.5 μM) induced an inward current that can be potentiated by Amp (300 μM) and fully blocked by bicuculline (10 μM) (Left panel). The right panel shows the structure of Amp. **b)** Quantified results from 9 cells. Amp (300 μM) significantly potentiated the amplitude of GABA (1 μM) evoked current (158.0±5.6% of control, $P<0.05$, n=9, Student's *t*-test). **c)** Amp (300 μM) have no effect on AMPAR-mediated current (Left panel) and NMDAR-mediated current (Right panel).

As an agonist at the glutamate-binding site, we expected that Amp was able to potentiate both phasic currents (i.e. mIPSCs) mediated by synaptic $GABA_A$Rs and the tonic currents mediated by extrasynaptic currents. Indeed, bath application of Amp (300 μM) significantly increased the mIPSC amplitude (128.8±8.4% of control; $P<0.05$; n=10; Figure 5.4b, Left), without altering the mIPSCs frequency (94.8±2.9% of control; $P>0.05$; n=10; Figure 5.4b, Right), suggesting its potentiation effects via a postsynaptic modulation of $GABA_A$Rs, but not a presynaptic alteration of GABA release. Similarly, as shown in Figure 5.5, Amp (300μM) also significantly enhanced the amplitude of tonic GABA currents (170.7±15.8% of control; $P<0.05$; n=11, Figure 5.5b). Taken together, our results demonstrated that Amp-induced positive modulation was associated with both synaptic and extrasynaptic $GABA_A$Rs, being of profound pharmacological and therapeutic significance.

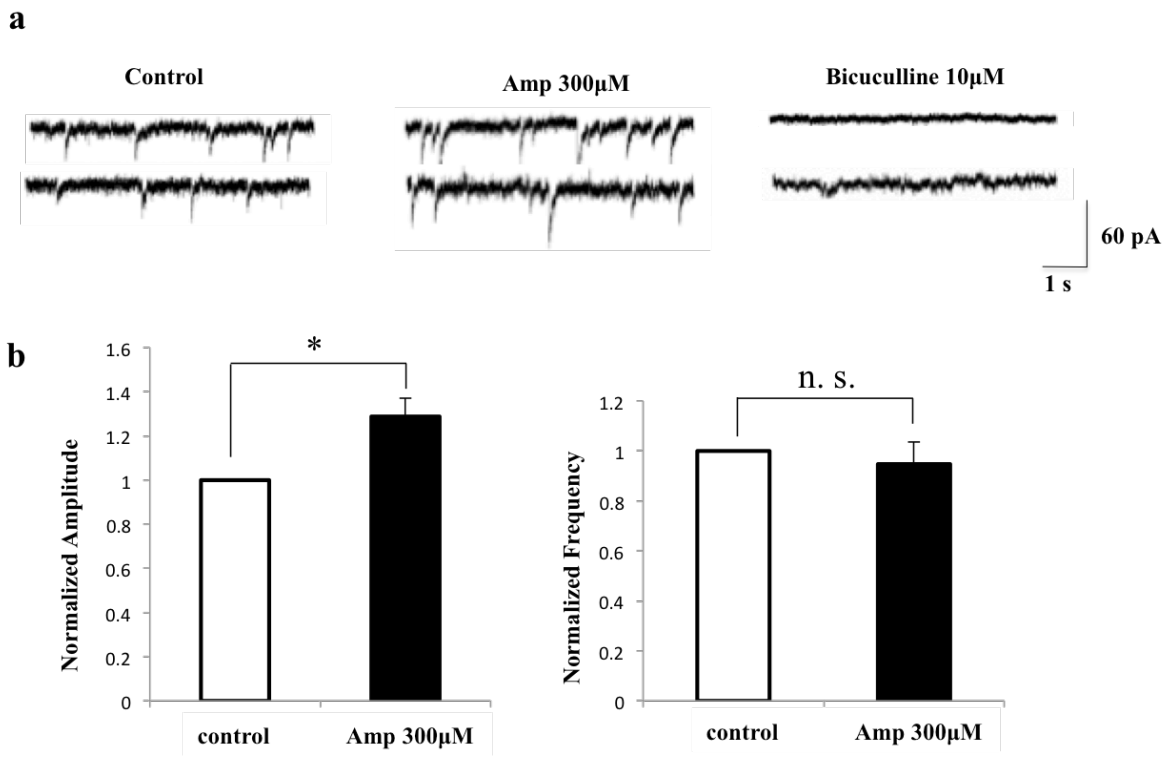

Figure 5.4 Ampicillin potentiates the $GABA_AR$-mediated phasic inhibition

a) Representative trace of mIPSCs before and after Amp (300 μM) application in hippocampal neurons. The mIPSC can be blocked by 10 μM bicuculline. **b)** Quantified results from 10 neurons. Amp (300 μM) significantly increased the mIPSC amplitude (128.8±8.4% of control; $P<0.05$, Student's *t*-test; left panel) but induced no significant change in mIPSC frequency (94.8±2.9% of control; $P>0.05$, n=10, Student's *t*-test; right panel).

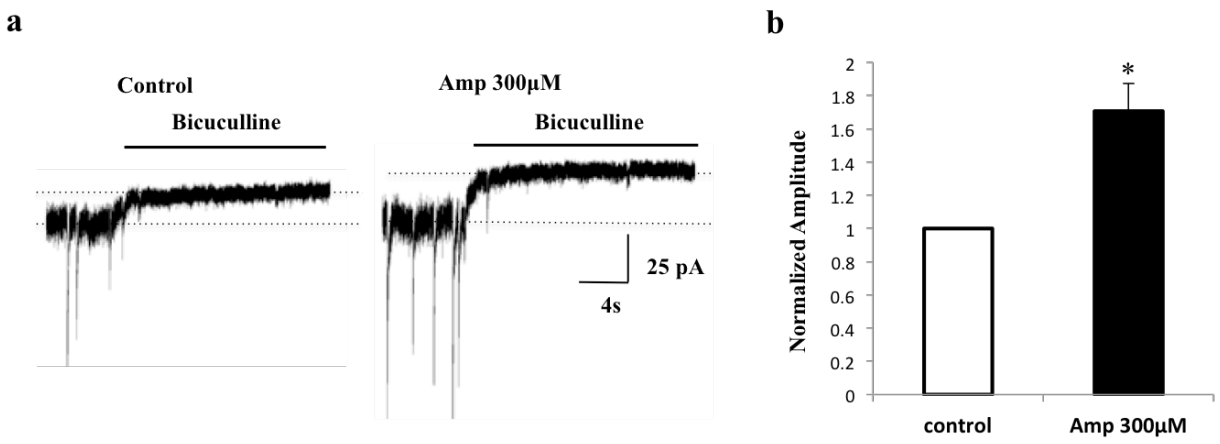

Figure 5.5 Ampicillin potentiates the $GABA_AR$-mediated tonic inhibition

a) Representative trace of tonic GABA current. Amp (300 µM) application increased the amplitude of tonic current in hippocampal neurons. **b)** Quantified results from 11 neurons. Amp (300 µM) significantly enhanced tonic GABA current (170.7±15.8% of control, $P<0.05$, Student's *t*-test).

Structural comparison of glutamate and Amp showed that Amp is structurally similar to glutamate, having both negatively and positively charged groups to respectively interact with the charged residues of the glutamate-binding pocket (Figure 5.6a). Using homology model docking analysis, we found that 3 of the 4 identified glutamate-binding residues (α1K155, α1E137, β2E181) were also critical in forming receptor-ligand interaction with Amp. To further confirm that Amp indeed exerts its potentiation effects on $GABA_A$Rs through acting at the glutamate-binding site on the $GABA_A$R, we expressed glutamate-binding deficient $GABA_A$R bearing β2E181G mutation in HEK 293 cells. Bath application of Amp failed to potentiate the GABA-induced currents in those cells (Figure 5.6b), indicating Amp might occupy the same binding site as glutamate.

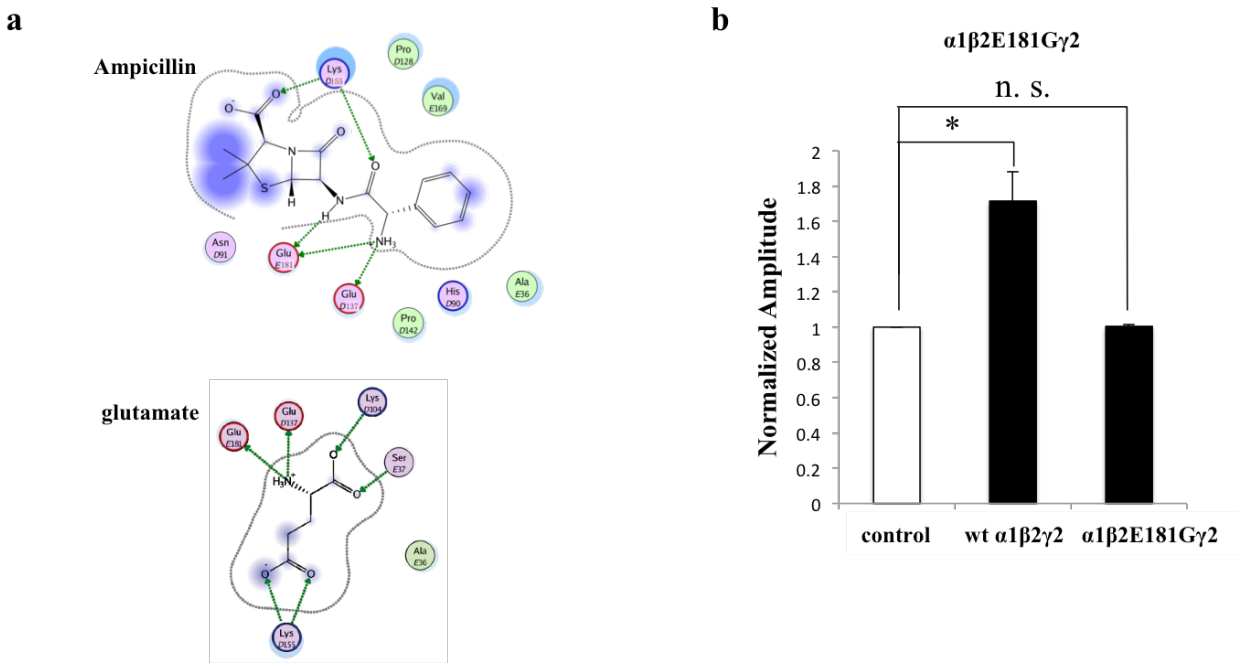

Figure 5.6 Ampicillin actes at glutamate-binding site on the $GABA_AR$

a) Structural and docking comparison of glutamate and Amp showing that similar as glutamate, Amp also has both negatively and positively charged groups to respectively interact with the charged residues of the glutamate-binding pocket. **b)** Mutating the critical residue (β2E181) in glutamate-binding site abolished Amp-induced potentiation of $GABA_AR$ function (α1β2E181Gγ2 100.3±1.2% of control, n=6, P>0.05, one-way ANOVA test).

Ampicillin belongs to penicillin family and shares almost the same structure with Penicillin-G, except for an additional amino group (Figure 5.7a). Interestingly, previous studies have shown that Penicillin-G is a potent $GABA_AR$ antagonist and is widely used to create experimental models of epilepsy (Fujimoto et al., 1995; Pickles and Simmonds, 1980). To compare the penicillin-G's effect on the $GABA_AR$ with that of ampicillin, we applied penicillin-G and ampicillin to the same HEK 293 cell expressing $\alpha1\beta2\gamma2$-containing $GABA_AR$. penicillin-G (PNG, 300 μM) itself did not evoke any noticeable current, but it significantly inhibited the GABA (1 μM)-induced currents (13.2±1.3%, $P<0.05$, n=7; Figure 5.7b & c). In the same cell, following wash out of PNG, bath application of Amp was still capable to produce a dramatic potentiation of GABA current (172.9±9.7%, $P<0.05$, n=7; Figure 5.7b & c). Notably, mutating the critical residue for glutamate binding (β2E181) had no effect on the PNG-induced inhibition of GABA current (12.0±1.8% v.s.13.2±1.3% in the presence or absence of β2E181G mutation, Figure 5.7d). Thus, our results clearly demonstrated the difference between PNG and Amp in modulation to $GABA_ARs$ and highlighted the importance of the additional amino group in mediating Amp binding on $GABA_ARs$.

Taken together, these results suggest that the glutamate binding site is a novel therapeutic target upon which new class of $GABA_AR$ modulators can be developed, and Amp may, by specifically functioning as a specific agonist at this $GABA_AR$ glutamate binding site without affecting ionotropic glutamate receptors, represent an example therapeutic compound from this class.

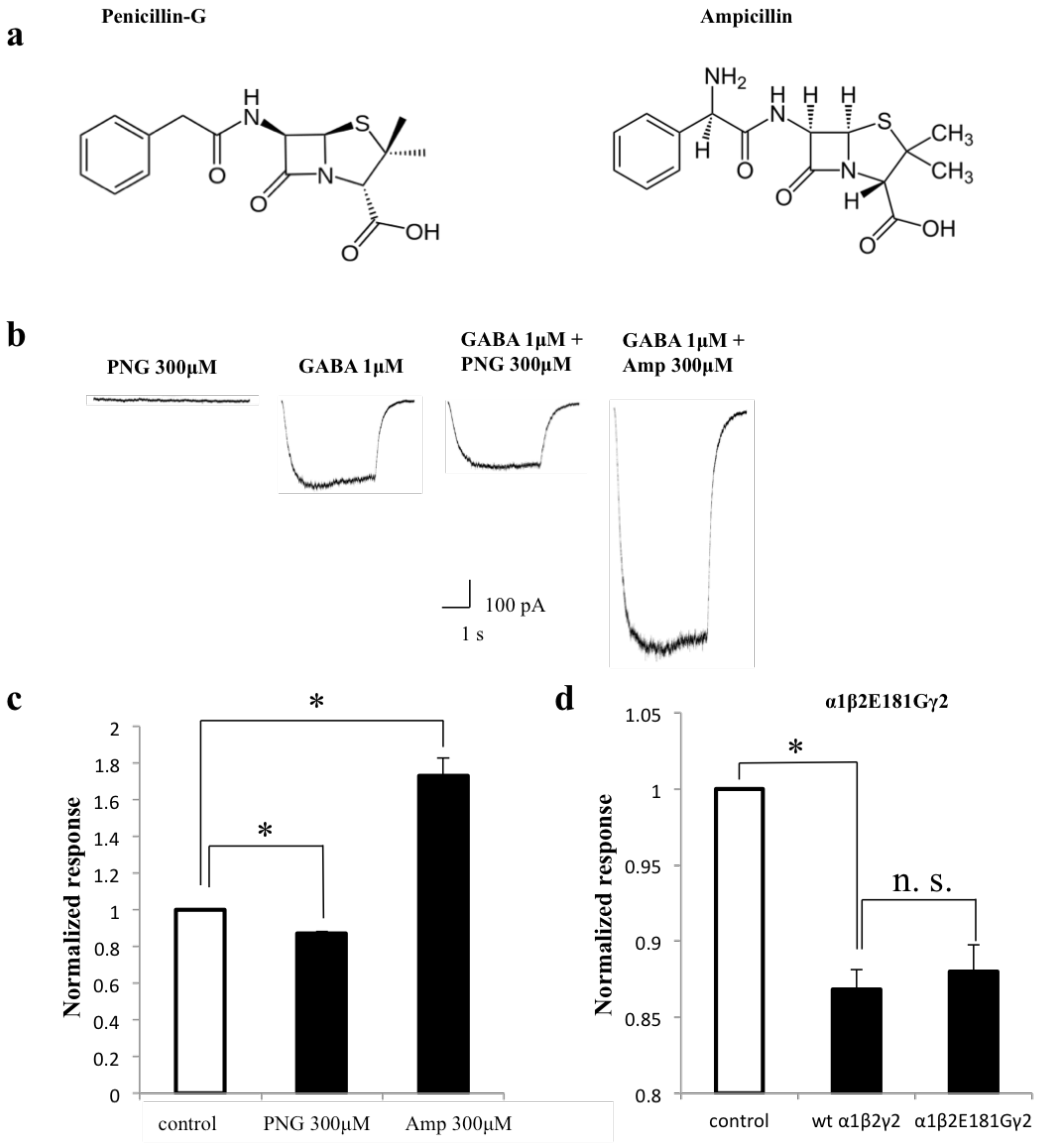

Figure 5.7 Ampicillin and penicillin-G exert distinct modulation effects on the $GABA_AR$

a) Structural comparison of Amp (Right) and penicillin-G (PNG, Left). Those two molecules share the similar structure except for the additional amino group of Amp. **b)** Representative trace showing PNG as a negative modulator but Amp as a positive modulator of the $GABA_AR$. **c)** Quantitative results from 7 cells. PNG (300 μM) decreased the amplitude of GABA current (86.8±1.3% of control, $P<0.05$, n=7, Student's *t*-test) while Amp increased the amplitude (172.9±9.7%, $P<0.05$, n=7, Student's *t*-test). **d)** Mutating glutamate-binding site has little effect on PNG-mediated negative modulations (wild-type 86.8±1.3% vs α1β2E181Gγ2 87.9±1.8% of control, $P>0.05$, n=6, one-way ANOVA test).

5.2.3 Identification of BRC640 as a novel $GABA_AR$ positive modulator targeting the glutamate-binding site

Among the 20 drugs we have tested in the initial screening, a drug named BRC640 showed drastic improvement in positive modulation effect on the $GABA_AR$. We then conducted a detailed characterization of this drug as the lead compound targeting glutamate-binding site on the $GABA_AR$. As shown in Figure 5.8, BRC640 dramatically potentiated GABA-induced currents in HEK 293 cells expressing either α1β2 containing $GABA_ARs$ or α1β2γ2 containing $GABA_ARs$ (α1β2: 377.6±56.1%, $P<0.05$, n=6; α1β2γ2: 282.8±21.5%, $P<0.05$, n=7; Figure 5.8b). Similarly as that of glutamate, BRC640-induced potentiation effect was also compromised by the incorporation of γ2 subunit into $GABA_ARs$, indicating BRC640 may also bind to the α+/β- interface. Concentration-response analysis revealed that the EC_{50} of BRC640 is around 17.5± 1.11 μM (Figure 5.8c, Left). Bath applying BRC640 at a fixed concentration of 100 μM produced a leftward shift of the GABA does-response curve (11.34±1.28 μM and 1.04±1.06 μM in the absence and presence of 100 μM BRC640, respectively; Figure 5.8c, Right), indicating BRC640 may increase GABA binding affinity on $GABA_ARs$.

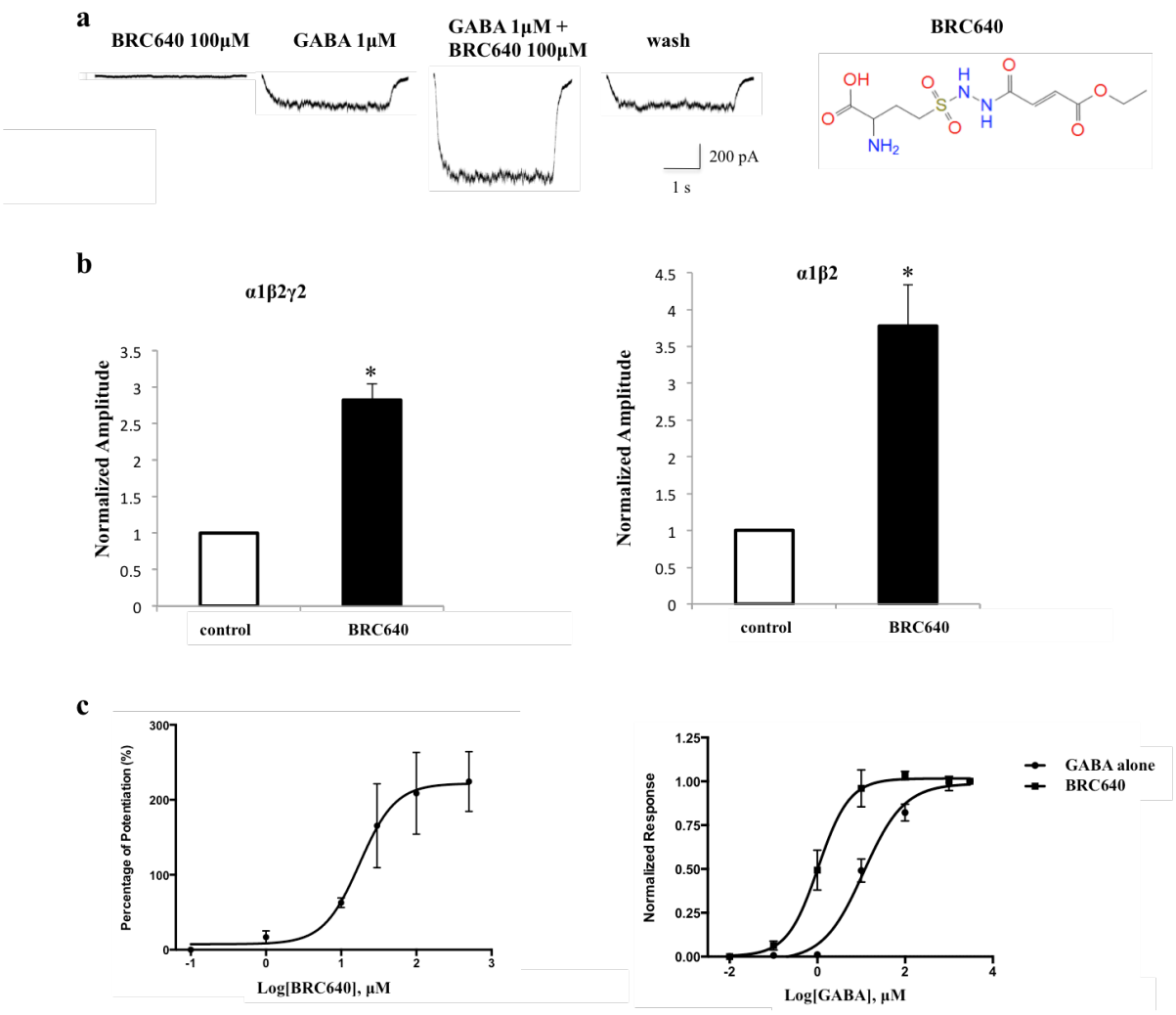

Figure 5.8 BRC640 positively modulates the $GABA_AR$-mediated current in HEK293 cells

a) Representative traces showing BRC640 (100 μM) alone did not induce any noticeable current but it reversibly enhanced the GABA (1 μM)-induced current in HEK293 cells expressing α1β2γ2 $GABA_ARs$ (Left panel). The right panel shows the structure of BRC640. **b)** Quantified results showing BRC640 (100 μM) significantly potentiated the amplitude of GABA (1 μM) evoked current in HEK293 cells expressing either α1β2γ2 $GABA_ARs$ (282.8±21.5% of control, n=7, $p<0.05$, Student's *t*-test), or α1β2 $GABA_ARs$ (377.6±56.1% of control, n=6, $p<0.05$, Student's *t*-test). **c)** Concentration-response curve of BRC640 potentiation effect on GABA (1 μM) evoked current (Left panel, n=6). BRC640 (100 μM) left shifted the GABA concentration-response curve (Right panel, n=6).

Native $GABA_A$Rs were also sensitive to BRC640, as shown in Figure 5.9. 100 μM BRC640 did not evoke any noticeable current in cultured hippocampal neurons (Figure 5.9a), suggesting that BRC640 was not capable of activating any ionotropic receptor on its own. Bath application of BRC640 (100 μM) significantly potentiated the neuronal current induced by GABA (0.5 μM) (211.4±10.9%, $P<0.05$, $n=4$; Figure 5.9B). The potentiated current was fully blocked by bicuculline (10 μM), confirming the current was mediated by $GABA_A$Rs (Figure 5.9a). Furthermore, BRC640 showed no effect on AMPA and NMDA induced currents (Figure 5.9c), suggesting that BRC640 might be a selective positive modulator of $GABA_A$Rs.

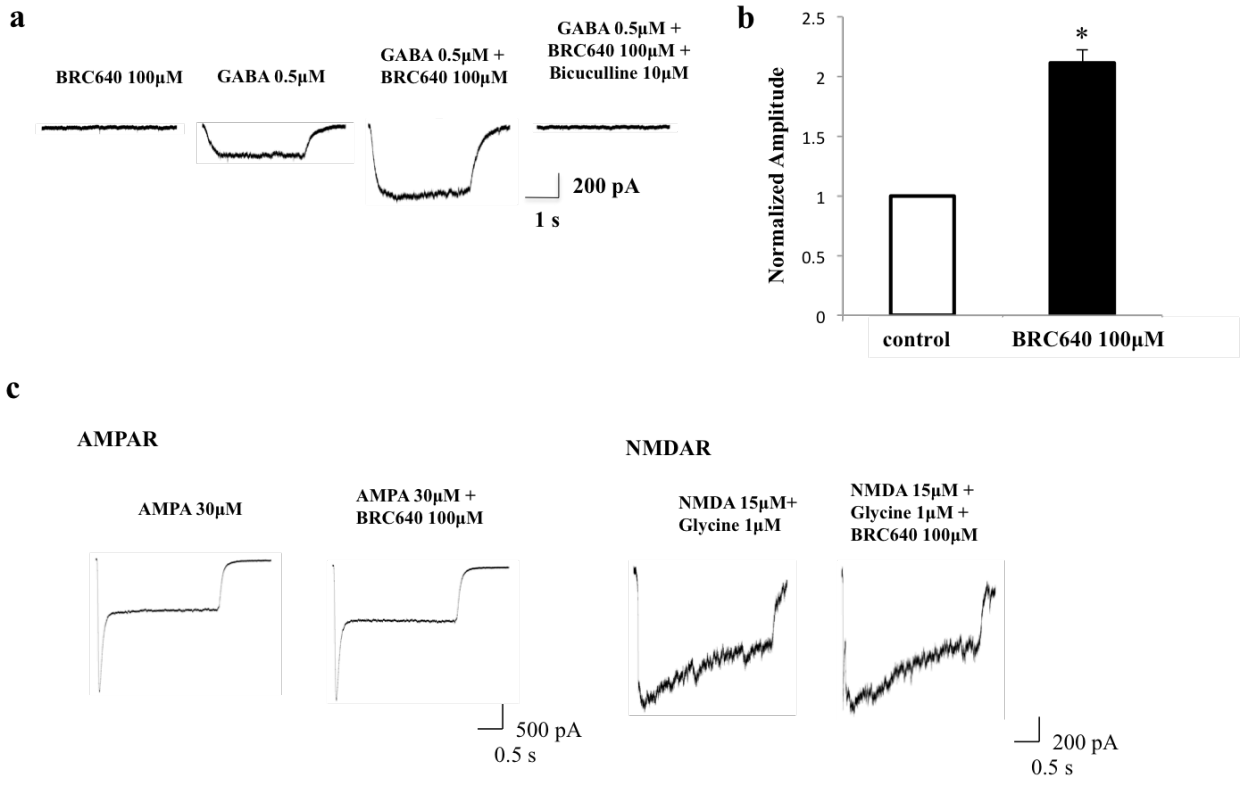

Figure 5.9 BRC640 positively modulates the $GABA_AR$-mediated current in cultured hippocampal neurons

a) Representative traces showing BRC640 (100 μM) did not evoke any current in cultured hippocampal neuron. GABA (0.5 μM) induced an inward current that can be potentiated by BRC640 (100 μM) and fully blocked by bicuculline (10 μM) (Left panel). The right panel shows the structure of BRC640. **b)** Quantified results from 4 cells. BRC640 (100 μM) significantly potentiated the amplitude of GABA (1 μM) evoked current (211.4±10.9% of control, $P<0.05$, n=4, Student's *t*-test). **c)** BRC640 (100 μM) have no effect on AMPAR-mediated current (Left panel) and NMDAR-mediatd current (Right panel).

We also tested the effect of BRC640 on $GABA_AR$-mediated phasic and tonic inhibition. Incubating hippocampal neurons in 100 μM BRC640 significantly increased the amplitude of mIPSC (143.4±17.1%, $P<0.05$, n=3; Figure 5.10B, Left), but not mIPSC frequency (108.3±21.0%, n=3; Figure 5.10B, Right), indicating that BRC640 modulated postsynaptic $GABA_ARs$ but not the presynaptic GABA releasing events. Similarly as glutamate analogs APV, BRC640 also increased the amplitude of the $GABA_AR$-mediated tonic currents (176.3±15.5%, $P<0.05$, n=3; Figure 5.11). Thus, BRC640 may be a good candidate drug modulating both fast inhibitory synaptic transmission, as well as the slow but persistent activation of extrasynaptic receptors.

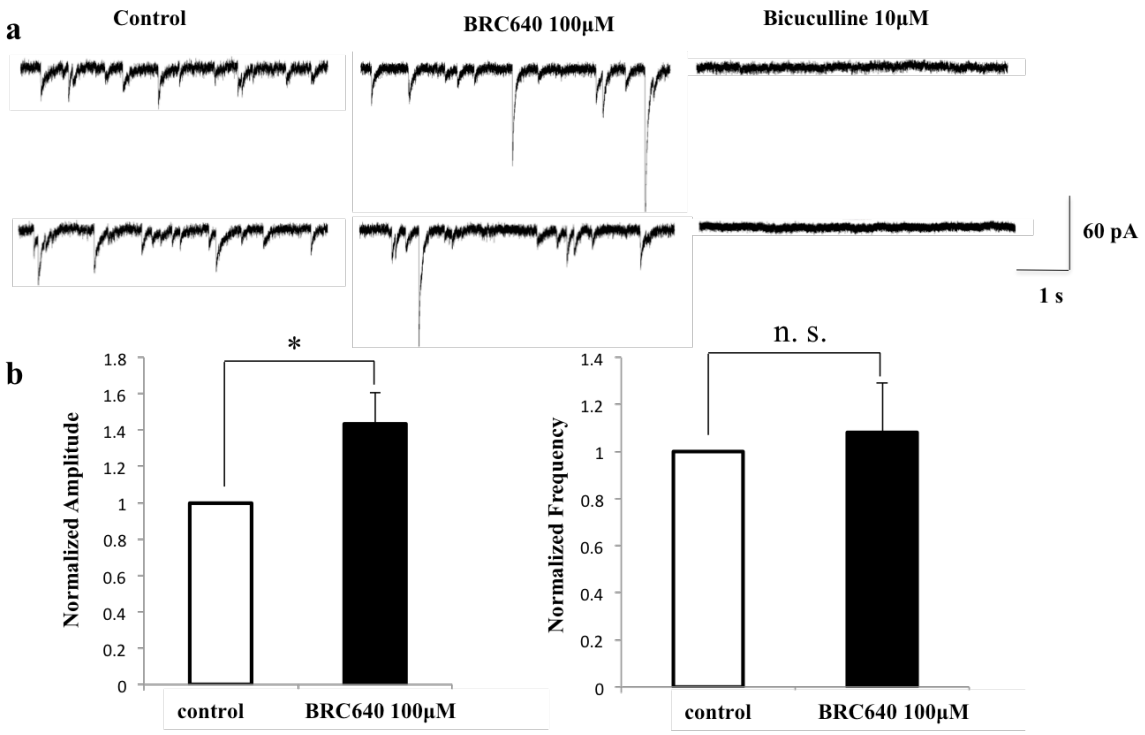

Figure 5.10 BRC640 potentiates the $GABA_AR$-mediated phasic inhibition

a) Representative trace of mIPSCs before and after BRC640 (100 µM) application in hippocampal neurons. The mIPSC can be blocked by 10 µM bicuculline. **b)** Quantified results from 3 neurons. BRC640 (100 µM) significantly increased the mIPSC amplitude (143.4±17.1% of control; $P<0.05$, Student's *t*-test) **c)** BRC640 (100 µM) induced no significant change in mIPSC frequency (108.3±21.0% of control; $P>0.05$, n=3, Student's *t*-test).

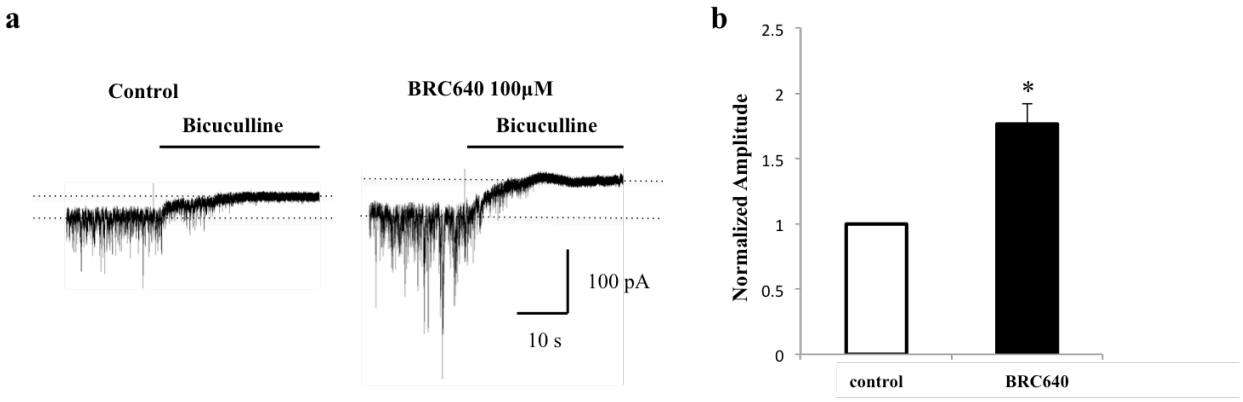

Figure 5.11 Ampicillin potentiates the $GABA_AR$-mediated tonic inhibition

a) Representative trace of tonic GABA current. BRC640 (100 μM) application increased the amplitude of tonic current revealed by 10 μM bicuculline in hippocampal neurons. **b)** Quantified results from 3 neurons. BRC640 (100 μM) significantly enhanced tonic GABA current (176.3±15.5% of control, $P<0.05$, Student's *t*-test).

As shown in Figure 5.12A, BRC640 has similar structure features as glutamate, bearing both positive and negative charged groups to form tight interactions with the charged residues in the glutamate-binding pocket. Docking analysis indicated that two residues, α1K155 and β2E181, were required for BRC640 binding on $GABA_A$Rs. Notably, as my previous results have showed, those two residues were also critical for glutamate binding. To confirm the binding site of BRC640, we transfected HEK 293 cells with α1β2γ2 $GABA_A$R containing β2E181G mutation. One single mutation at the glutamate-binding site almost eliminated BRC640-induced potentiation (Figure 5.12B). Taken together, our results suggested BRC640 as a novel $GABA_A$R positive modulator targeting the allosteric glutamate-binding site on $GABA_A$Rs.

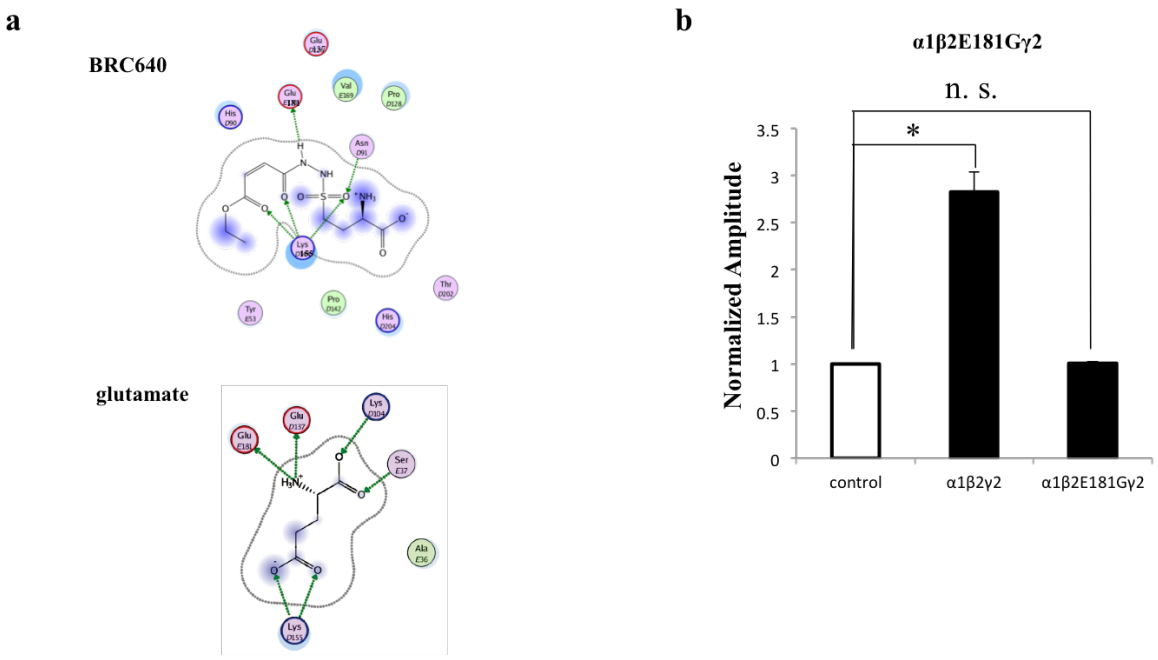

Figure 5.12 BRC640 actes at glutamate-binding site on the $GABA_AR$

a) Structural and docking comparison of glutamate and BRC640 showing BRC640 shares the same critical binding residues with glutamate. **b)** Mutating the critical residue (β2E181) in glutamate-binding site eliminated BRC640-induced potentiation of $GABA_AR$ function (α1β2E181Gγ2 100.6±2.2% of control, n=6, P>0.05, one-way ANOVA test).

5.3 Discussion

$GABA_ARs$ contain targeting sites for various therapeutic drugs including benzodiazepines, barbiturates and anesthetics. In particular, benzodiazepines have been one of the safest and most popular therapeutics for anxiety, sedation, and as anticonvulsants for the treatment of seizures (Vinkers and Olivier, 2012). However, their utility has been limited by strict subunit-specificity and rapidly declining efficacy (i.e. drug tolerance) (Browne and Penry, 1973; Deeb et al., 2012; Schneider-Helmert, 1988). The classic benzodiazepines allosterically modulate GABA-induced synaptic inhibition by binding to the benzodiazepine site located in the interface of α+/γ- and therefore require the presence of a γ subunit in the receptor (Figure 4.6) (Sigel, 2002; Sigel and Steinmann, 2012). Many extrasynapatic $GABA_ARs$, such as αβδ-composed receptors, do not contain a γ subunit and are insensitive to benzodiazepines. Thus benzodiazepines can only partially regulate the $GABA_AR$-mediated tonic inhibition. In great contrast, the novel glutamate binding site we identified in the present study only requires the α and β subunits, therefore is presented on almost all known native $GABA_ARs$ in the mammalian brain, including those located extracellularly (Mohler, 2006). Thus, this newly identified site may represent a more preferable therapeutic target upon which new $GABA_AR$ positive modulators with a broader receptor spectrum can be developed.

In this regard, in the present study, we were able to identify ampicillin as an agonist at this site. Most importantly, consistent with our prediction, the ampicillin indeed potentiated both synaptic $GABA_AR$-mediated mIPSCs and extrasynaptic $GABA_AR$-mediated tonic currents. Admittedly, ampicillin is far from a clinically applicable positive $GABA_AR$-based therapeutic due to its side effects and poor blood-brain-barrier (BBB) and plasma membrane permeability. However, recent

studies have reported that enhancing pancreatic $GABA_AR$ functions might facilitate insulin release and prevent β cell loss (Bansal et al., 2011; Soltani et al., 2011). Based on these findings, despite its impermeability to blood-brain-barrier, ampicillin may exert a previously unknown effect on the regulation of glucose metabolism.

Using the same screening criteria, we also identified compound BRC640 as a potent positive modulator of $GABA_ARs$ targeting the glutamate-binding site. Similar as glutamate and ampicillin, BRC640 was also capable to regulate both phasic and tonic inhibition, making it a good candidate for developing GABA-related drugs. Additional assessments on cell toxicity, drug stability and BBB permeability of BRC640 are needed to fully characterize it before testing with *in vitro* and *in vivo* disease models.

Nonetheless, our discovery of ampicillin and BRC640 using a combination of *in-silicon* screening and electrophysiological characterization provides the proof-of-concept support for this glutamate-binding site as a novel therapeutic target. Coupled with further medicinal optimization, it will undoubtedly facilitate the development of a novel class of $GABA_AR$ positive modulators acting at the glutamate-binding site as effective therapeutics for various neurological disorders such as anxiety, epilepsy and stroke.

Chapter 6: The role of glutamate/$GABA_AR$ interaction in inhibitory synaptic plasticity of cerebellar Purkinje cells

6.1 Introduction

Purkinje cells (PCs) play dominant roles in the cerebellar circuits and the dysfunctions of PCs are associated with motor deficits (Giuliani et al., 2011; Kishore et al., 2014; Redondo et al., 2015; Ruegsegger et al., 2016; Tu et al., 1997) as well as psychiatric disorders such as autism (Fujita et al., 2012; Lotta et al., 2014; Piochon et al., 2014; Skefos et al., 2014). PC receives two types of afferents, the climbing and mossy fibers and sends inhibitory projections to the deep cerebellar nuclei (Purves et al., 2001; Shepherd, 2004) (Figure 1.3). PC activity is also refined by the inhibitory inputs from stellate cells (SCs) and basket neurons (BCs) (Ito, 1987; 2008), with BCs synapsing on the Purkinje cell axon initial segment and SCs onto the dendrites (Hirano et al., 2002; Konnerth et al., 1990; Shepherd, 2004; Southan and Robertson, 1998).

Plasticity at inhibitory synapses of Purkinje cells seems to contribute to refined information processing in the cerebellar cortex (Mapelli et al., 2015). Rebound potentiation (RP) and depolarization-induced potentiation of inhibition (DPI) are two major types of plasticity occur at SC-PC synapses (Hirano et al., 2002). RP is a long-lasting potentiation of postsynaptic response to GABA, which is induced by postsynaptic depolarization and the following calcium influx in PCs (Kano et al., 1992). Late phase of RP is ascribed to the phosphorylation cascades through CaMKII and PKA, activated by elevated levels of calcium, and subsequent modification of the $GABA_AR$ (Kano and Konnerth, 1992; Kano et al., 1996; Kawaguchi and Hirano, 2000; 2002). However, the mechanism of the early phase (up to 10 min) of RP remains unknown.

DPI is, by contrast, thought to be a presynaptic event, which characterized by the increase of frequency of miniature inhibitory postsynaptic currents (mIPSCs) (Duguid and Smart, 2004). It is also induced by an elevation of calcium after depolarization, which is believed to trigger dendritic glutamate release near the inhibitory SC-PC synapses. The dendritic-released glutamate then diffuses to the presynaptic compartment of SC-PC synapses, and activates presynaptic NMDARs as a retrograde signal and enhances presynaptic GABA release probability (Duguid and Smart, 2004; Glitsch, 2008; Tzingounis and Nicoll, 2004) (Figure 1.4).

The dendritic glutamate release in PCs is also supported by several other studies. Although PCs are purely GABAergic, they also express vesicular glutamate transporter 3 (VGluT3), which is responsible for the uptake of glutamate into releasing vesicles (Gras et al., 2005). In VGLUT3 knockout mice, DPI was almost abolished, suggesting the participation of retrograde glutamate release in DPI (Crepel et al., 2011; Duguid et al., 2007). Dendritic glutamate release was also found to produce autocrine activation of mGluR1 in PCs (Crepel, 2007; 2009; Crepel et al., 2011; Duguid et al., 2007; Shin et al., 2008).

Considering the spatial proximity between dendritic glutamate release and $GABA_A$Rs in PCs, it is highly possible that the dendritic released glutamate not only activates presynaptic NMDA receptor but also allosterically binds to postsynaptic $GABA_A$Rs and potentiates its function. We hypothesize that this fast autocrine potentiation of $GABA_A$R may be the underlying mechanism of the early phase of RP (see proposed model in Figure 1.5).

6.2 Results

6.2.1 Activation of glutamate-binding site potentiates the function of the $GABA_AR$ in cerebellar Purkinje cells

To investigate if the glutamate/$GABA_AR$ crosstalk plays a role in the early phase of rebound potentiation, we first tried to determine if the phenomenon of glutamate-induced potentiation of $GABA_ARs$ also present in cerebellar Purkinje cells. Whole cell patch clamp recording was performed on cultured PCs and the GABA current was evoked by fast perfusion of exogenous GABA (0.5μM) to the cell. Glutamate analog, APV (200μM), was used to activate the glutamate-binding site on $GABA_AR$ but not those on glutamate receptors in PCs. Bath application of APV induced a reversible potentiation of $GABA_AR$-mediated current in PCs (180.9 ± 14.7% of the control, $P<0.05$, n=6; Figure 6.1b), which was fully blocked by bicuculline (10 μM; Figure 6.1a). To distinguish the synaptic $GABA_AR$ potentiation from extrasynaptic potention, we assessed APV's effect on phasic inhibition by recording mIPSCs in PCs. APV (200μM) significantly increased the amplitude of mIPSCs (140.9 ± 7.8% of the control, $P<0.05$, n=6; Figure 6.1d), confirming the presence of glutamate-induced potentiation effect on PC synaptic $GABA_AR$.

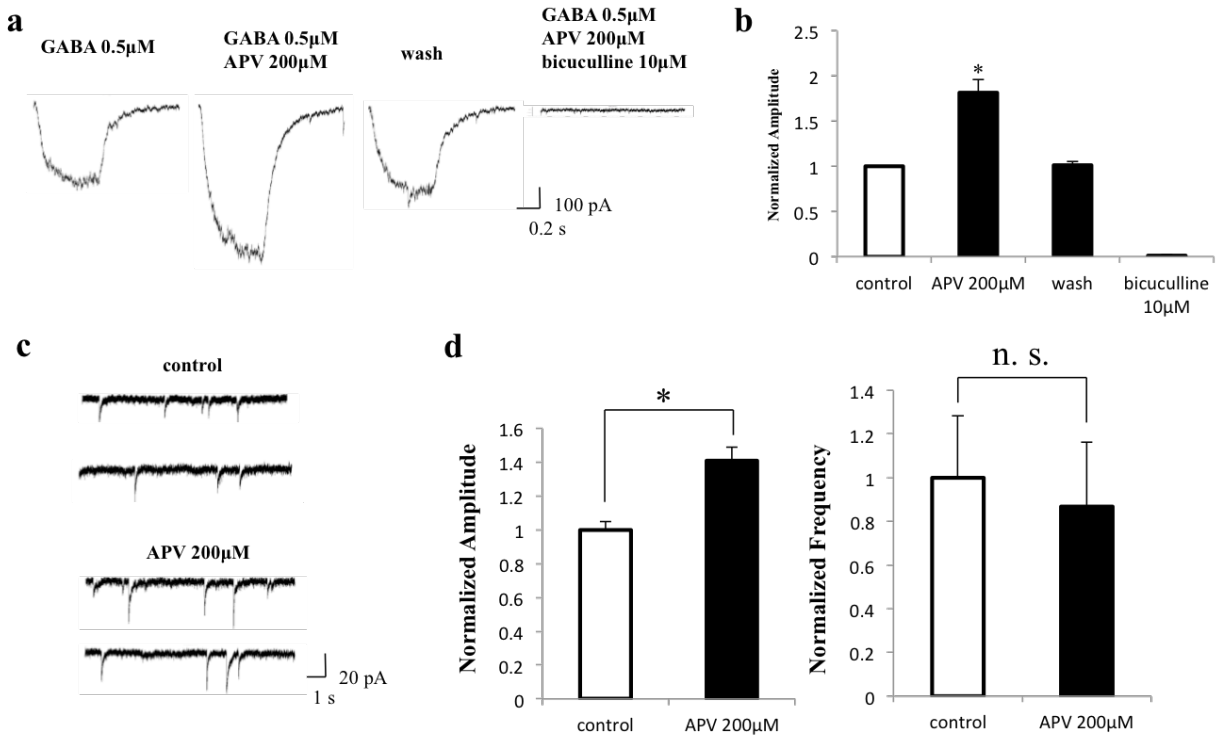

Figure 6.1 APV enhances the $GABA_AR$-mediated current in cultured Purkinje cells

a) Representative traces showing GABA (0.5 μM) evoked an inward current in cultured Purkinje cells. APV (200μM) reversibly potentiated the GABA-induced current. Bicuculine (10μM) completely blocked the APV-enhanced current. **b)** Quantified results from 6 neurons. APV (200μM) significantly potentiated GABA (0.5 μM)-induced current in Purkinje cells (180.9 ± 14.7% of the control, $P < 0.05$, Student's *t*-test). **c)** Representative trace of mIPSCs before and after APV (200 μM) application. **d)** Quantified results from 6 neurons. APV (200 μM) significantly increased the mIPSC amplitude (140.9 ± 7.8% of the control, $P<0.05$, Student's *t*-test, Left panel). There is no significant change in mIPSC frequency (86.8 ± 29.3% of the control, $P>0.05$, Student's *t*-test).

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In the previous chapter, we have identified ampicillin as a positive allosteric modulator targeting glutamate-binding site on $GABA_A$Rs. Here we tested if Amp exerts a similar effect on $GABA_A$Rs in Purkinje cells. Bath application of Amp (300 μM) produced a potentiation effect on $GABA_A$Rs in PCs at a comparable level of that on $GABA_A$Rs in hippocampal neurons (180.1 ± 14.8% of the control, $P<0.05$, n=6; Figure 6.2b). Amp (300 μM) also significantly increased the amplitude of mIPSC recorded in cultured PCs (124.1 ± 5.9% of the control, $P<0.05$, n=6; Figure 6.2c), which was likely through potentiating postsynaptic $GABA_A$R function.

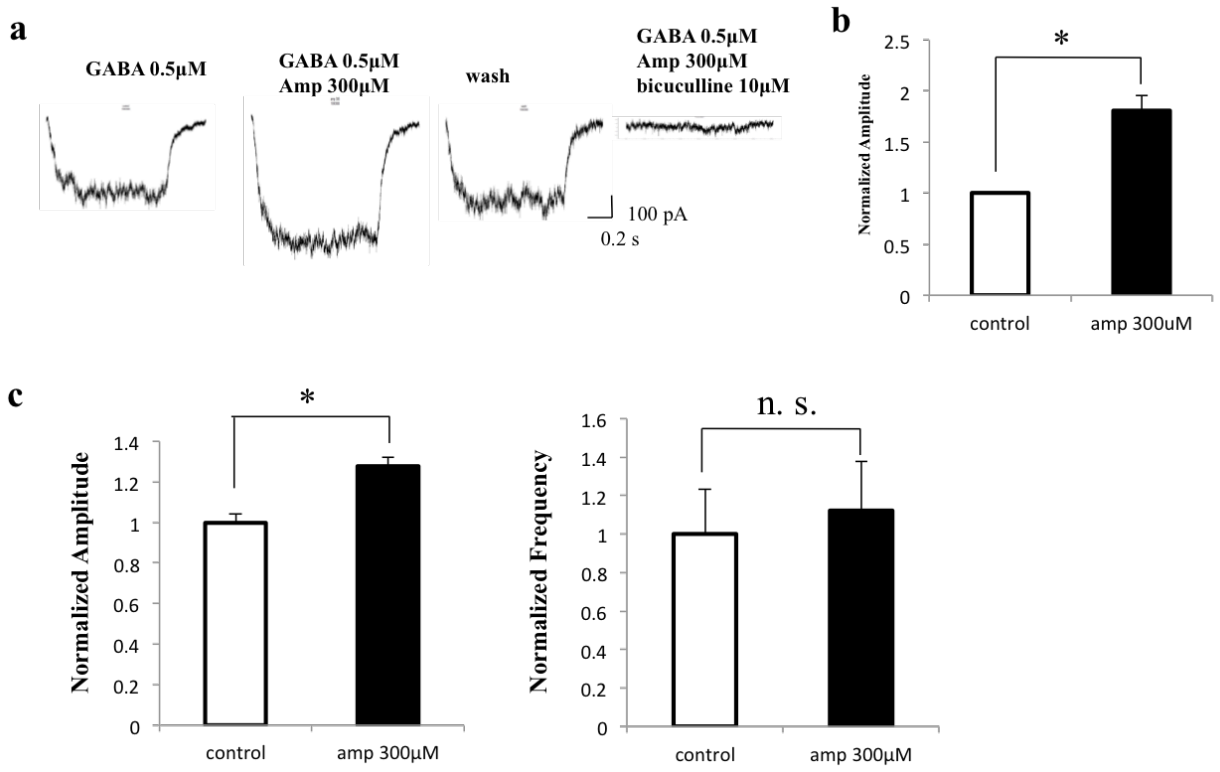

Figure 6.2 Ampicillin potentiates the $GABA_AR$-mediated current in cultured Purkinje cells

a) Representative traces showing the reversible potentiation of GABA (0.5 μM)-evoked current by Amp (300μM) in cultured Purkinje cells. Bicuculine (10μM) blocked the Amp-enhanced current. **b)** Quantified results from 6 neurons. Amp (300μM) significantly increased the amplitude of GABA (0.5 μM)-induced current in Purkinje cells (180.1 ± 14.8% of the control, $P < 0.05$, Student's *t*-test). **c)** Amp (300μM) significantly potnetiated the mIPSC amplitude (124.1 ± 5.9% of the control, $P<0.05$, n=6, Student's *t*-test, Left panel) but not the mIPSC frequency (112.2 ± 25.5% of the control, $P>0.05$, n=6, Student's *t*-test).

6.2.2 Ampicillin occludes the early phase of rebound potentiation in Purkinje cells

We hypothesized that the activation of the glutamate-binding site on $GABA_A$Rs by the dendritic released glutamate contributes to the early phase of rebound potentiation. Therefore, it is rationale to predict that if we use one of our previously identified positive modulators to fully occupy the glutamate-binding site, we could partially or even fully occlude the early phase of rebound potentiation. Here, we chose to use Amp instead of APV as the occlusion ligand to avoid the presynaptic effect of APV. we first assessed if Amp was capable to occlude the further activation of glutamate-binding site on PC expressed $GABA_A$Rs. Pre-incubation of PCs with Amp (500 μM) compromised APV (500 μM, saturate concentration) induced potentiation of GABA current. Thus, Amp is capable to saturate the glutamate-binding sites on $GABA_A$Rs and consequently prevent further potentiation of $GABA_A$Rs induced by glutamate or other glutamate-like ligands.

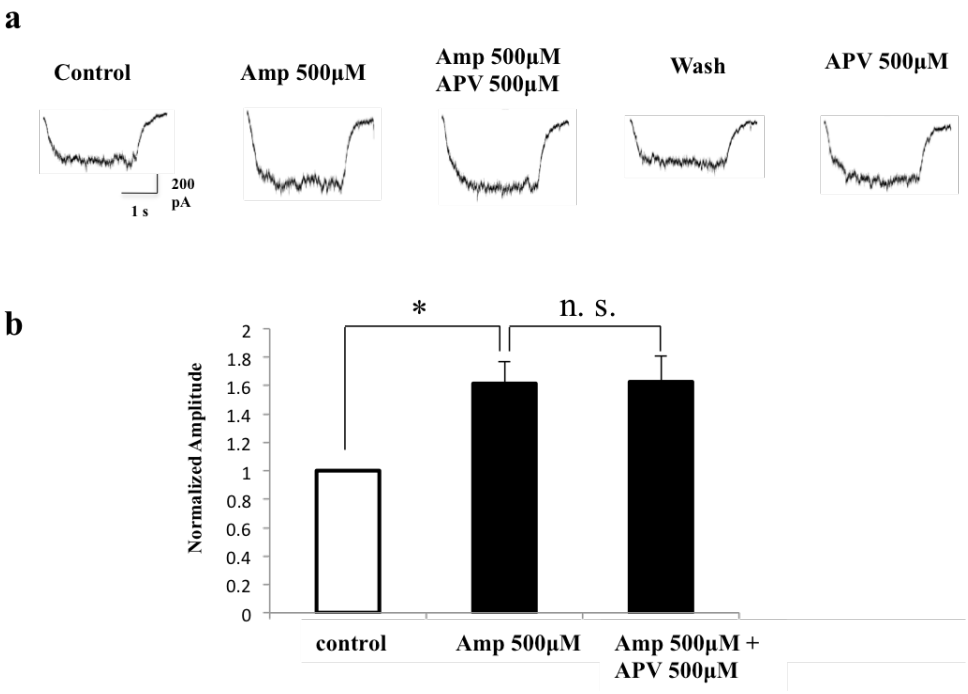

Figure 6.3 Ampicillin occludes the APV-induced activation of glutamate-binding site in Purkinje cells

a) Representative traces showing Amp (500 µM) enhanced GABA current and prevented the further potentiation induced by APV (500 µM). Washout of the Amp restored the PC's sensitivity to APV (500 µM). **b)** Quantified results showing Amp saturated the glutamate-binding site and occluded the further activation of this site (Amp 500 µM: 177.1±13.3% vs Amp 500 µM + APV 500 µM: 174.9±13.7% of control, n=6, P>0.05, one-way ANOVA test).

To test the occlusion effect of Amp on rebound potentiation, we established a RP model using cultured Purkinje cells. Cerebellar neurons were isolated from E18 rat brain and cultured at high density. PCs were identified by their unique morphology at 7-9 DIV. Under whole-cell patch recording configuration, PCs were stimulated by depolarization from -70 mV to 0 mV for 5s, an optimized protocol to produce largest rebound potentiation. sIPSCs of PCs was recorded instead of their mIPSCs because the absence of TTX in sIPSC recording solutions ensured a better depolarization of PCs. Immediately after the stimulation, the amplitude of sIPSCs in PCs increased significantly, representing the rebound potentiation of $GABA_AR$ functions.

To occlude the early phase of rebound potentiation, PCs were incubated in Amp (500 μM) for 3 mins before stimulation, which produced a significant increase of sIPSCs amplitude. Under this condition, stimulation of PCs by the same protocol as described above failed to induce any additional potentiation of sIPSCs amplitude in the first 60s after stimulation, suggesting Amp occluded the very early phase of rebound potentiation. After 60s, the potentiation of sIPSCs amplitude slowly increased to a level comparable to that of the control condition. Taken together, our results showed potentiation of $GABA_ARs$ function by Amp was sufficient to prevent the occurrence of the very early phase (0-60s) of rebound potentiation, suggesting that those two phenomenon may share the same mechanism, i.e. activation of glutamate-binding site on the $GABA_AR$.

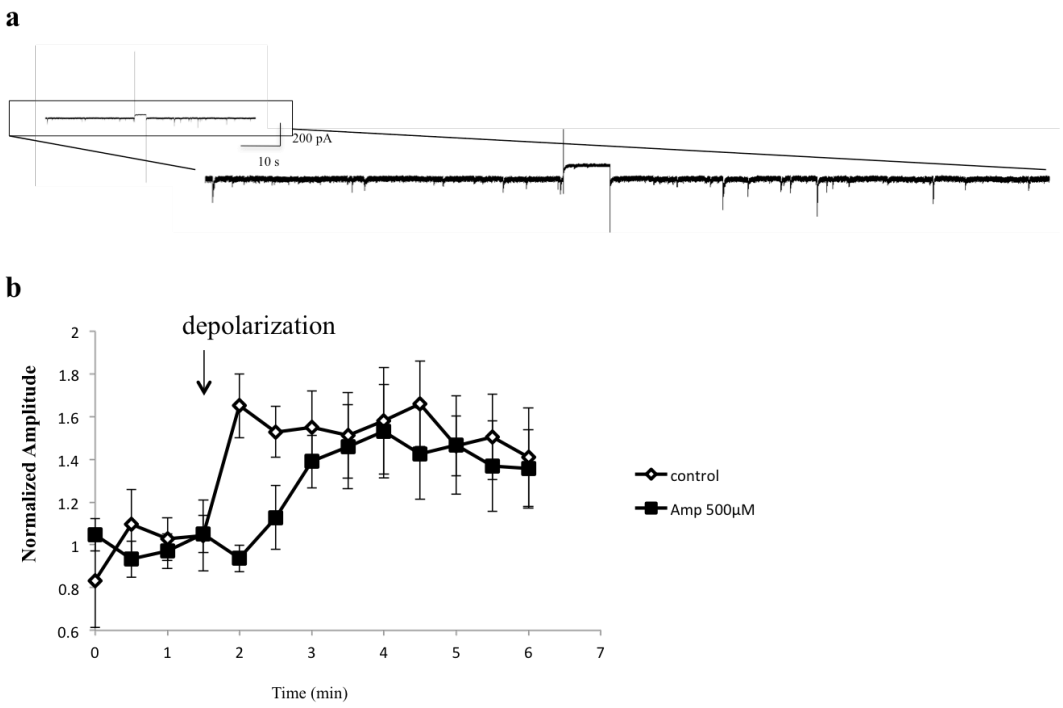

Figure 6.4 Ampicillin occludes the early phase of rebound potentiation in Purkinje cells

a) Representative traces showing under control condition, depolarization of the Purkinje cell from -70 mV to 0 mV induced an instant rebound potentiation of the sIPSC amplitude. **b)** Amp (500 µM) prevented the occurrence of RP in the first 60 s after stimulation (n=6).

6.3 Discussion

The $GABA_AR$ in Purkinje cell is comprised of α1, β2 or 3 and γ2 subunits (Laurie et al., 1992b). Since these subunits contain the critical residues for glutamate binding (see Chapter 4), it is highly likely that the GABA response in Purkinje cells is also susceptible to glutamate, similarly as those in hippocampal neurons. As expected, our results showed that glutamate analog APV and the newly identified glutamate-binding site targeting compound Amp enhanced the GABA-induced current in Purkinje cells. This result demonstrated the existence of functional glutamate-binding site on the Purkinje cell $GABA_AR$ and strongly suggested that glutamate/$GABA_AR$ interaction may contribute to the inhibitory plasticity of Purkinje cells.

During DPI, depolarization of Purkinje cells triggers dendritic glutamate release at a site close to the $GABA_AR$ (Crepel et al., 2011; Duguid and Smart, 2004; Duguid et al., 2007). Thus, the spatial proximity between glutamate and the $GABA_AR$ may enable their interaction and subsequent potentiation of the $GABA_AR$ function. In our experiment, depolarization of Purkinje cell induced an instant increase of postsynaptic GABA response, which is consistent with previous RP studies (Kano et al., 1992; 1996; Kawaguchi and Hirano, 2000; 2002). Using Amp to pre-occupy the glutamate-binding site on the $GABA_AR$ occluded the very early phase (0-60s) of RP, indicating that the activation of $GABA_AR$ glutamate-binding site, presumably by dendritic released glutamate, may involve in the induction of RP.

The dendritic glutamate release in Purkinje cell is thought to be an early development phenomenon. In cerebellar slices, P11-14 Purkinje cells need stronger stimulation but exhibit smaller DPI comparing with that of P6-8 cells (Duguid and Smart, 2004). Thus, we use 7-9 DIV

cells in our experiment to obtain more profound dendritic glutamate release. Notably, in early development, the GABA response is excitatory due to the elevated intracellular chloride concentration (Achilles et al., 2007; Ben-Ari, 2002; Kakazu, 2000; Obata et al., 1978). The developmental switch of $GABA_AR$ function happens within the first postnatal week (P7) in Purkinje cells (Eilers et al., 2001), which means dendritic glutamate may potentiate both excitatory and inhibitory GABA responses, but with much higher thresholds for the latter one. Therefore, the dendritic glutamate release may have dual roles in Purkinje cell plasticity: at early development stage when GABA response is excitatory, dendritic glutamate serves as a top-up of the small excitation and potentially contribute to the neuronal growth and synapse formation; at the later stage after the $GABA_AR$ functional switch, dendritic glutamate can only be evoke by the strong excitation and hence prevent Purkinje cell from over-excitation.

A recent study revealed the first physiological role of RP as contributing to the adaptation of vestibulo-ocular reflex (VOR) (Tanaka et al., 2013). Remarkably, this experiment was conducted on P8-10 weeks old mice, in which the early phase of RP was not detected possibly due to the development-related diminishment of dendritic glutamate release (Tanaka et al., 2013). Thus, it is unlikely that the glutamate/$GABA_AR$ crosstalk contribute to the VOR adaptation in P8-10 weeks young adult rat. However, its potential effect on motor learning in juvenile or even younger animals has not been determined.

It is important to note the study presented in this Chapter is still at a preliminary stage. There may be alternative explanations for the results of the occlusion experiment. For example, although unlikely, Amp might activate an unknown pathway that diminished the early phase of

RP. It is also possible that the conformational change after Amp binding made the $GABA_AR$ insensitive to another modulation factor, which is responsible for early phase of RP. Future investigations are needed to obtain more direct evidence for the involvement of glutamate/$GABA_AR$ crosstalk in the early phase of RP. For example, since we have already known the critical residues for glutamate binding, we can swap the endogenous $GABA_ARs$ with glutamate-binding deficient $GABA_ARs$ in Purkinje cell using shRNA knockdown, followed by receptor overexpression. This receptor substitution approach will allow us to test if the presence of glutamate-binding site on the $GABA_AR$ is necessary for RP induction.

In previous study, the time length of early phase (i.e. CaMKII/PKA independent phase) of RP varies from 60 s to 10 min depending on the stimulation protocols (He et al., 2015; Kawaguchi and Hirano, 2002). Since we have not determined the duration of early phase of RP under our experimental conditions, thus we do not know if Amp fully occluded the early phase of RP or just partially. Co-application of CaMKII blockers, such as KN93 or KN62, with Amp will enable us to answer this question. It will be interesting if we observe a third pathway involved in RP induction. Notably, besides glutamate, depolarization of Purkinje cell can also induce postsynaptic release of endocannabinoids, which is the mechanism underlying another types of Purkinje cell inhibitory plasticity, termed depolarization induced suppression of inhibition (DSI) (Yoshida et al., 2002). Meanwhile, one type of endocannabinoids 2-AG was found to directly interact with the $GABA_AR$ as a positive allosteric modulator (Baur et al., 2013; Sigel et al., 2011). Future investigation is needed to clarify whether 2-AG is released from Purkinje cells following depolarization and if its concentration is sufficient to induce the $GABA_AR$ potentiation.

Nevertheless, our results indicate that the early phase of rebound potentiation may share the same mechanism with Amp-induced potentiation of $GABA_AR$ function, since saturate this mechanism with Amp prevent the occurrence of the early phase of RP. Taken together, our results showed that occupation of glutamate-binding site on $GABA_ARs$ with Amp was sufficient to prevent the very early phase (0-60s) of rebound potentiation, suggesting that activation of glutamate binding site may be vital for this type of inhibitory synaptic plasticity in PCs.

Chapter 7: Conclusion

In this dissertation, we presented a novel glutamate-binding site at the α+/β- interface of the $GABA_AR$. By binding on this site, the excitatory neurotransmitter glutamate is able to potentiate function of $GABA_ARs$, the principal inhibitory receptors in the CNS. This excitation/inhibition interaction may contribute to Purkinje cell rebound potentiation, a type of inhibitory plasticity that promotes motor learning. This newly identified site also presents a novel target for future drug development. Here, we demonstrated that a combination of *in silicon* and electrophysiological screening could be a beneficial approach to identify and characterize the ideal therapeutics.

7.1 Glutamate-induced potentiation of the $GABA_AR$

In the CNS, glutamate is the major excitatory neurotransmitter that depolarizes of the target cells and increases the neuronal firing probability through activation of the glutamate receptors. In contrast, the $GABA_AR$ is the principal inhibitory receptor that counteracts the effect of glutamate receptors by chloride influx that hyperpolarizing the postsynaptic cell membrane. Following Stelzer and Wong's work in 1989 (Stelzer and Wong, 1989), we identified a novel glutamate binding site on the $GABA_AR$. Electrophysiological study showed that, binding of glutamate or glutamate analogs on this site induced a potentiation of both phasic and tonic inhibition mediated by the $GABA_AR$. Using recombinant $GABA_ARs$ expressed in HEK293 cells, we confirmed that the glutamate/analogs-induced effect is through direct interaction with the $GABA_AR$ but not any known glutamate receptor/protein. To further investigate the mechanism of this interaction, we performed a [^{3}H]-glutamate binding assay and confirmed the physical binding of glutamate on an allosteric site on the $GABA_AR$. These findings proved our hypothesis that glutamate-induced

potentiation of $GABA_AR$ function observed in previous and present work is through allosteric interaction between glutamate and the $GABA_AR$.

Our findings revealed a previous unknown mechanism of excitation/inhibition crosstalk and blurred the traditional distinction between the excitatory and inhibitory neurotransmitters. The balance between excitatory and inhibitory systems in the CNS is crucial for maintaining normal brain functions as well as preventing neurological disorders. Previous studies have identified several indirect pathways involved in the interaction between these two systems. For example, activation of NMDAR by glutamate enhanced GABA response by promoting postsynaptic $GABA_AR$ membrane expression as well as increasing presynaptic GABA releasing probability (Marsden et al., 2007; Xue et al., 2011). Here, we presented a direct interaction between glutamate and the $GABA_AR$. The instant feedback mechanism is capable to provide fast homeostatic regulation in order to sustain the proper excitability of the neurons. Recently our lab also illustrated that glutamate allosterically modulated the function of the glycine receptor, the major inhibitory receptor in spinal cord and brainstem (Liu et al., 2010). Thus, the glutamate-induced potentiation may be a universal phenomenon in the inhibitory system. The participation of glutamate in both the excitatory and inhibitory neurotransmissions emphasized the complexity of nerves system and largely challenged our traditional categorization of neurotransmitter.

7.2 Glutamate/$GABA_AR$ crosstalk in Purkinje cell rebound potentiation

A previous study showed that depolarization of cerebellar Purkinje cell induces dendritic glutamate release near GABAergic synapses (Duguid and Smart, 2004). The spatial proximity between the dendritically released glutamate and the $GABA_AR$ makes it an excellent model for

studying their interaction. The present study demonstrated that depolarization of Purkinje cell by current injection led to an instant enhancement of the amplitude of sIPSC, which is termed as rebound potentiation in previous studies (Kano et al., 1992; 1996). Application of ampicillin, a newly identified compound targeting the glutamate-binding site, was able to occlude the early phase of rebound potentiation. Since we have confirmed the existence of the glutamate-binding site on Purkinje cell $GABA_AR$ using ampicillin and glutamate analog APV, it is highly likely that the glutamate/$GABA_AR$ interaction contribute to the early phase of rebound potentiation, a major type of Purkinje cell inhibitory plasticity.

Combining with our previous finding in hippocampal neurons, the presence of glutamate-induced potentiation effect on Purkinje cells GABA response strongly supports it as a common regulatory mechanism in different brain regions. In the CNS, the extrasynaptic glutamate concentration is tightly controlled by glutamate reuptake through glutamate transporters (Anderson and Swanson, 2000; Moussawi et al., 2011). However, our study reveals that the dendritic glutamate release at the site close to the $GABA_ARs$ provides a chance for glutamate to access the $GABA_AR$ under physiological condition. The observation that preoccupation of the glutamate-binding site occluded the early phase of rebound potentiation shed a light on the physiological role of the glutamate/$GABA_AR$ interaction in motor learning. Since the phenomenon of dendritic glutamate release also exists at the inhibitory synapses in several other brain regions such as neocortex and midbrain (Penzo and Peña, 2011; Zilberter, 2000; Zilberter et al., 2005), this nontraditional form of transmitter release and its interaction with the $GABA_AR$ may bear larger significance in cognition, motivation and other brain functions.

7.3 Glutamate-binding site on the $GABA_AR$

Following our discovery of the physical binding between glutamate and the $GABA_AR$, we then utilized homology model docking and systematic mutagenesis analysis to identify the glutamate-binding site on the $GABA_AR$. Our result illustrated that the glutamate-binding site was located at the α+/β- interface with four charged critical residues, α1K104, α1E137, α1K155 and β2E181. This finding is consistent with previous studies on glutamate receptors showing that glutamate binding requires charged residues to form electrostatic interaction with the charged amino and carboxyl moieties of glutamate molecules (Chen et al., 2005a; Wellendorph and Bräuner-Osborne, 2009). By sequence alignment and subsequent mutation, we demonstrated the conservation of those four critical residues in their respective subunit families. We further assured our previous findings by creating artificial glutamate-binding site through mutation of the corresponding residues on γ2 subunit. The above results support our hypothesis that, the glutamate-binding site may locate at the subunit interface, where a small molecule like glutamate can induce sufficient conformational change and subsequent functional alteration of the receptor (Hibbs and Gouaux, 2011).

The α+/β- location of glutamate-binding site is interesting. Since 95% of $GABA_AR$ contains α and β subunits (Mohler, 2006), glutamate may affect most, if not all $GABA_ARs$, especially when considering the subfamily conservation of this binding site. For the allosteric modulators, the location of their binding sites determines the direction and extent of their regulatory effects. This newly identified binding site is distinct with other known allosteric binding site, such as benzodiazepine binding site, on the $GABA_AR$. Although this new site shares two residues with a minor binding site of zinc, it just slightly overlap with the zinc site at the pocket edge and

activation of each of these two site induces opposite modulation effects on the $GABA_AR$ function. There is a small possibility that glutamate exerts its potentiation effect by relieving the $GABA_AR$ from zinc inhibition. Further investigation is needed to address this possibility, which will enhance our understanding on how the allosteric modulators interact with each other and facilitating $GABA_AR$ function. Nevertheless, our study revealed a previous unidentified allosteric modulation site on the $GABA_AR$, which may display unique physiological properties and therapeutic potential.

7.4 Glutamate-binding site as a novel drug target

The distinctive location of the glutamate-binding site indicates its potential as the target for developing novel $GABA_AR$ modulators. In our study, using a technique combining *in silicon* screening and electrophysiological testing, we identified ampicillin and BRC640 as the novel allosteric modulators of the $GABA_AR$. These two compounds exhibited the similar effects on the $GABA_AR$ as glutamate by potentiating both the phasic and tonic inhibition. Mutation at the glutamate-binding site compromised the potentiation effects of Amp and BRC640, indicating they may share the same binding site with glutamate.

The traditional benzodiazepine drugs bind to the α+/γ- interface, thus their effect depends on the presence of γ subunits (Sigel, 2002; Sigel and Steinmann, 2012). In contrast, the two novel drugs, ampicillin and BRC640, only requires the α and β subunits to form the binding site, therefore have broader-spectrum effects, e.g. potentiating the δ containing extrasynaptic $GABA_ARs$. This property may be vital for counteracting severe neuronal over-excitation as well as treating benzodiazepine-insensitive neurological disorders, such as prolonged seizures (Deeb et al., 2012).

The independence of γ subunit may also enable those compounds to overcome the difficulties encountered by traditional benzodiazepine drugs. For example, patients with insomnia and epilepsy often develop drug tolerance after long-term exposure to benzodiazepines due to the uncoupling of benzodiazepine site and loss of γ subunit expression (Ali and Olsen, 2001; Browne and Penry, 1973; Schneider-Helmert, 1988; Vinkers and Olivier, 2012). Thus, with their binding site at the α+/β- interface, those novel compounds are theoretically not susceptible to these changes and may have potential for long-term treatment.

It is interesting to see ampicillin, a classic antibiotic, also potentiated the $GABA_AR$ response. Since ampicillin has poor permeability to pass the blood brain barrier (Feng, 2002), it is unlikely that oral or intravenous administration of ampicillin will have a significant influence on the CNS excitation. However, it may affect the peripheral $GABA_ARs$ such as those expressed in pancreas and influence diverse physiological events, such as insulin release (Bansal et al., 2011; Soltani et al., 2011). The conformation comparison between ampicillin and the previous known $GABA_AR$ inhibitor penicillin reveals that the additional amino group on the ampicillin molecule is crucial for its docking at the glutamate-binding site, offering a strategic reference for future drug screening and optimization. Taken together, we demonstrated that the newly identified glutamate-binding site could be a more preferable therapeutic target upon which new $GABA_AR$ positive modulators with a broader receptor spectrum can be developed. And the combination of *in silicon* and electrophysiological screening is a beneficial approach to achieve this goal.

7.5 Future directions

Our study has identified glutamate as a novel positive allosteric modulator of the $GABA_AR$. In future investigations, single channel recording is needed to demonstrate the glutamate effect on $GABA_AR$ activation, deactivation, channel open duration and desensitization. Purkinje cell rebound potentiation may be one of the physiological events that require glutamate-induced potentiation of the $GABA_AR$. However, more direct evidence is needed to clarify if or to what extent the glutamate/$GABA_AR$ interaction is involved in this type of plasticity. Receptor substitution experiment to change to endogenous $GABA_ARs$ into glutamate-insensitive $GABA_ARs$ may be a good approach.

The extrasynaptic glutamate concentration at resting condition is not sufficient to induce significant potentiation of the $GABA_AR$ function (Herman and Jahr, 2007; Jacobson et al., 1985). Thus, the glutamate/$GABA_AR$ crosstalk may happen only if the glutamate is released in close proximity with the $GABA_AR$ or when there is glutamate spillover due to massive glutamate release or impaired reuptake machinery. The former condition can be fulfilled in several types of unconventional glutamate release events such as dendritic glutamate release and GABA/glutamate co-release at the inhibitory synapses (Beltrán and Gutiérrez, 2012; Duguid and Smart, 2004; Zilberter, 2000; Zimmermann et al., 2015). The latter one is usually observed in many pathological conditions, including epilepsy, stroke and traumatic brain injury (During and Spencer, 1993; Hillered et al., 1989; Nilsson et al., 1990; Zhou et al., 2013). Those brain conditions are excellent candidates for studying the physiological and pathological roles of glutamate/$GABA_AR$ interaction.

To investigate those roles, a feasible approach is to develop a glutamate antagonist that blocks the glutamate binding on the $GABA_AR$ without affecting the basic channel function. Previous development of benzodiazepine antagonist that competitively inhibits benzodiazepine binding can shed some light on our current attempt (Hoffman and Warren, 1993; Longmire and Seger, 1993). Since, we have discovered the critical residues for glutamate binding, another approach is to generate transgenic mouse strain expressing glutamate-insensitive $GABA_ARs$ by mutating the critical residues at the binding site. The behavior phenotypes of the wild type and transgenic mice and their thresholds for developing pathological conditions can be compared to reveal the significance of the this novel type of excitation/inhibition crosstalk.

As I have discussed above, the glutamate-binding site can be a good target for developing novel $GABA_AR$ modulators. However, due to the time and technique limitation, the two compounds presented in this dissertation are still far from a clinically applicable therapeutic. Structure optimization is needed to increase their potency, stability, solubility and blood-brain-barrier permeability and minimize the off-target effect and cell toxicity. Then their therapeutic effectiveness for treating neurological disorders, such as anxiety, epilepsy and stroke can be tested using *in vitro* and *in vivo* disease models.

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