$GABA_B$ Receptors Regulate Extrasynaptic $GABA_A$ Receptors

Wucheng Tao

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Reading Committee:

William J Spain, Chair

Bertil Hille

Rachel Wong

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Abstract

GABA$_B$ Receptors Regulate Extrasynaptic GABA$_A$ Receptors

Wucheng Tao

Chair of the Supervisory Committee:

Professor William J Spain

Department of Physiology and Biophysics

Classically, GABA$_B$ receptors regulate neurotransmission primarily through presynaptic mechanisms that inhibit neurotransmitter release, and thus inhibit GABA$_A$ receptor function. Many studies have shown that postsynaptic GABA$_B$ receptors have no direct functional effects on GABA$_A$ receptors. In this thesis, I describe novel results that indicate postsynaptic GABA$_B$ receptors activation enhances GABA$_A$ receptor function in dentate gyrus granule cells (DGGCs).

During my early experiments on DGGCs, I made the surprising observation that inhibition of GABA$_B$ receptors reduced the amplitude of currents mediated by GABA$_A$ receptors. Intrigued by this exciting and unexpected result, I shifted my research to address the following three questions: 1) Do GABA$_B$ receptors regulate GABA$_A$ receptor

function? 2) What are the molecular mechanisms responsible for enhancement of $GABA_A$ currents following $GABA_B$ receptor activation? and 3) What are the signaling pathways involved in this modulation? Based on these questions, I divide my thesis contents into four chapters. Chapter 1 presents an introductory review of $GABA_A$ receptors and $GABA_B$ receptors and a summary of my thesis. The main parts of this thesis with descriptions of results addressing the three questions above are presented in Chapter 2 – 4: In Chapter 2, I found that in dentate gyrus granule cells, postsynaptic $GABA_B$ receptor activation enhanced extrasynaptic $GABA_A$ receptor function with no effect on synaptic $GABA_A$ receptors. Specially, this modulation was cell type specific and only occurs in cell type with delta subunit-containing $GABA_A$ receptors. Also, this modulation did not occur at resting condition and required increase of ambient GABA concentration; in Chapter 3, I found $GABA_B$ receptor activation increased surface expression of delta subunit-containing $GABA_A$ receptors with no change in its total protein expression or single channel conductance or channel kinetics of $GABA_A$ receptors; in Chapter 4, I found that $GABA_B$ receptor modulation of $GABA_A$ receptors required two signaling pathways, one was mediated by PKA and other one was PKC; and these two signaling pathways worked in opposite directions to modulate surface expression of delta subunit-containing $GABA_A$ receptors and GABA currents. As DGGCs act as a gate for hippocampus to prevent excessive excitation inputs from entorhinal cortex, the mechanisms (enhancement of tonic inhibition by membrane trafficking of delta subunit-containing $GABA_A$ receptors) I found here maybe utilized by DGGCs to enhance their gating role.

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PREFACE

Some of the work presented in this dissertation has been published in the following manuscript:

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Chapter 1

Review of $GABA_A$ Receptors and $GABA_B$ Receptors

GABA is the major inhibitory neurotransmitter in the mammalian brain and serves many important functions. Besides its main functional role in balancing excitable signals, GABA also influences development of neuronal networks, refines synaptic integration, and reshapes dendrite outgrowth (Represa and Ben-Ari, 2005; Ge et al., 2006). These actions of GABA are mainly mediated by its three receptors: $GABA_A$, $GABA_B$, and $GABA_C$ receptors. Because $GABA_C$ receptors are mainly distributed in retina, and $GABA_A$ and $GABA_B$ receptors are widely distributed in hippocampus, where my work is mainly done, my review focuses on $GABA_A$ and $GABA_B$ receptors.

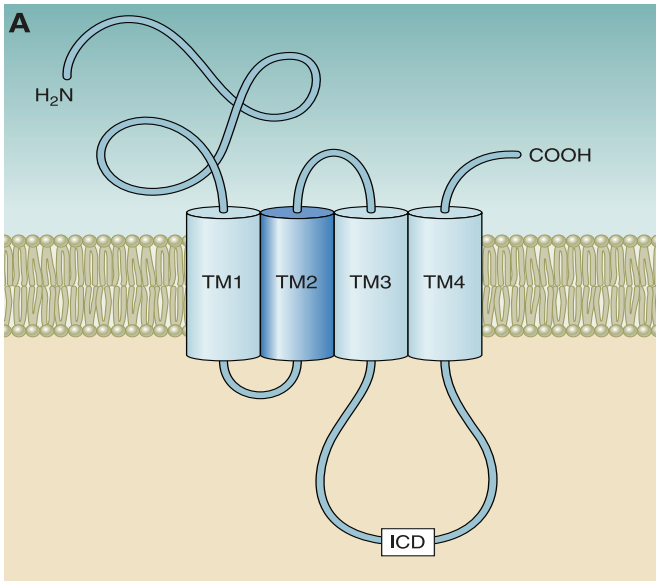
1.1 $GABA_A$ receptors

1.1.1 General introduction to $GABA_A$ receptors

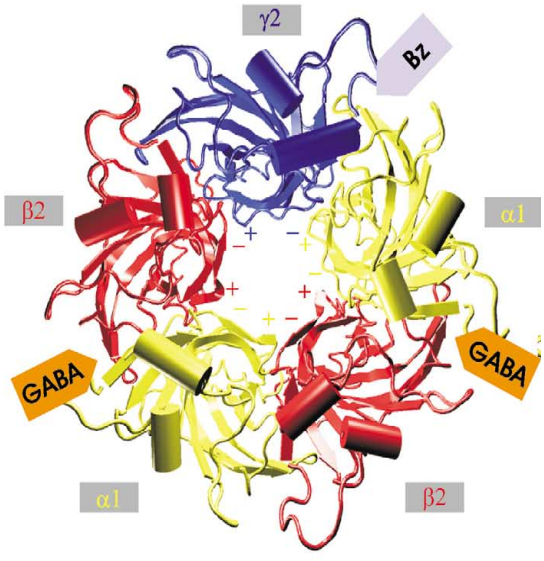
$GABA_A$ receptors are ligand-gated ion channels permeable to Cl^- (and HCO_3^-) that are widely distributed in the central nervous system. They exist as hetero-pentameric complexes, formed from 19 different types of subunits: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π and ρ_{1-3} (Barnard et al., 1998). A typical subunit consists of extracellular N-terminal, C-terminal, four trans-membrane domains (M1-M4), and a large intracellular loop between M3 and M4 (Fig 1.1 A). The pore is formed by M2 domains from each subunit (Leonard et al., 1988). The most common $GABA_A$ receptor complex consists of five subunits, including two α, two β, and one γ subunit (Chang et al., 1996; Farrant and Nusser, 2005). $GABA_A$

receptors are activated when two GABA molecules bind to the interface between α and β subunits (Fig 1.1 B). Many clinically relevant drugs, most notably benzodiazepines, bind to different regions of $GABA_A$ receptors and modulate their function (structure and pharmacology of $GABA_A$ receptors is extensively reviewed in Sieghart, 2006). Although hetero-pentameric receptors are most common, ρ subunits can form functional homomeric receptors in retina (Bormann, 2000).

The biophysical and pharmacological properties of $GABA_A$ receptors are dictated by their subunit composition, and this subunit composition varies between brain regions, cell types, and even on the subcellular level. For example, γ_2 subunit-containing receptors cluster at synaptic sites and are sensitive to diazepam while δ-subunit containing receptors exist at extrasynaptic sites, have a higher affinity for GABA, and are insensitive to diazepam (Saxena and MacDonald, 1996; Farrant and Nusser, 2005). In this review, I will focus on δ-containing $GABA_A$ receptors.



(A)



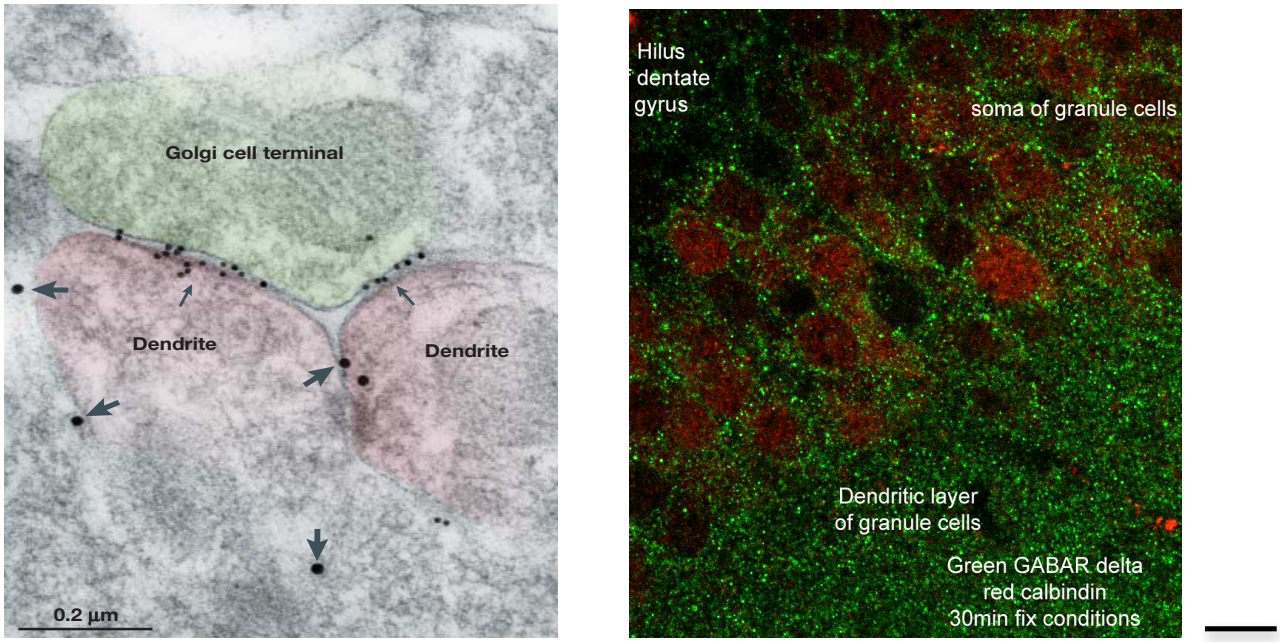
(B)

Fig 1.1 Cartoon model of $GABA_A$ receptor structure. **(A)** A typical $GABA_A$ receptor subunit consists of extracellular N-terminal, C-terminal, four trans-membrane domains (M1-M4), and a large intracellular loop between M3 and M4 (Taken from Vithlani et al., 2011) **(B)** Top view of model structure of $GABA_A$ receptor and its binding sites for GABA and benzodiazepines (BZ) (Taken from Ernst et al., 2003)

1.1.2 Distribution and molecular components of δ-containing $GABA_A$ receptors

In many brain regions and cell types, δ subunits form functional receptor complexes with different subunits, predominantly with α_4 and α_6. $\alpha_4\beta\delta$ receptors are mainly expressed in dentate gyrus granule cells (DGGC) and thalamocortical relay neurons (Brickley and Mody, 2012). In cerebellar granule cells, δ subunits combine primarily with α6 subunits (Jones et al., 1997). Additionally, some interneurons also express δ subunits but assemble with α_1 subunits (Glykys et al., 2007). These different combinations of receptor subunits will shape the structure and function of δ subunit containing $GABA_A$ receptors in very dynamic ways. $GABA_A$ receptors containing δ subunits have a unique subcellular distribution, and are exclusively located at extrasynaptic/perisynaptic sites. In DGGCs, δ subunits are concentrated in dendrites (i.e. molecular layer of dentate gyrus) (Wei et al., 2003; Tao et al., unpublished data; Fig1.2) Interestingly, some groups show that expression of δ subunits can also modulate the expression and distribution of other subunits. For example, in the pilocarpine model of temporal lobe epilepsy: the expression of δ subunits is reduced, but the expression of γ_2 subunits in extrasynaptic sites is increased (Peng et al., 2002).

This may compensate for the loss-of-function of δ subunits. The factors that determine how and when δ subunits assemble with other $GABA_A$ receptor subunits and how their subcellular trafficking is directed and regulated remain unknown.



(A) (B)

Fig 1.2 Subcellular distribution of δ subunits. **(A)** Electron micrograph of $GABA_A$ receptors in mouse cerebellum granule cells. Gold particles labeling delta subunits (large arrows) and beta subunits (small arrows) (taken from Farrant and Nusser, 2005, which is adapted from Nusser et al.,1998) **(B)** Immunostaining of delta subunits of dentate gyrus granule cells in hippocampal slice. Much denser staining of delta subunits in molecular layer than other regions of DG (Tao et al., unpublished data). Scale bar, 10 µm.

1.1.3 Biophysical properties of δ-containing $GABA_A$ receptors

Whole-cell current recordings show that δ subunit-containing $GABA_A$ receptors have high affinity for ambient GABA (at resting condition, GABA concentration in the extracellular space is around 100 nM to 1 μM), with EC_{50} of 0.3-0.7 μM (Saxena and Macdonald, 1996; Mtchedlishvili and Kapur, 2005). Another important property of δ-containing $GABA_A$ receptors is that this receptor shows little desensitization (Farrant and Nusser, 2005). Single-channel recordings showed that δ-containing $GABA_A$ receptors have relatively lower conductance (around 10 pS; Bai et al., 2001; Yeung et al., 2003; Brickley et al., 1999) than synaptic $GABA_A$ receptors (around 25 pS; De Koninck and Mody, 1994; Mortenson and Smart, 2006) Also, they have a lower channel open probability and mean open time, and modeling suggests a different number of open states (Fisher and Macdonald, 1997; Akk et al., 2004).

A consequence of the high-affinity and incomplete desensitization of extrasynaptic δ-containing $GABA_A$ receptors is that they are tonically activated by the low levels of ambient GABA that exists in brain extracellular space, estimated to be in the range of 90-250 nM (Santhakumar et al., 2006). This tonic form of inhibition is seen experimentally as the change in holding current produced by $GABA_A$ receptor antagonists. Tonic inhibition has important effects on both cellular and network excitability (Farrant and Nusser, 2005). The contribution of δ-containing $GABA_A$ receptors to tonic inhibition in DGGCs is well-established through studies demonstrating similar biophysical and pharmacological properties of tonic GABA currents to the properties of δ-containing $GABA_A$ receptors in heterologous expression systems and δ

subunit knockout mice that have greatly reduced tonic currents in DGGCs. Interestingly, several studies show that extrasynaptic $GABA_A$ receptors can be spontaneously active even in the absence of agonist (Wall 2002; Birnir et al., 2000; Wlodarczyk et al., 2013).

1.1.4 Pharmacological properties of δ-containing $GABA_A$ receptors

δ-containing $GABA_A$ receptors have distinguishing pharmacology. The agonist THIP, or gaboxadol, selectively activates δ-containing $GABA_A$ receptors at concentrations of 1 μM (Meera et al., 2011). Neurosteroids, such as pregnenolone and allopregnanolone, are also strong modulators selective for δ-containing $GABA_A$ receptors (Stell et al., 2003). As neurosteroids are endogenous modulators for δ-containing $GABA_A$ receptors, and their endogenous concentrations do not affect synaptic $GABA_A$ receptors, neurosteroid regulation of δ-containing $GABA_A$ receptors is a unique mechanism to regulate tonic inhibition independent of protein trafficking/synthesis machines. Neurosteroids are progesterone metabolites and their level changes predictably during the ovarian cycle and the activation and expression of δ-containing $GABA_A$ receptors is enhanced when neurosteroid levels are elevated (Maguire et al., 2005). This suggests a new functional role of δ-containing $GABA_A$ receptors in physiological conditions. Some clinical anxiolytic and anticonvulsive agents, such as pyrazolopyridines, also strongly potentiate δ-containing $GABA_A$ receptors (Young et al., 1987). Finally, socially relevant concentrations of ethanol are believed to act primarily by increasing tonic current mediated by δ-containing $GABA_A$ receptors (Wallner et al., 2003; Wei, et al., 2004; Choi et al., 2008**)**. Because δ-containing $GABA_A$ receptors are insensitive to benzodiazepines,

a $GABA_A$ receptor allosteric modulator, this agent can be used to differentiate extrasynaptic from synaptic $GABA_A$ receptors. Although pharmacological tools exist to activate or potentiate δ-containing $GABA_A$ receptors, there is no specific antagonist for δ-containing $GABA_A$ receptors.

1.1.5 Physiological properties of δ-containing $GABA_A$ receptors

δ subunits are the major component of tonic current in DGGCs and tonic inhibition is significantly reduced in δ subunit knockout mice (Glykys et al., 2008). This indicates an important physiological role of δ-containing $GABA_A$ receptors in these cells. Compared with phasic inhibition, tonic inhibition mediated by δ-containing $GABA_A$ receptors have very different physiological properties. Unlike phasic inhibition, that produces rapid and transient voltage changes to provide temporally precise signals, tonic inhibition produces a slow and steady signal. This is due to different anatomical distribution and different patterns of activation by presynaptic vesicular release of GABA or ambient GABA levels in these two types of inhibition. During early postnatal development of neuronal circuits, tonic inhibition is present even before synapse formation (Demarque et al., 2002) Although we still do not know exact functional roles of extrasynaptic $GABA_A$ receptors in early development, they seem to be important for synaptic integration and dendrite outgrowth (Represa and Ben-Ari 2005; Ge et al., 2006). In mature neurons, the main role of tonic inhibition is to limit excitability by shunting excitatory synaptic inputs, making the cell less likely to fire action potentials (Farrant and Nusser, 2005). This shunting mechanism can affect neuronal firing by offsetting the input threshold for action

potentials and affecting the gain of neuronal input-output relationships (Chance et al., 2002; Mitchell and Silver, 2003; Pavlov et al., 2009).

1.1.6 Physiological modulation of δ-containing $GABA_A$ receptors

Because extrasynaptic $GABA_A$ receptors are significantly affected by ambient GABA concentration, any mechanism that affects GABA concentration in the extracellular space can affect tonic inhibition. These mechanisms include vesicular release of GABA (Glykys and Mody, 2007), anion-channel mediated release of GABA from glial cells (Lee et al., 2010), and both uptake and release of GABA by GABA transporters (Farrant and Nusser, 2005; Ransom et al. 2013). The regulation of δ-containing $GABA_A$ receptors is activity-dependent; thus, TTX treatment increases surface expression of delta subunits in cultured hippocampal neurons (Joshi and Kapur, 2009). Additionally, some postsynaptic mechanisms can also modulate δ-containing $GABA_A$ receptors (Tao et al., 2013; Connelly et al., 2013).

The modulation of synaptic $GABA_A$ receptors by kinases has been investigated intensely (Tretter and Moss, 2008; Connelly et al., 2013), but the influence of signaling pathways/kinases on δ-containing extrasynaptic $GABA_A$ receptors remains unknown.

1.1.7 δ-containing $GABA_A$ receptors in disease

Alterations of tonic inhibition is associated with many neurological diseases, such as epilepsy, sleep disorders, stress, learning and memory (Brickley and Mody, 2012). Due to personal interest and the scope of this review, I will focus on the involvement of tonic inhibition in epilepsy.

Several point mutations in δ subunits have been reported in epilepsy patients, including E177A, R220H (Macdonald et al., 2004; 2010). In the pilocarpine/status epilepticus model of epilepsy, Peng et al., (2004) reported a reduction in the expression of δ subunits and tonic currents. Thus, tonic inhibition may contribute to epileptogenesis and network excitability and is a potential therapeutic target. Indeed, several drugs targeting δ subunits to increase tonic currents have been put into clinical use. For example, gaboxadol, a δ-subunit selective agonist (Pollack et al., 2005), and two positive allosteric modulators of δ-containing $GABA_A$ receptors, ganaxolone and alphaxalone, are being studied in clinical trials (Biagini et al., 2010; Winter et al., 2003). However, enhancing tonic inhibition may not be a good strategy for treating all types of epilepsy. For example, Cope et al. (2009) showed that tonic current is enhanced in typical Absence Epilepsy, a type of primary generalized epilepsy, and this enhancement promoted burst firing of thalmocortical neurons and increased Absence Seizures. Perucca et al. (1998) found that some drugs that are used to treat focal epilepsy and enhance tonic inhibition (i.e. tiagabine and vigabatrin), exacerbated Absence Seizures. Thus, we still do not know how tonic inhibition is regulated in epilepsy. To answer this

question, more basic mechanistic studies of tonic inhibition in different types of epilepsy are necessary.

1.2 $GABA_B$ receptors

In addition to ionotropic $GABA_A$ receptors, GABA also activates $GABA_B$ receptors. $GABA_B$ receptors are G-protein coupled receptors that are easily distinguished from $GABA_A$ receptors by their effects and pharmacology (Bowery and Hudson, 1979). Although $GABA_B$ receptors were first identified in the 1980s, they have received relatively less attention than $GABA_A$ receptors. The properties of $GABA_B$ receptors will be reviewed below, with special focus on the structure and function of $GABA_B$ receptors.

1.2.1 Structure and distribution of $GABA_B$ receptors

Like all classical G protein coupled receptors (GPCRs), $GABA_B$ receptors consist of an extracellular N terminal, 7 transmembrane domains, and an intracellular C terminus. A fully functional $GABA_B$ receptor is a heterodimer, formed by $GABA_{B1}$ and $GABA_{B2}$ subunits (Kaupmann et al., 1997; White et al., 1998), each of which has the structure and topology of a GPCR. $GABA_{B1}$ subunits are important for agonist binding and are sub-divided into the splice variants $GABA_{B1a}$ and $GABA_{B1b}$ (Kaupmann et al., 1997); $GABA_{B1a}$ is distributed predominantly at presynaptic terminals, wheras $GABA_{B1b}$ is mostly expressed at postsynaptic sites. $GABA_{B2}$ is important for the coupling of downstream signaling pathways and for trafficking receptors to the membrane. The C terminus of $GABA_{B2}$ subunits is especially important for receptor trafficking (Robbins et

al., 2001; Calver et al., 2001). $GABA_B$ receptors are distributed in many regions of the brain, including the hippocampus and cerebral cortex. Subcellularly, they are found on soma and dendrites (spine and shaft) of both excitatory and inhibitory synapses. Scanziani (2000) showed that postsynaptic $GABA_B$ receptor activation requires strong presynaptic activity, indicating the need for GABA spillover consistent with the extrasynaptic distribution of $GABA_B$ receptors (Kulik et al., 2003). Recent work has shown that $GABA_B$ receptors interact with auxiliary binding proteins, including KCTD (potassium channel tetramerization domain-containing) proteins (Schwenk et al., 2010; Bartoi et al., 2010), Mupp1 (Balasubramanian et al., 2007) and GISP (Kantamneni et al., 2007). These auxiliary binding proteins have profound influence on agonist potency and the kinetics of the receptor response. Interestingly, $GABA_B$ receptors are already present and functional at the embryonic stage of development, even before synapse formation (Obrietan and Van den Pol, 1998, 1999; Fritschy et al., 2004).

In addition to the different cellular/subcellular localization of the different $GABA_B$ receptor subunits, there is compelling evidence for a functional difference between presynaptic (i.e. $GABA_{B1a}$) and postsynaptic (i.e. $GABA_{B1b}$) $GABA_B$ receptors: 1) higher concentrations of antagonist are required to block presynaptic effects of $GABA_B$ receptors compared to postsynaptic effects (Pozza et al., 1999; Cruz et al., 2004), 2) presynaptic $GABA_B$ receptors are functional at birth, but postsynaptic $GABA_B$ receptors are not functional during early postnatal developmental stages (Gaiarsa et al., 1995), and 3) Dutar and Nicoll (1988) reported that pre-and post-synaptic $GABA_B$ actions have different sensitivities to pertussis toxin.

1.2.2 Function of $GABA_B$ receptors

$GABA_B$ receptors exert diverse effects on brain function through coupling to several types of downstream effector molecules. $GABA_B$ receptor activation is linked to $G_{i/o}$ α subunits, which inhibit adenylate cyclase (AC), thereby reducing cAMP and PKA activity. This modulates many downstream targets, including NMDA receptors (Chalifoux and Carter, 2010). Additionally, $GABA_B$ receptor activation causes $G_{\beta/\gamma}$ subunits to dissociate from α subunits. These dissociated $G_{\beta/\gamma}$ subunits also produce downstream effects, including direct activation of K_{ir} channels and inhibition of voltage gated Ca^{2+} channels (VGCCs) (Bowery et al., 2002). The activation of presynaptic $GABA_B$ receptors, acting as autoreceptors, inhibits transmitter release; an effect likely mediated by K^+ channel opening, inhibition of VGCCs, and PKA pathways (Bowery et al., 2002). Interestingly, Blackmer et al., (2001) found that $GABA_B$ receptors also inhibit transmitter release by targeting SNARE proteins and other molecules involved in release of synaptic vesicles. Through these multiple mechanisms, $GABA_B$ receptors have diverse effects on pre- and post-synaptic neurons. In GABAergic neurons, presynaptic $GABA_B$ receptors increase the excitability of postsynaptic cells (by reducing GABA release). Conversely, postsynaptic $GABA_B$ receptors in glutamatergic neurons reduce their excitability (by opening K^+ channels). Beside these well-established roles, $GABA_B$ receptors also enhance metabotropic glutamatergic signaling (Tabata and Kano 2010), modulate inhibitory synaptic strength (Davies et al., 1991; Huang et al., 2005), and play important roles in neuronal-network construction (Gaiarsa et al., 2011).

Because of the varied signaling pathways that are influenced by $GABA_B$ receptors (AC/PKA, PLC/PKC, and possibly PI3K), it is likely that $GABA_B$ receptors have other effects that are not yet appreciated. For example, do $GABA_B$ receptors affect $GABA_A$ receptors and what are the signaling pathways involved in this modulation? In the following chapters, I will examine these questions.

Chapter 2

Postsynaptic $GABA_B$ Receptors Enhance Extrasynaptic $GABA_A$ Receptor Function in Dentate Gyrus Granule Cells

2.1 Introduction

The inhibitory neurotransmitter gama-aminobutyric acid (GABA) activates both ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors. $GABA_A$ receptors are Cl^- ion channels that produce electrical signals when activated. $GABA_A$ receptors respond transiently to GABA released from synaptic vesicles and, in many areas of the brain including the hippocampus, high-affinity $GABA_A$ receptors at extrasynaptic sites are activated tonically by ambient GABA (Farrant and Nusser, 2005; Glykys and Mody, 2007). Activation of presynaptic and postsynaptic $GABA_B$ receptors stimulates intracellular G-protein signaling cascades that activate K^+ channels, inhibit voltage-gated Ca^{2+} channels, and regulate cyclic AMP (cAMP) and protein kinase A (PKA) (Padgett and Slesinger, 2010). Because postsynaptic $GABA_B$ receptors are located at extrasynaptic sites away from GABA release sites, their activation is limited by GABA uptake and requires patterns of presynaptic activity that lead to GABA spillover and elevations of ambient GABA (Scanziani, 2000; Kulik et al., 2003).

Under conditions of increased ambient GABA, such as occur with ischemia, epileptic seizures, or drugs that increase GABA concentration, coactivation of $GABA_A$ receptors and postsynaptic $GABA_B$ receptors will occur (Scanziani et al., 1991; During and

Spencer, 1993; Wu et al., 2003; Allen et al., 2004).

In dentate gyrus granule cells (DGGCs), electron microscopy with immunogold labeling has identified $GABA_B$ receptors at perisynaptic sites on dendritic and somatic membranes (Kulik et al., 2003), a distribution pattern that has remarkable overlap with the distribution of extrasynaptic $GABA_A$ receptor subunits that mediate tonic inhibition in DGGCs (i.e., delta subunits) (Wei et al., 2003). The proximity of postsynaptic $GABA_B$ receptors to extrasynaptic $GABA_A$ receptors on DGGCs suggests that $GABA_A$ receptors will be exposed to intracellular signaling pathways activated by $GABA_B$ receptors. This potential interaction has likely been overlooked, because studies of $GABA_A$ receptors are routinely done in the presence of $GABA_B$ receptor antagonists.

We investigated the interaction between $GABA_B$ receptors and $GABA_A$ receptors in DGGCs. Our data show that activation of postsynaptic $GABA_B$ receptors enhances $GABA_A$ currents caused by exogenous GABA. This newly identified interaction was not present in CA1 pyramidal neurons or layer 2/3 cortical pyramidal neurons. In DGGCs, tonic GABA currents and currents mediated by delta subunit-containing receptors were also modulated by $GABA_B$ receptor activation. Our results indicate that extrasynaptic $GABA_A$ receptor function will be enhanced when postsynaptic $GABA_B$ receptors are activated, increasing the inhibitory tone of DGGCs.

2.2 Materials and Methods

2.2.1 Brain slice preparation

Brain slices were prepared from 4–6 week old Sprague Dawley rats of both sexes. Rats

were anesthetized with 4% isoflurane, decapitated, and the brain dissected free. Transverse hippocampal slices (300 μm) were prepared. These slices contained portions of temporal cortex that were used for experiments on cortical neurons. Slices were cut and stored in a solution containing (in mM): 125 NaCl, 3 KCl, 26 $NaHCO_3$, 1.2 NaH_2PO_4, 0.5 $CaCl_2$, 4 $MgCl_2$, 20 dextrose, and 1 kynurenic acid. Slices were cut in ice-cold solution and stored at room temperature. Solutions were continuously gassed with 95% O_2/5% CO_2. Slices were allowed to recover for 1 h before recording. All animal use protocols were approved by the local Institutional Animal Care and Use Committee.

2.2.2 Electrophysiology

Membrane currents were recorded using whole-cell patch clamp techniques. Neurons were visualized with an Axioskop 2 upright microscope with fixed stage (Carl Zeiss). Recordings were made using an Axopatch 200B amplifier, a Digidata 1200 series A-D converter, and pClamp 9 software (Molecular Devices). Data were acquired at 2 kHz and low-pass filtered at 1 kHz. Series resistance was compensated by 50–70% online. If series resistance exceeded 20 MΩ or changed by 20%, the experiment was discarded. Focal application of GABA or bicuculline was made by pressure ejection (Picospritzer II, General Valve) from a patch pipette containing (in mM): 150 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 dextrose, and 10 HEPES with pH adjusted to 7.4 with NaOH. The pressure ejection pipette was positioned 20–30 μm from the soma. The recording chamber was continuously superfused at 2–2.5 ml/min with a bath solution containing (in mM): 134 NaCl, 3 KCl, 1.4 NaH_2PO_4, 24 $NaHCO_3$, 10 dextrose, 2 $MgCl_2$, 2 $CaCl_2$, and 1 kynurenic acid (pH 7.35–7.4 when bubbled with 95% O_2/5% CO_2). Osmolarity

was adjusted to 300–305 mOsm with H_2O. Patch pipettes were pulled from borosilicate glass with filament (Sutter Instrument). Pipettes had resistances of 3–4 MΩ when filled with intracellular solution containing (in mM): 125 CsCl, 10 QX-314 chloride, 10 HEPES, and 1 EGTA (pH corrected to 7.25 with CsOH). Osmolarity was adjusted to 275–285 mOsm with H_2O as needed. Data acquisition was started 4–6 min after establishing a whole-cell recording. Experiments were performed at room temperature (23°C). All chemicals were purchased from Sigma except SKF 89976a (Tocris-Cookson) and QX-314 (Alomone Labs). Baclofen was used at 20 μM (Dutar and Nicoll, 1988), and CGP55845 was used at 10 μM (Chen and Regehr, 2003).

2.2.3 Analysis

Data analysis was performed with Clampfit (pClamp 10) and Origin (v6.1, Microcal Software) software. Tonic currents were measured as the change in holding current caused by the $GABA_A$ receptor antagonist bicuculline methiodide (40 μM), based on Gaussian fits to all-points current amplitude histograms constructed from 2–10 s of data with a bin width of 1 pA. Fits were performed using a Levenberg–Mar-quardt algorithm in Clampfit, and the holding current was taken as the center of the Gaussian curve. IPSCs were analyzed using template matching event detection. Data are presented as mean ± standard error of the mean (SEM), and all error bars represent SEM. Statistical analyses were performed using Microsoft Excel. A two-tailed, paired or homoscedastic Student's *t* test was used with a p value < 0.05 considered as significant.

2.3 Results

2.3.1 $GABA_B$ receptor activation enhanced $GABA_A$ receptor currents in a cell type-specific manner

To investigate an interaction between metabotropic $GABA_B$ receptors and ionotropic $GABA_A$ receptors, we made focal applications of GABA (10 μM) to activate $GABA_A$ currents in DGGCs (V_m = 60 mV). Bath application of the $GABA_B$ receptor agonist baclofen (20 μM) increased GABA-evoked currents by 83 ± 18% (control, -358 ±143 pA vs baclofen, -577 ± 184 pA, n = 5, p< 0.01) (Fig. 2.1*A*). Currents in the presence of baclofen were inhibited by bicuculline (20 μM) to a similar extent as under control conditions (control, 91 ± 2% inhibition, baclofen, 95 ± 1% inhibition, n = 3-4, p = 0.26), indicating that currents enhanced by baclofen were also mediated by $GABA_A$ receptors (data not shown). Modulation of $GABA_A$ currents by baclofen was blocked by the $GABA_B$ receptor antagonist CGP55845 (CGP, 10 μM). In the presence of CGP, $GABA_A$ currents were -521 ± 120 pA at baseline and -486 ± 138 pA after application of baclofen (n = 5, p = 0.38) (Fig. 2.1*B*). These data indicate that activation of $GABA_B$ receptors augment $GABA_A$ receptor function in DGGCs. CGP by itself reduced $GABA_A$ currents, demonstrating baseline activation of $GABA_B$ receptors (and modulation of $GABA_A$ receptors) during application of GABA. On average, CGP alone reduced $GABA_A$ currents from -309 ± 87pA to -187 ± 62pA (n = 5, p < 0.05) (Fig. 2.1*C*).

We tested the effects of baclofen on GABA-evoked currents in hippocampal CA1 pyramidal cells and cortical layer 2/3 pyramidal cells. In contrast to results from DGGCs,

baclofen did not modulate $GABA_A$ currents in these other cell types (CA1: control -669 ± 173 pA vs baclofen -647 ± 163pA, *n* = 4, *p* = 0.19; Layer 2/3: control -457 ± 103 pA vs baclofen -445 ± 96 pA, *n* = 6, *p*= 0.28) (Fig. 2.1*D*).

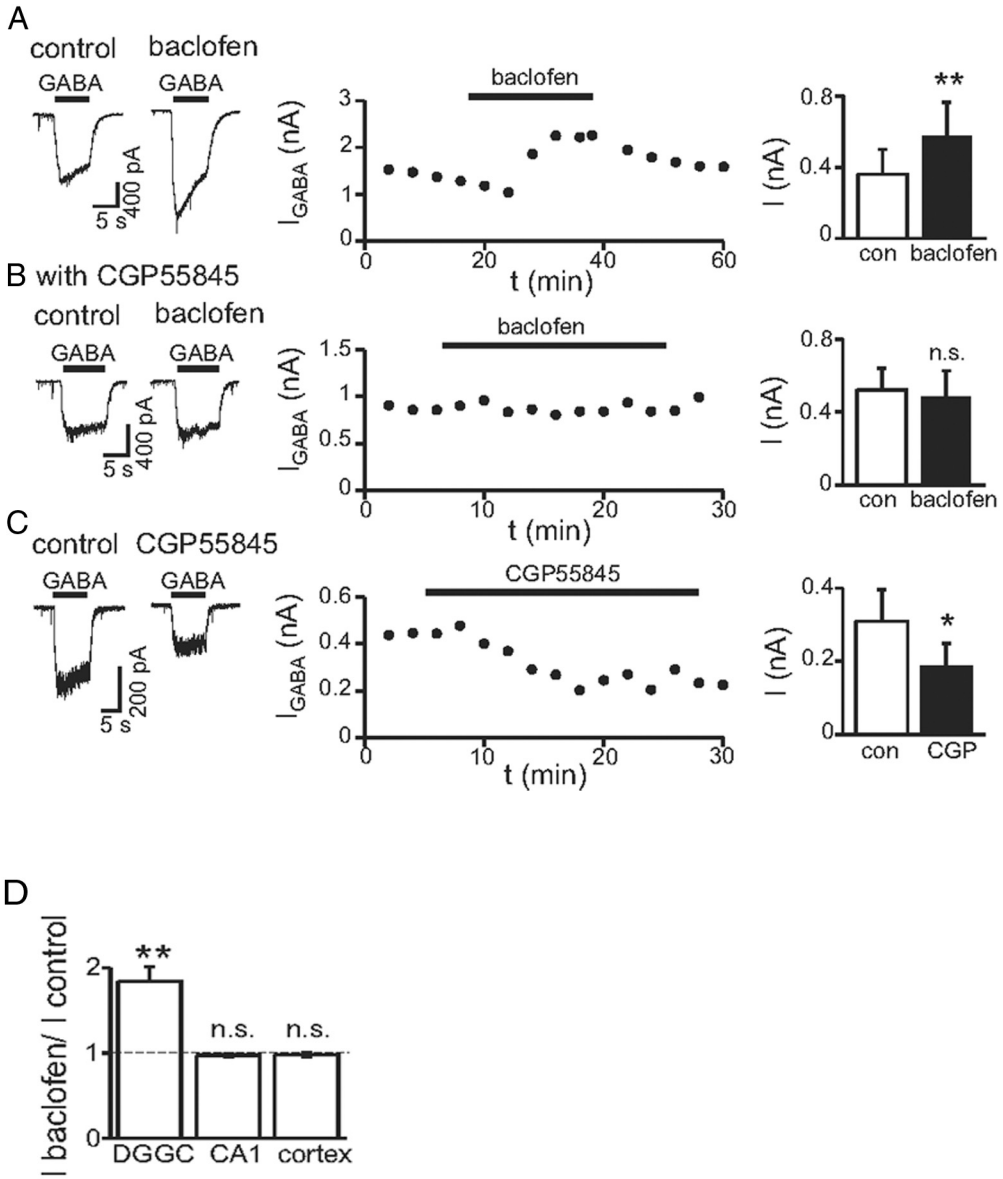


Figure 2.1. $GABA_B$ receptor activation enhanced $GABA_A$ currents in DGGCs, but not CA1 pyramidal neurons or cortical layer 2/3 pyramidal neurons. **(A)** $GABA_A$ currents evoked by focal application of GABA (10 μM) before and during bath application of

baclofen (20 μM) (left panel). Middle panel shows time course of current change during baclofen application for this cell. Horizontal bars indicate the period of drug application here and in subsequent figures. Right panel shows mean current amplitudes under control conditions (con) and in the presence of baclofen. **(B)** GABA currents in the presence of the $GABA_B$ antagonist CGP 55845 (10 μM) before and during baclofen application (left), time course of current change for this cell (middle), and mean current under control conditions (con) and in the presence of baclofen (right panel). CGP prevented baclofen effects on $GABA_A$ currents. **(*C*)** GABA currents before and after application of CGP alone (left), time course for this cell (middle), and mean current amplitude (right). **(*D*)** Normalized currents (baclofen/control) for experiments on DGGCs, CA1 pyramidal cells, and layer 2/3 pyramidal cells. Error bars represent SEM; *$p < 0.05$, **$p< 0.01$, n.s., nonsignificant.

2.3.2 Modulation of $GABA_A$ current required postsynaptic G-protein activation

Bath-applied baclofen activates both presynaptic and postsynaptic $GABA_B$ receptors, raising the possibility of an indirect action (e.g., via modulation of GABA release). Because $GABA_A$ receptors are G-protein coupled, transduction of receptor activation requires guanine nucleotide exchange. This process involves dissociation of GDP from inactive G proteins and binding of GTP. Thus, postsynaptic $GABA_B$ receptor signaling can be prevented by intracellular GDP-β-S, a nonhydrolyzable GDP analog (Harayama et al., 1998; Lin and Dun, 1998). We recorded $GABA_A$ currents from DGGCs with intracellular solutions containing GDP-β-S (0.5 mM) and no GTP (Fig. 2. 2*A*).

Intracellular GDP-β-S prevented baclofen-induced potentiation of $GABA_A$ currents (control, -615 ± 141 pA vs baclofen, -532 ± 118 pA, n = 5, p = 0.12) (Fig. 2. 2*A*). These results indicate that baclofen modulates $GABA_A$ currents via postsynaptic $GABA_B$ receptors and a G-protein signaling pathway.

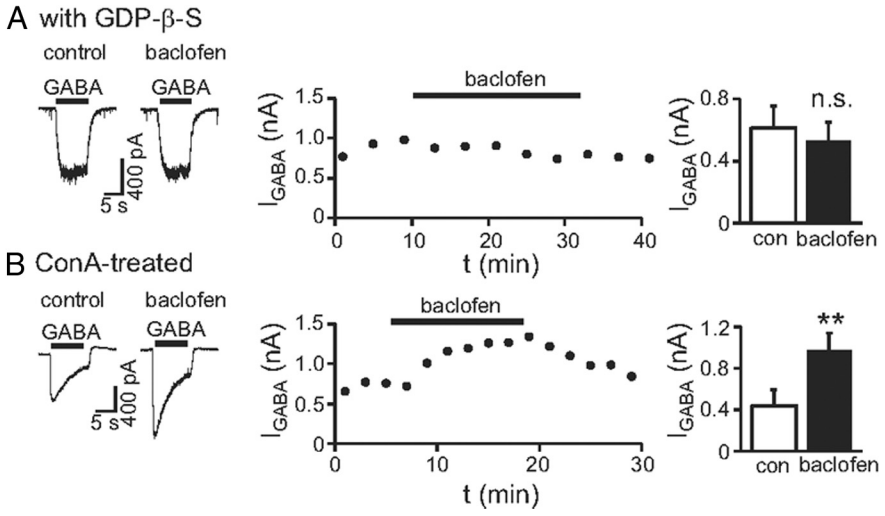


Figure 2.2. Modulation of $GABA_A$ currents by baclofen required postsynaptic G-protein activation and was independent of presynaptic vesicle release. **(A)** $GABA_A$ currents recorded with intracellular GDP-β-S to inhibit postsynaptic G proteins (left), time course of current change for this cell (middle), and mean current under control conditions (con) and in the presence of baclofen (right panel). Intracellular GDP-β-S blocked the effect of baclofen. **(B)** Currents recorded from a DGGC pretreated with concanamycin A to inhibit vesicular GABA release (left). Baclofen increased $GABA_A$ currents after ConA treatment (middle, current time course; right, mean current). *p < 0.05, **p < 0.01.

2.3.3 Modulation of $GABA_A$ current was independent of synaptic vesicle release

Although changes in GABA release were not predicted to affect responses to exogenous GABA, we wished to exclude a contribution of vesicular GABA release to the effects of baclofen. To address this issue, we inhibited vesicular release of GABA by pretreating slices with the H^+-ATPase inhibitor concanamycin A (ConA, 0.5 μM, 2 h). ConA treatment reduced the frequency of spontaneous IPSCs (sIPSCs) by 75% (n = 9, $p < 0.05$) (data not shown). However, ConA treatment did not affect the baclofen-induced potentiation of $GABA_A$ currents (control, -437 ± 157 pA vs baclofen, -974 ± 167 pA, n = 5, $p < 0.01$) (Fig. 2.2 *B*).

2.3.4 Baclofen potentiated tonic $GABA_A$ receptor currents, but not synaptic $GABA_A$ receptor currents

The data presented above show that postsynaptic $GABA_B$ receptors can potentiate $GABA_A$ currents elicited by exogenous GABA. Distinct types of $GABA_A$ receptors with unique subunit compositions are transiently activated at synapses by vesicular GABA release or tonically activated by ambient GABA at extrasynaptic sites (Farrant and Nusser, 2005). To determine whether extrasynaptic $GABA_A$ receptors were subject to modulation by postsynaptic $GABA_B$ receptors, we measured tonic $GABA_A$ current as the change in holding current caused by focal application of bicuculline (40 μM). Similar to its effect on exogenous GABA currents, baclofen potentiated tonic currents (control, -7.5 ± 1 pA vs baclofen, -15.2 ± 0.1 pA, n = 5, $p < 0.01$) (Fig. 2.3A,B). Tonic currents were

unaffected by baclofen in the presence of CGP (CGP, -5.5 ± 1.2 pA vs baclofen/CGP, -4.8 ± 0.9, p = 0.44, n = 5) (Fig. 2.3*C*). In contrast to GABA-evoked currents, CGP alone did not affect tonic currents (control, -13.6 ± 3.0 pA vs CGP, -15.3 ± 3.4, n = 4, p = 0.17) (Fig. 2.3*C*). This indicates that $GABA_B$ modulation of tonic currents is not basally active and requires periods of increased ambient GABA (such as periodic application of exogenous GABA, i.e., Fig. 2.1*C*). Following inhibition of GABA uptake by SKF 89976a (SKF, 30 μM) to increase ambient GABA and tonic current, blocking $GABA_B$ receptors with CGP significantly reduced tonic current (from -86.8 ±17.9 pA to -45.9 ± 9.6 pA, n = 5, p <0.01) (Fig. 2.3*D*,*E*). These data indicate that $GABA_B$ receptors modulate $GABA_A$ receptors that mediate tonic currents.

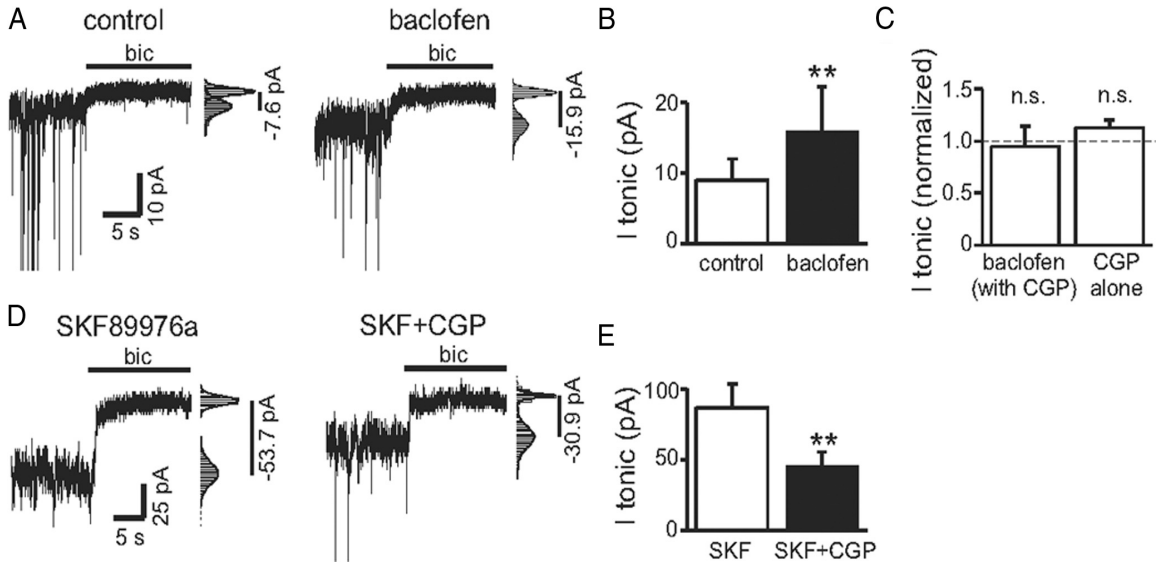


Figure 2.3. Postsynaptic $GABA_B$ receptors increased tonic $GABA_A$ currents. **(A)** Tonic currents under baseline conditions (left) and during baclofen application (right). The histograms and Gaussian fits used to measure holding current before and during focal application of bicuculline (bic, 40 μM) are shown to the right of each trace, and each

tonic current amplitude is indicated. (**B)** Mean tonic current under control conditions and in the presence of baclofen. **(C)** Tonic currents during application of baclofen (with CGP) or CGP alone normalized to control. **(D)** Tonic currents in the presence of the GABA uptake inhibitor SKF 89976a (30 μM) before and during CGP application. **(E)** Mean tonic currents in the presence of SKF or SKF + CGP. $GABA_B$ antagonism by CGP reduced the large tonic current seen in the presence of SKF; $^{**}p < 0.01$, n.s., nonsignificant.

Tonic currents in DGGCs are primarily mediated by delta subunit-containing $GABA_A$ receptors (Glykys et al., 2008). To confirm that baclofen potentiates currents produced by delta subunit-containing $GABA_A$ receptors, we used the delta subunit- selective agonist THIP (10 μM). Baclofen increased currents evoked by THIP (control, -103 ± 19 pA vs baclofen, -167 ± 32 pA, $n = 5$, $p < 0.05$) (Fig. 2.4*A*). In the presence of CGP, baclofen had no effect on THIP-induced currents (CGP, -48 ± 13 pA vs baclofen/CGP, -46 ± 14 pA, $p = 0.56$, $n = 5$) (Fig. 2.4*B*). Similar to tonic currents, CGP alone did not affect THIP currents (control, -85.5 ± 18.9pA vs CGP, -88.0 ± 19.3pA, $n = 4$, $p = 0.50$) (Fig. 2.4*B*). These results demonstrate that delta subunit-containing $GABA_A$ receptors are a target of postsynaptic $GABA_B$ receptors.

Measurements of sIPSCs showed that baclofen reduced their frequency to 52 ± 6% of control values ($n = 5$, $p < 0.05$) but did not significantly affect sIPSC amplitude, 10–90% rise times, or decay times ($n = 5$, $p = 0.94$, 0.44, and 0.30, respectively) (data not shown). CGP alone did not affect sIPSC frequency, amplitude, or decay times ($p = 0.53$,

0.52, and 0.33, respectively; *n* = 4). These data indicate that $GABA_B$ receptors reduce presynaptic release of GABA but do not alter the properties of $GABA_A$ receptors activated at synapses (Otis and Mody, 1992).

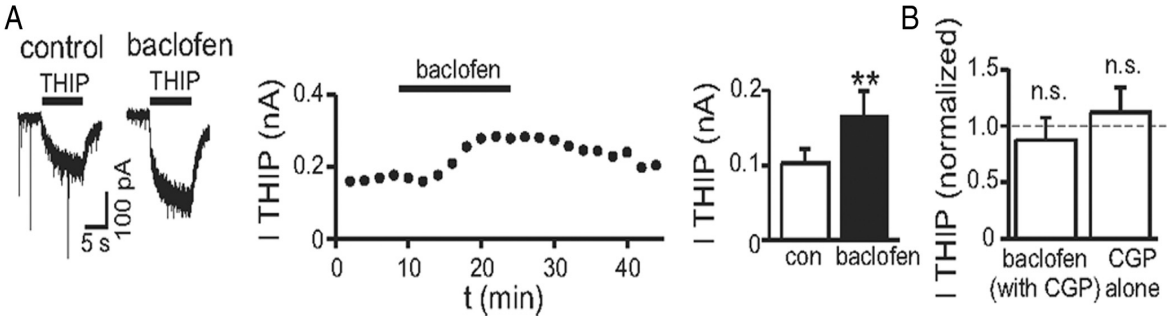


Figure 2.4. $GABA_B$ receptors increased currents evoked by the $GABA_A$ receptor delta subunit-selective agonist THIP. **(A)** Currents evoked by THIP (10 μM) (left), the time course of current change for this cell (middle), and the mean THIP-induced currents under control conditions (con) and during baclofen application (right). **(B)** THIP currents during application of baclofen (with CGP) or CGP alone normalized to control; ** $p < 0.01$, n.s., nonsignificant.

2.4 Discussion

Our results show for the first time that postsynaptic $GABA_B$ receptors can modulate $GABA_A$ receptor function. Specifically, our data indicate that postsynaptic $GABA_B$ receptors enhance the function of $GABA_A$ receptors that produce tonic currents in DGGCs (including delta subunit-containing receptors). Because postsynaptic $GABA_B$ receptors and the $GABA_A$ receptors they modulate are located extrasynaptically (Kulik

et al., 2003; Wei et al., 2003), both types of receptors will experience similar levels of ambient GABA. Coactivation of these receptors during periods of increased ambient GABA associated with intense neural activity may represent a feedback mechanism to increase inhibitory tone of DGGCs (Scanziani et al., 1991; During and Spencer, 1993; Scanziani, 2000).

2.4.1 Mechanism of $GABA_B$ receptor modulation of $GABA_A$ currents

Several lines of evidence indicate that $GABA_A$ currents were modulated by postsynaptic $GABA_B$ receptors. The use of exogenous GABA to activate $GABA_A$ currents would minimize effects of altered GABA release due to presynaptic $GABA_B$ activation, and $GABA_B$ effects were seen after inhibition of vesicular GABA release with ConA. Altered GABA uptake caused by presynaptic $GABA_B$ receptor activation could potentially account for our observations. However, this is unlikely because $GABA_B$ effects were seen in the presence of the GAT1 antagonist SKF. Additionally, currents produced by the nontransported $GABA_A$ agonist THIP were potentiated by baclofen, indicating that $GABA_B$ receptors enhance $GABA_A$ currents independently of GABA uptake mechanisms (including GAT3). Finally, selectively inhibiting postsynaptic G proteins by including GDP-β-S in our whole-cell pipette solutions blocked the effects of $GABA_B$ receptor activation, confirming a postsynaptic site of action.

Our data show that tonic currents caused by ambient GABA are modulated by postsynaptic $GABA_B$ receptors. Experiments using the delta subunit-selective agonist THIP (Mortensen et al., 2010; Meera et al., 2011) confirmed that receptors containing delta subunits were potentiated by $GABA_B$ receptors. $GABA_B$ receptor activation did not

change sIPSC amplitude or kinetics but reduced sIPSC frequency, indicating that postsynaptic $GABA_B$ receptors did not modulate $GABA_A$ receptors activated at synapses. Because a large number of "synaptic-type" $GABA_A$ receptors (i.e., delta subunit containing) are located away from synapses in extra-synaptic membranes (near postsynaptic $GABA_B$ receptors) (Farrant and Nusser, 2005), our data do not exclude the possibility that $GABA_B$ receptors modulate these types of $GABA_A$ receptors in addition to delta subunit-containing receptors.

Signals mediated by postsynaptic $GABA_B$ receptors are transduced by the G proteins $G_{\alpha i}/G_{\alpha o}$ and $G_{\beta\gamma}$ (Padgett and Slesinger, 2010). A downstream effect of $G_{\beta\gamma}$ is K^+ channel activation; however, this is unlikely to have contributed significantly to our results because control currents and currents in the presence of baclofen were both inhibited >90% by $GABA_A$ antagonists, and intracellular QX314 effectively blocks $GABA_B$-activated K^+ channels (Nathan et al., 1990; Andrade, 1991). Activation of G_i/G_o inhibits adenylate cyclase, with subsequent reduction in cAMP levels and reduced activation of PKA. Our data show that inhibition of G-protein signaling prevented $GABA_B$ receptor modulation of $GABA_A$ currents. The final effect on $GABA_A$ receptors is not known, but the possibilities include increased single channel conductance, open probability, or surface expression. Future studies using different experimental methods are required to distinguish between these possibilities.

2.4.2 Cell type specificity of the postsynaptic $GABA_B$–$GABA_A$ interaction

In DGGCs, activation of $GABA_B$ receptors substantially increased $GABA_A$ currents by 83% on average. This strong modulation was absent in CA1 pyramidal neurons and

cortical layer 2/3 pyramidal neurons. One salient difference between DGGCs and CA1 pyramidal cells is that tonic inhibition in CA1 pyramidal cells is mediated by alpha 5 subunit-containing $GABA_A$ receptors, whereas delta subunit-containing receptors are dominant in DGGCs (Caraiscos et al., 2004; Glykys et al., 2008). In layer 2/3 pyramidal neurons there is little, if any, functional expression of delta subunits (although delta subunit mRNA is present) (Yamada et al., 2007). Thus, we speculate that signals produced by postsynaptic $GABA_B$ receptors may preferentially affect delta subunits. Our results showed that baclofen did not affect sIPSC characteristics but potentiated tonic currents and THIP-evoked currents, consistent with a selective modulation of delta subunit-containing receptors by postsynaptic $GABA_B$ receptors. However, other factors in addition to differences in $GABA_A$ receptor subunit expression may also contribute to the cell type specificity of $GABA_B$ receptor effects.

2.4.3 Functional implications

Tonic inhibition affects neuronal excitability and network behavior (Chadderton et al., 2004; Glykys and Mody, 2006; Pavlov et al., 2009; Duguid et al., 2012), thereby influencing many physiologic and pathophysiologic processes, including synaptic plasticity and epileptic seizures (Maguire et al., 2005; Martin et al., 2010). The effect of tonic inhibition is generally inhibitory, but in some cell types (i.e., thalamocortical cells) increasing tonic inhibition is maladaptive and contributes to pathologic patterns of neuronal firing (Cope et al., 2009). Our results add to the ways that tonic inhibition can be modulated, including GABA uptake and release by transporters, drugs that increase GABA concentrations, voltage-dependent modulation of extrasynaptic $GABA_A$ receptors, and neurosteroids (Overstreet and Westbrook, 2001; Nusser and Mody,

2002; Stell et al., 2003; Wu et al., 2003; Pavlov et al., 2009; Ransom et al., 2010). The enhancement of tonic inhibition by postsynaptic $GABA_B$ receptors described here is predicted to reduce cellular excitability of DGGCs by shunting excitatory synaptic currents and raising action potential threshold (Stell et al., 2003; Holter et al., 2010; Arima-Yoshida et al., 2011; Gupta et al., 2012).

Chapter 3

Molecular Mechanisms Responsible for Potentiation of $GABA_A$ Current by $GABA_B$ Receptor Activation

3.1 Introduction

In the second chapter, I described the experiments that led to the conclusion that postsynaptic $GABA_B$ receptor activation increased tonic $GABA_A$ current. In this chapter, I will present experiments aimed at determining the molecular mechanisms responsible for the potentiation of $GABA_A$ current by $GABA_B$ receptor activation. Several possibilities may be responsible for this increase of $GABA_A$ current, including a change in single channel properties and/or a change in the number of $GABA_A$ receptors in the cell surface membrane. Thus, in this chapter, I will examine whether $GABA_B$ receptor activation affects single channel properties of $GABA_A$ receptors, $GABA_A$ receptor protein synthesis, or membrane trafficking of $GABA_A$ receptors.

3.2 Materials and Methods

3.2.1 stationary noise analysis

Whole-cell $GABA_A$ currents evoked by puffing GABA (using the same protocol as described in Chapter 2 and shown in Figure 2.1) were used for stationary noise analysis (Neher and Stevens, 1977; De Koninck and Mody, 1994). A segment of fixed length, typically 500 ms, was selected from the peak of the evoked current to the end of the decay. To avoid the contamination of sIPSC, segments containing sIPSC were

discarded. Within each segment, the mean amplitude and the variance were calculated. By plotting the mean current amplitude against its variance, and fitting this curve with equation (1), we can get the single channel current (slope).

For a cell containing N channels that each pass a current of amplitude i when open, with open probability p, the mean current I is:

$I = Npi;$

and the variance is

$\sigma^2 = i^2 Np(1–p)$

combining these two equations and substituting for p yields:

$\sigma^2 = iI–(I^2/N)$

At low open probability condition, the relationship between variance and mean current I is approximately linear, so we get

$\sigma^2 = iI$ (1)

Based on Ohm's law, we can calculate the single channel conductance:

$g = i/(Vm-Vre)$

where Vm = -60 mV, Vre of Cl = 0 mV.

Power spectral analysis

Power spectral of current trace was computed by fast Fourier transform (after subtracting the corresponding average spectral of baseline noise) and then it was fit by a single normalized Lorentzian equation:

$$S(f) = S(0) / [1+(f/f_c)^2]$$

Where S (f) is the spectral density at frequency f, S (0) is the spectral density at zero frequency, and f_c is the cut off frequency at which the spectral density is half of S (0). The corresponding time constants (tau) are obtained:

$$tau = 1 / (2\pi f_c)$$

At low p (open probability), the forward rate constant is negligible and tau is simply the reciprocal of the closing rate or the mean channel open time. Thus, we can calculate the single channel kinetics of $GABA_A$ receptors.

3.2.2 Brain slice preparation

The procedure of brain slice preparation here is the same as described in Chapter 2.2.1.

3.2.3 Electrophysiology recordings

The procedure of electrophysiology recordings here is the same as described in Chapter 2.2.2.

3.2.4 Biotinylation

Surface expression of delta subunits of $GABA_A$ receptors in acutely obtained hippocampal slices was examined as described in Goodkin et al., (2008). Briefly, hippocampal slices from each rat were pooled together, and incubated in 2 mg/ml sulfo-NHS-SS-biotin solution (Pierce Biotechnology, Rockford, IL, USA) for 30 min at 4 ℃ with gentle shaking. Unbound biotin was removed by Tris-buffered saline with Tween-20 (TBST, 25 mM Tris-Cl, 125mM NaCl, 0.1%Tween-20, PH 7.4). Slices were then homogenized and sonicated in lysis buffer (50 mM Tris-HCL, 150 mM Nacl, 1 mM EDTA, 1% NP-40). The non-soluble fraction was removed after centrifugation of the lysate at 13000 g for 30 min at 4 degree. The protein concentration was then measured with a BCA method and 30 µg total protein was used to calculate the surface/total protein ratio. The rest of the supernatant was incubated in neurtravidin-agarose beads (Pierce Biotechnology, Rockford, IL, USA) overnight at 4 ℃, to isolated biotin-labeled proteins. The pull-down proteins were eluted in a non-reducing sample buffer for 5 min at 95 ℃ following three washes in lysis buffer.

3.2.5 Western blot

The proteins were separated by 10% SDS-PAGE (sodium dodecyl sulfate ploy acrylamide gel electrophoresis) gels and transferred to hy-bond-P polyvinylidine fluoride (PVDF) membranes (Amersham Bio-Sciences, Piscataway, NJ, USA). The membranes were blocked in 5% non-fat dry milk in TBST for 2 hours at room

temperature, and then incubated with antibodies (anti-delta subunit antibody, Santa Cruz Biotechnology, Santa Cruz, CA; anti-gamma2 antibody, PhosphoSolutions, Aurora, CO) overnight at 4 ℃ with gentle shaking. After that, the membrane was incubated with secondary antibody (anti-rabbit IgG-AP, Santa Cruz Biotechnology, Santa Cruz, CA) and bands were detected by ECF detection kit (GE Healthcare, Little Chalfont, UK), exposed to x-ray film under nonsaturating conditions, and imaged with the Bio-Rad gel imaging system. To insure the absence of contamination of the surface pool of protein with the intracellular pool of protein and the same amount of proteins loaded in different lanes, the membranes were reprobed with GADPH antibody (Fisher Scientific, Pittsburgh, PA). The signal intensity was determined by densitometric scanning of the western blots. Total expression of the delta subunits was normalized with GADPH expression, and surface expression was normalized to total protein expression.

3.2.6 Hippocampal neuronal cultures and transfection

Hippocampal neurons were isolated from E18 rat fetuses, plated on poly-D-lysine coated cover glass with a density of 0.2×10^5 cells per cm^2. Neurons were grown in vitro for 7 days then were collected for DNA transfection. cDNA of rat super-ecliptic phluorin (SEP)- tagged delta subunits (gift from Dr. Trevor G. Smart, U of College London) and SEP-tagged alpha1 subunits (gift from Dr. Tija C. Jacob, U of Pittsburgh) were used in this study (Bright and Smart, 2013). Cultured hippocampal neurons were transfected with SEP-tagged delta subunits or SEP-tagged alpha1 subunits by chemical method (Lipofectaimin LTX and Plus, Invitrogen). After 1-2 days, the cells were used for live-cell

imaging and electrophysiology recordings.

3.2.7 Live-cell imaging

Transfected neurons were perfused with external solution (bubbled with 5% CO_2 and 95% O_2, pH = 7.3-7.4) at room temperature. To confirm the pH sensitivity of SEP-delta subunits, the quench solution with pH = 5.5 used in this study was made from external solution except replacing equal amount of $NaHCO_3$ with 4-Morpholineethanesulfonic acid sodium salt (MES).

To image fluorescence, two-photon laser-scanning microscopy (TPLSM) was used in this study. The cell body of the neuron was chosen for data acquisition. Single plane images were collected on a custom-built instrument based on a Fluoview laser-scanning microscope (Olympus America, Melville, NY). The light source was a mode-locked Ti: sapphire laser (Mira 900F; Mira, Santa Clara, CA) running at 910 nm. We used a LUMPlanFI/IR 40-0.80 numerical aperture objective. The images were analyzed by Image J software. The average fluorescence intensity of the soma of the neuron was calculated after subtraction of background fluorescence. To insure the consistency of imaging focus plane over time, we also include Alexa 594 dye (0.5 μM) in the pipette solution.

3.2.8 Statistical analysis

Data analysis was performed with Clampfit (pClamp 10), Origin (v6.1, Microcal Software) and Matlab (R2012a, MathWorks, Inc) software. Data are presented as mean ± standard error of the mean (SEM), and all error bars represent SEM. Statistical

analyses were performed using Microsoft Excel. A two-tailed, paired or homoscedastic Student's *t* test was used with a *p* value ≤ 0.05 considered as significant.

3.3 Results

3.3.1 $GABA_B$ receptor activation did not change single-channel current or channel kinetics of $GABA_A$ receptors

The enhancement of $GABA_A$ current by $GABA_B$ receptor activation has been reported in Tao et al., 2013, but the molecular mechanisms for this modulation remain unclear. Several possibilities may be responsible for this increase of $GABA_A$ current, including a change in single-channel properties and/or a change in the number of $GABA_A$ receptors in the cells surface membrane. We first used stationary noise analysis to examine whether $GABA_B$ receptor activation affected the single-channel properties of $GABA_A$ receptors. Figure 3.1 shows that bath application of the $GABA_B$ receptor agonist baclofen, did not have a significant effect on single channel $GABA_A$ current (control, 1.2 ± 0.03 pA vs baclofen, 1.3 ± 0.07 pA; n = 6, p = 0.68) (calculation according to Equation 1 in Methods). According to Ohm's law, we calculated the conductance (control, 20.7 ± 0.6 pS vs baclofen, 21.3 ± 1.2 pS, n = 6, p = 0.68) and these conductance were well within the range of $GABA_A$ conductance reported by different groups (around 20-25 pS; see noise analysis in De Koninck and Mody, 1994; see single channel recordings in Brickley et al., 1999), confirming the validity of stationary noise analysis in this study. Also, we found no significant change in the mean open lifetime of $GABA_A$ receptors after the application of baclofen (control, 15.9 ± 2.3 ms vs baclofen, 17.4 ± 3.5 ms; n = 7, p =

0.59) (Figure 3.1 F) (calculation according to equations in Methods, power spectral analysis) Thus, these results suggested that $GABA_B$ receptor activation did not change single-channel current or channel kinetics of $GABA_A$ receptors.

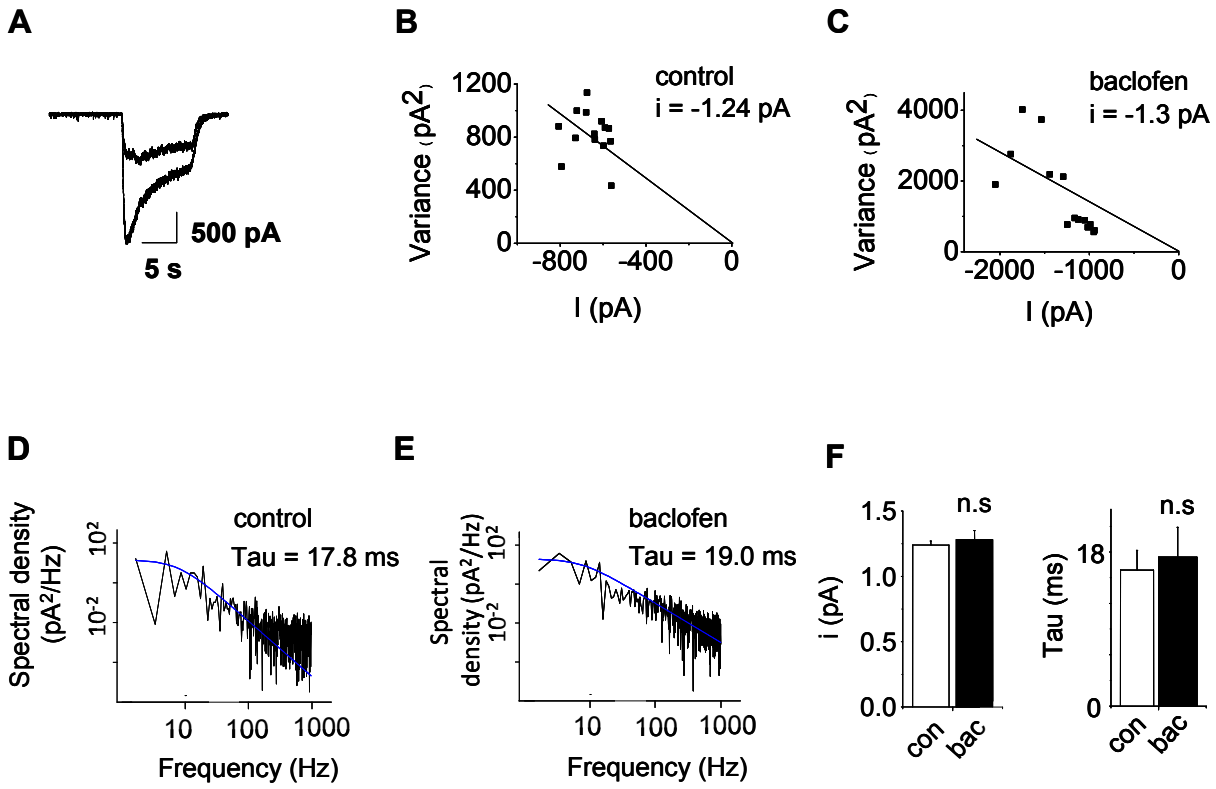


Fig 3.1 Baclofen did not change single-channel current or channel kinetics of $GABA_A$ receptors. **(A)** Representative $GABA_A$ currents elicited by exogenous GABA (10 μM) were used for stationary noise analysis; **(B, C)** single-channel current estimated from noise analysis in control and baclofen (20 μM) groups; **(D, E)** Mean open lifetime estimated from spectral density in control and baclofen groups; **(F)** Summary data of single channel current (left) and mean open life time (tau, right). V_m = -60 mV (E_{Cl} = 0 mV). *p < 0.05; **p < 0.01; n.s. nonsignificant. Error bars are SEM, and horizontal bars indicate the period of drug application.

3.3.2 $GABA_B$ receptor activation did not change total protein expression of delta subunits of $GABA_A$ receptors, but increased surface expression of delta subunits of $GABA_A$ receptors

Stationary noise analysis indicated that some other mechanisms might contribute to the enhancement of $GABA_A$ current by baclofen. One of the mechanisms could be that baclofen increases protein synthesis of delta subunits. To test this idea, we measured the total protein expression of delta subunits of $GABA_A$ receptors by western blot. From Fig 3.2, we found that baclofen did not have significant effect on the total protein expression of delta subunits of $GABA_A$ receptors (99.2 ± 5 % of control, n = 4, p = 0.5). This propelled us to examine another possibility that baclofen may change only the surface expression of delta subunits of $GABA_A$ receptors without changing the synthesis of delta subunits of $GABA_A$ receptors. To examine this, we measured the surface expression of delta subunits of $GABA_A$ receptors by biotinylation assay. Figure 3.2 showed that surface expression of delta subunits of $GABA_A$ receptors increased significantly after the application of baclofen (190 ± 23% of control, n = 4, $p < 0.05$). To test whether the baclofen effect was specific to delta subunits, we also measured the effect of baclofen on gamma 2 subunits, which is the major component of synaptic $GABA_A$ receptors. We found that neither surface nor total protein expression of gamma2 subunits of $GABA_A$ receptors were significantly changed by baclofen (total protein expression of gamma2: 115 ± 6% of control, n = 4, p = 0.1; surface expression of gamma2: 90 ± 7% of control, n = 4, p = 0.08), indicating that $GABA_B$ modulation is specific to extrasynaptic $GABA_A$ receptors but not to synaptic $GABA_A$ receptors.

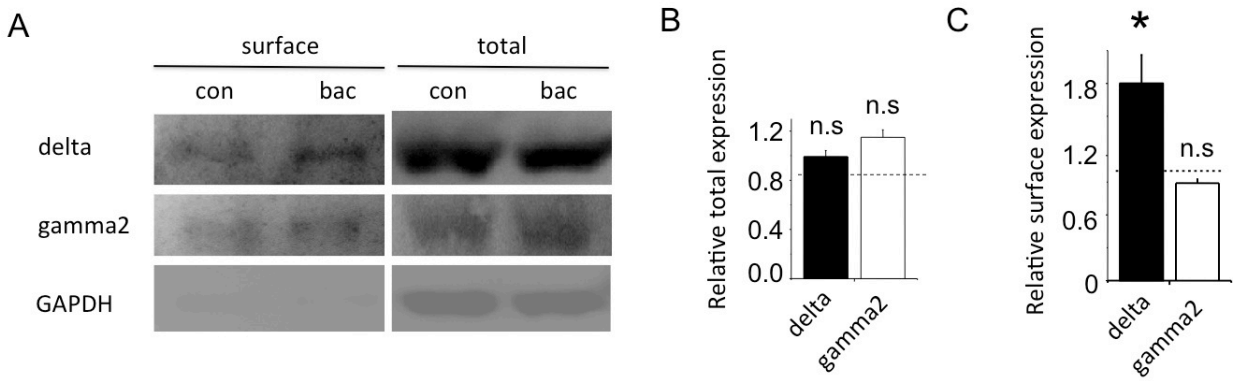


Fig 3.2 $GABA_B$ receptor activation increased surface expression of delta subunits of $GABA_A$ receptors in hippocampal slices. **(A)** Representative western blot samples from control and baclofen groups (20-25 min treatment of 20 μM balcofen) using indicated anti-bodies; **(B, C)** Summary data of relative change in total and surface expression of delta and gamma2 subunits by baclofen.

To further confirm this result, live-cell imaging of SEP-tagged delta subunits of $GABA_A$ receptors over-expressed in cultured hippocampal neurons were used to examine the surface expression of delta subunits. Consistent with the whole-cell recordings from DGGC in slices (Tao et al., 2013), we found that baclofen also increased the $GABA_A$ current in transfected cultured neurons (200 ± 40% of control, n = 3, $p < 0.05$) (Fig 3.5), indicating that the cultured cell system could be used to study the $GABA_B$ modulation of $GABA_A$ receptors. The SEP moiety is a pH sensitive variant of GFP that displays bright fluorescence emission at pH> 6.0 (e.g., when tagged delta subunit is in plasma membrane, the pH sensitive GFP tag is exposed to the extracellular fluid), while at pH< 6.0 (e.g., in an intracellular compartment) it displays little emission. Thus, by monitoring the fluorescence intensity, we can infer the surface expression of proteins. To confirm

the validity of this method, we replaced the external solution with pH 5.5 MES solution (see Methods) for 5 to 10 min and found that the majority of fluorescence was quenched (relative change: 30 ± 3% of control, n = 11, $p < 0.01$) (Fig 3.3 A, B), indicating that the majority of the fluorescence was from the surface expression of proteins. Using this assay, we found that baclofen increased fluorescence intensity of the cell body significantly (143 ± 1% of control, n = 6, $p < 0.01$) (Fig 3.3 C, D); also, this effect was blocked by $GABA_B$ receptor antagonist, CGP 55845 (100 ± 3% of control, n = 5, $p = 0.8$) (Fig 3.3 E, F), indicating the effect of baclofen is specific to $GABA_B$ receptors. We also found that CGP55845 did not change basal surface expression of delta subunits (104 ± 1%, n = 5, $p = 0.2$) (Fig 3.3 G), which is consistent with the idea that $GABA_B$ receptors are not activated at resting condition (Tao et al., 2013; Scanziani, 2000).

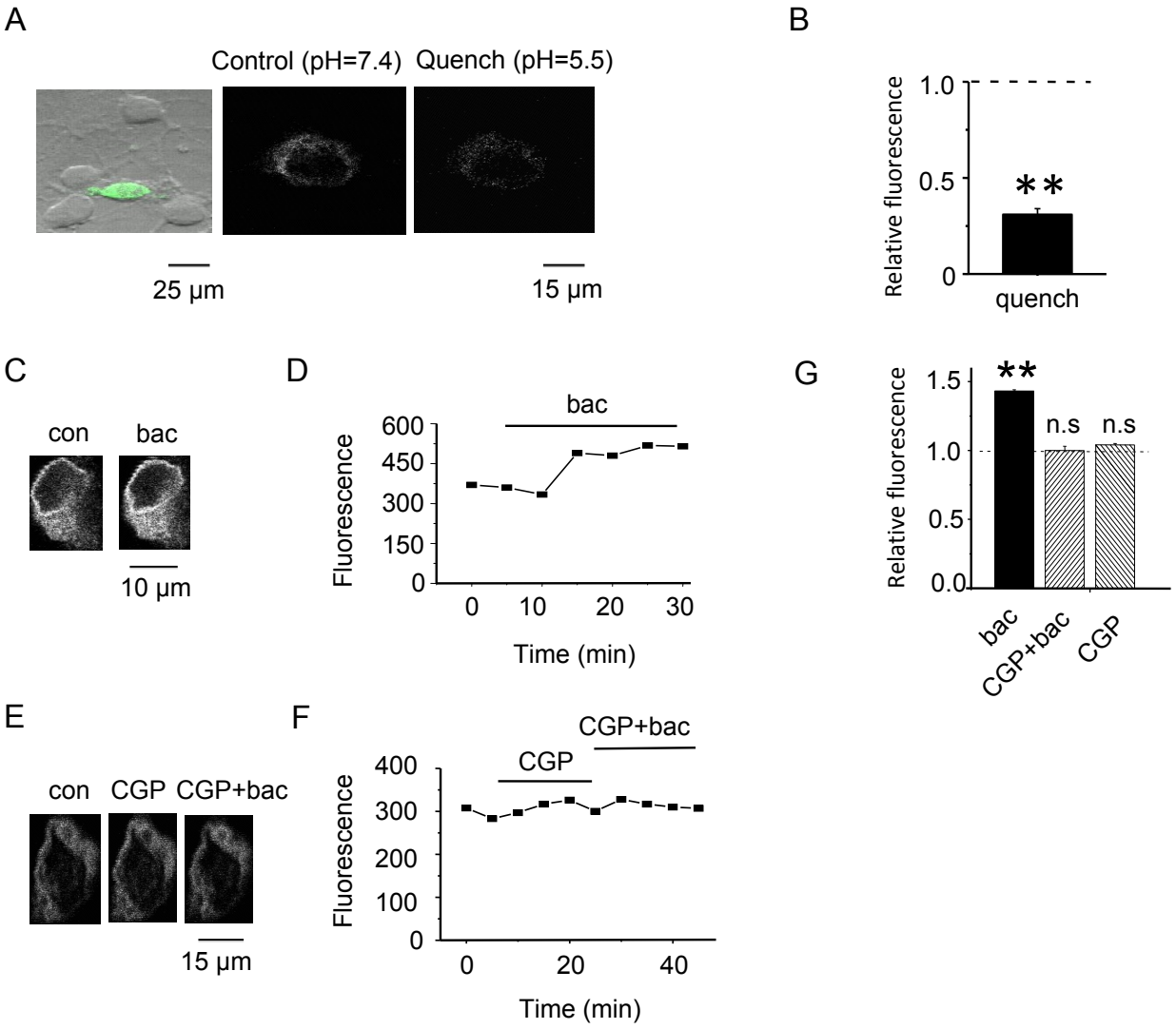
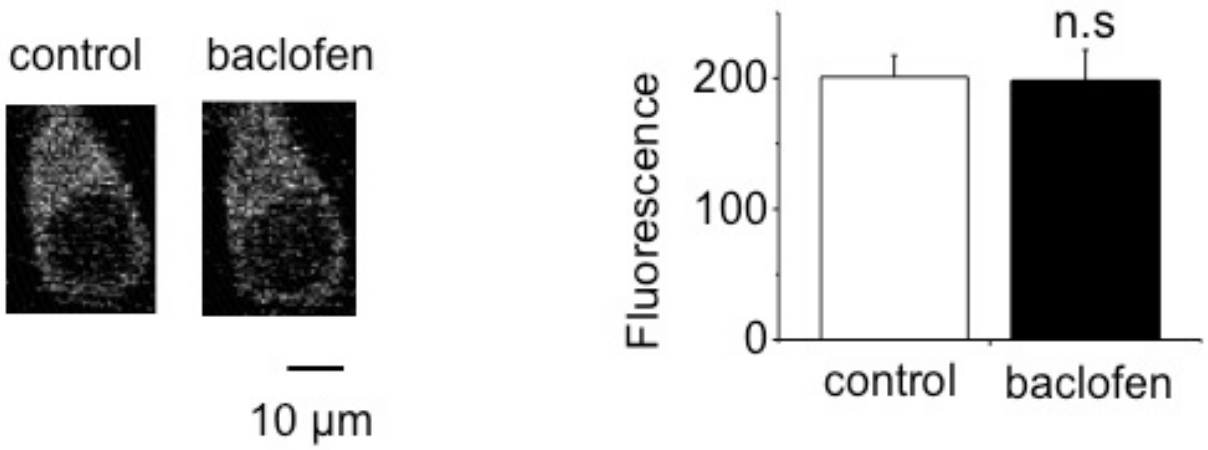


Fig 3.3 $GABA_B$ receptor activation increased surface expression of delta subunits of $GABA_A$ receptors in cultured hippocampal neurons. **(A)** Representative pictures showing cultured hippocampal neurons transfected with SEP-tagged delta subunits (green cell, left panel); Representative pictures of live-cell imaging of SEP-tagged delta subunits of $GABA_A$ receptors, in the presence of control solution (ACSF, pH = 7.3-7.4, middle panel), and quench solution (normal ACSF solution, except equal amount of $NaHCO_3$ was replaced by MES, pH = 5.5, right panel); **(B)** summary data of relative change in fluorescence intensity after quench treatment. **(C)** Representative pictures

from live cell imaging of SEP-tagged delta subunits of $GABA_A$ receptors, during application of control solution (left panel), baclofen solution (dissolved in ACSF, 20 μM, pH = 7.3-7.4, middle panel), quench solution (right panel); **(D)** Time course of fluorescence intensity before and after baclofen application for cell showed in (C); **(E)** Representative pictures from live cell imaging of SEP-delta, during the application of control, CGP (1 μM), CGP (1 μM) plus baclofen (20 μM), quench solution; **(F)** Time course of experiment; **(G)** Relative change of SEP-delta fluorescence intensity in different treatment groups.

To test whether the baclofen effect was specific to delta subunits, we examined the effect of baclofen on SEP-alpha1 subunits (Fig 3.4). We found that surface expression of SEP-alpha1 subunits was not significantly affected by baclofen (98 ± 5%, n = 3, p = 0.84). Interestingly, we found that the increase of delta subunit surface expression and the increase of $GABA_A$ current by baclofen occurred with similar time course/ simultaneously (fluorescence intensity change: 200 ± 40% of control, n = 3, $p < 0.05$; $GABA_A$ current change: 150 ± 7% of control, n = 3, $p < 0.01$) (Fig 3.5). These results not only confirmed the biochemistry data that $GABA_B$ receptor activation increased surface expression of delta subunits of $GABA_A$ receptors, but also indicated that the increase of surface expression of delta subunits of $GABA_A$ receptors is most likely the reason for the increase of $GABA_A$ current.



(A) **(B)**

Fig 3.4 Baclofen did not increase surface expression of SEP-tagged alpha1 subunits of $GABA_A$ receptors. **(A)** Representative pictures of live-cell imaging of SEP-tagged alpha1 subunits of $GABA_A$ receptors, in the presence of control solution, and quench solution; **(B)** summary data of fluorescence intensity after baclofen treatment (20 μM, 20 min)

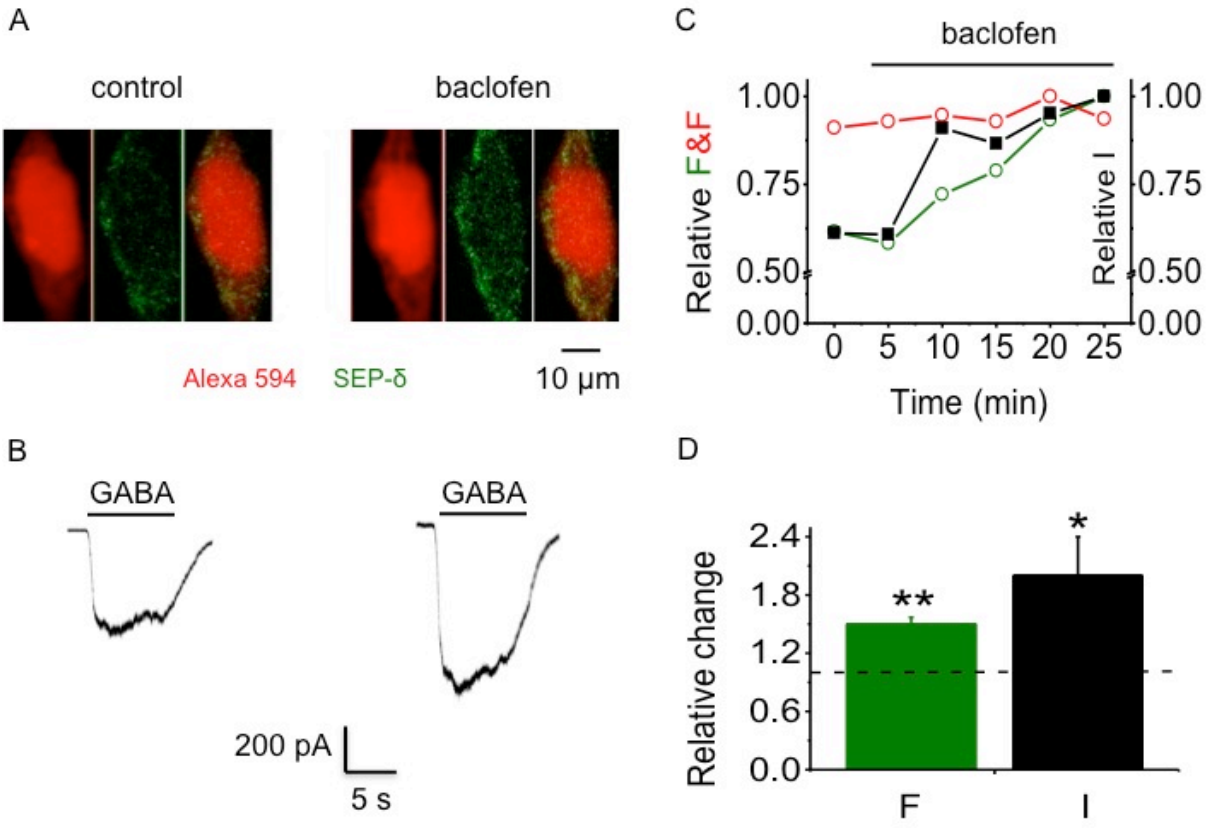


Fig 3. 5 Simultaneous increase of $GABA_A$ current and surface expression of delta subunits by $GABA_B$ receptor activation. **(A, B)** Simultaneous recording $GABA_A$ current and imaging of SEP-tagged delta subunits of $GABA_A$ receptors before (left panel) and after application of baclofen (right panel, 20 μM, 20 min treatment); Alexa 594 (red) is included in pipette solution as a reference for focus plane. Note that red signal did not have significant change over time, but GFP fluorescence intensity increased after application of baclofen. **(C)** Time course of change in $GABA_A$ current and fluorescence intensity for cell showed in (A, B); **(D)** summary data of relative change in $GABA_A$ current and fluorescence intensity.

3.4 Discussion

In this chapter, we found that $GABA_B$ receptor activation did not change single-channel $GABA_A$ receptor current or channel kinetics, but we did not exclude the possibility that baclofen may change open probability of $GABA_A$ receptors. Thus, change in single-channel properties (P_{open}) may also contribute to the increase of $GABA_A$ current. Also, because our noise analysis is based on whole cell recordings of $GABA_A$ current rather than tonic current, the estimation of these single-channel properties reflects the channel properties contributed by mixture of both synaptic and extrasynaptic $GABA_A$ receptors. However, we think this is predominately contributed by extrasynaptic $GABA_A$ receptors based on the facts: 1) the number of extrasynaptic $GABA_A$ receptors is far larger than the number of synaptic $GABA_A$ receptors; 2) extrasynaptic $GABA_A$ receptors are less desensitized than synaptic $GABA_A$ receptors; 3) extrasynaptic $GABA_A$ receptors have much higher affinity for GABA than that of synaptic $GABA_A$ receptors (Mtchedlishvili and Kapur, 2005). Thus, when 10 μM GABA solution is puffed onto the soma of the DG, the actual concentration of GABA reaching soma is much lower than 1 μM and this lower concentration is more likely detected by large number of extrasynaptic $GABA_A$ receptors.

Further, we showed that baclofen did not change total protein expression of delta subunits, but increased the surface expression of delta subunits of $GABA_A$ receptors. This suggested that $GABA_B$ receptor activation affected turnover of $GABA_A$ receptors in the surface membrane rather than synthesis of $GABA_A$ receptors. Trafficking of receptor in and out of the surface membrane is a common mechanism used by neurons to

change synapse strength, such as long-term plasticity (Nicoll and Roche, 2013). Although the studies of $GABA_A$ receptor trafficking are not that extensively, compared with AMPA receptors, recent data suggest that both groups of receptors use the common themes to regulate the trafficking of receptors. First, the insertion of receptors from the intracellular pool of receptors at ER compartment are regulated by some interacting proteins, such as GABA associated proteins, NSF, and kinases. Specially, these kinases are targeted by adaptor proteins, which bind to both receptors and kinases. Once these receptors reach the plasma membrane, they are randomly distributed at extrasynaptic sites. On the other hand, the removal of receptors from plasma membrane is often associated with phosphatases and clathrin- and dynamin-dependent internalization. Beside these in- and out-strategy, the receptors at the plasma membrane are also regulated by anchoring proteins/scaffolding proteins, such as gephyrin, which target the receptors to the synaptic sites and make them stabilized. Moreover, the number of receptors at synapses is also regulated by ubiqui-tination. More recent data also suggest that some interacting proteins that bind cytoskeletal elements regulate the trafficking of receptors. In all, the trafficking of receptors is a highly dynamic process, regulated by many interacting proteins and can be modulate by neuronal activity, occurring at the range from minutes to days.

Chapter 4

The Signaling Pathways Involved in $GABA_B$ Modulation of $GABA_A$ Receptors

4.1 Introduction

$GABA_B$ receptor activation induces a classical signal pathway that involves $G_{i/o}$ α subunit that inhibits adenylyl cyclase (AC) and thus reduces protein kinase A (PKA) activity (Padgett and Slesinger, 2010). Activation of $GABA_B$ receptors also activates $G_{\beta\gamma}$ subunits to inhibit Ca^{2+} channels and open K^+ channels (Bowery et al., 2002). Further, in some preparations, some additional signal molecules have been reported to be involved in $GABA_B$ receptor activation, including protein kinase C (PKC) (Dutar and Nicoll, 1988); these indicate that $GABA_B$ receptor activation may engage multiple signal pathways. In this chapter, I will describe the possible signaling pathways involved in the $GABA_B$ modulation of $GABA_A$ receptor described in Chapters 2 and 3.

4.2 Materials and Methods

4.2.1 Brain slice preparation

The procedure of brain slice preparation here is the same as described in Chapter 2.2.1.

4.2.2 Electrophysiology recordings

The procedure of electrophysiology recordings here is the same as described in

Chapter 2.2.2.

4.2.3 Hippocampal neuronal cultures and transfection

The procedure of neuronal cultures and transfection here is the same as described in Chapter 3.2.6.

4.2.4 Live-cell imaging

The procedure of live cell imaging here is the same as described in Chapter 3.2.7.

4.2.5 Statistical analysis

Data analysis was performed with Clampfit (pClamp 10), Origin (v6.1, Microcal Software) and Matlab (R2012a, MathWorks, Inc) software. Data are presented as mean ± standard error of the mean (SEM), and all error bars represent SEM. Statistical analyses were performed using Microsoft Excel. A two-tailed, paired or homoscedastic Student's *t* test was used with a *p* value ≤ 0.05 considered as significant.

4.3 Results

4.3.1 PKA and Ca^{2+}-independent PKC signaling pathways are involved in $GABA_B$ modulation of $GABA_A$ currents

To further examine the molecular mechanism involved in $GABA_B$ modulation of $GABA_A$ current, we analyzed the signaling molecules involved in this modulation (Fig 4.1).

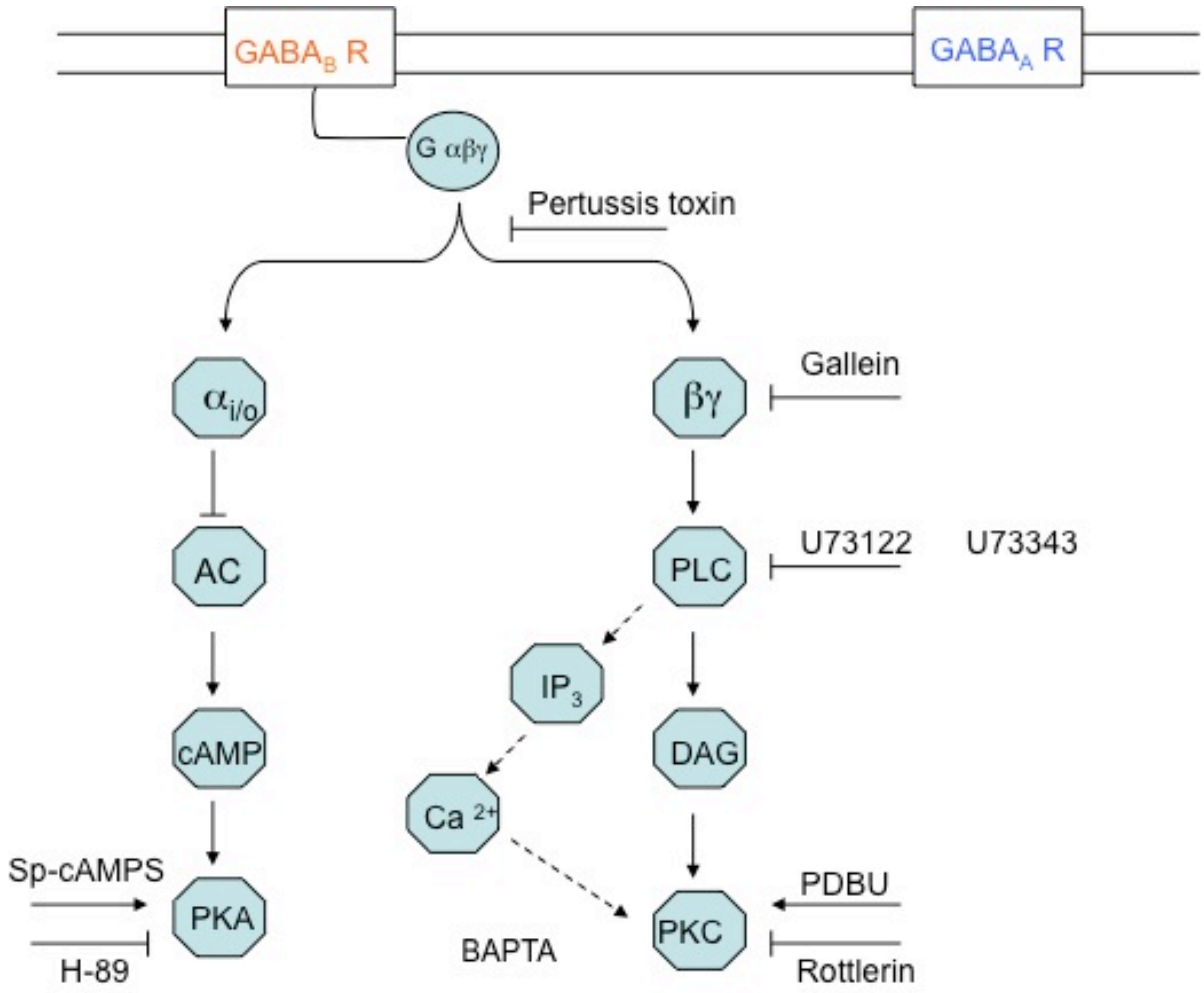


Fig 4.1 The potential signaling pathways involved in $GABA_B$ modulation of $GABA_A$ currents. Pharmacological agents tested in this study were shown in the signaling pathway diagram: $G_{i/o}$ specific inhibitor (pertussis toxin, PTX, 2 μg/ml, for 2 hours), PKA activator and inhibitor (Sp-cAMP, 10 μM; H-89, 10 μM; 30 min pretreatment), PKC activator and inhibitor (PDBU, 10 μM; rottlerin, 10 μM; 30 min pretreatment), $G_{\beta\gamma}$ specific inhibitor (gallein, 100 μM; 30 min pretreatment), PLC inhibitor (U73122, 10 μM; 30 min pretreatment), U73122 inactive analog (U73343, 10 μM; 30 min pretreatment), Ca^{2+} chelator (BAPTA, 10 mM in pipette solution).

We first tested the classical signaling pathway ($G_{i/o}$-cAMP-PKA) that is involved in $GABA_B$ receptor activation. In Figure 4.2, we found that pertussis toxin (PTX), a specific inhibitor of $G_{i/o}$ alpha subunits, blocked the effect of baclofen (96 ± 6% of control, n = 5, p = 0. 5), confirming that $G_{i/o}$ alpha subunits were involved in baclofen modulation. We

also found that PKA agonist Sp-cAMPS occluded and PKA antagonist H-89 blocked the baclofen effect (Sp-cAMPS: 97 ± 5% of control, n = 4, p = 0.4; H-89: 92 ± 4% of control, n = 5, p = 0.1) (Fig 4.3). This is consistent with the previous study that $GABA_B$ receptor activation involves $G_{i/o}$-PKA signaling pathway (Pinard et al., 2010).

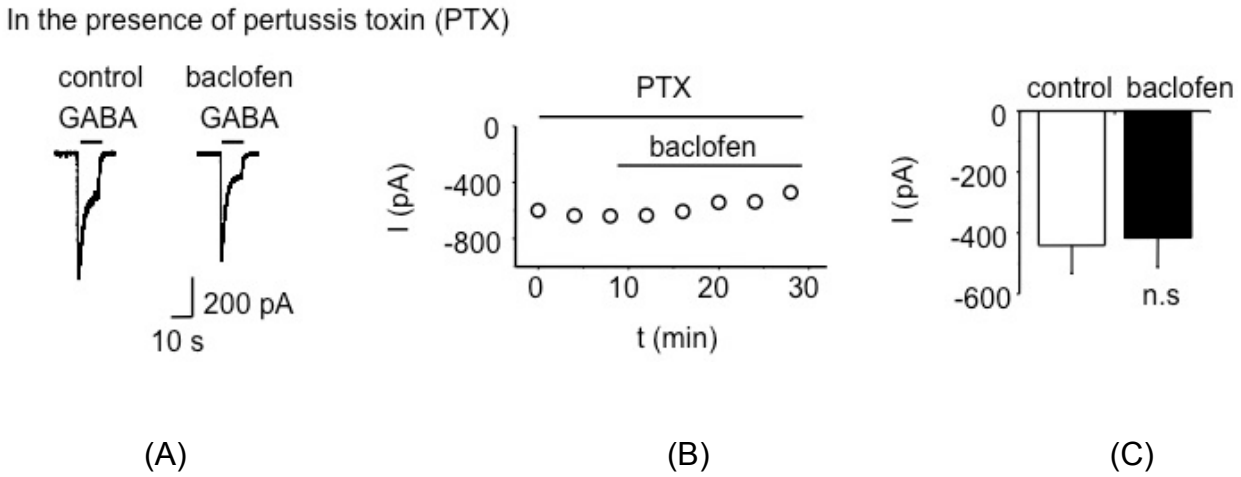


Fig 4.2 PTX (a specific inhibitor of $G_{i/o}$ alpha1 subunits) blocked the effect of baclofen on $GABA_A$ current. **(A)** Representative $GABA_A$ current traces before and after baclofen (20 μM, 20 min) treatment in the presence of PTX (2 μg/ml, 2 hours). **(B)** Time course of experiment for cell in (A); **(C)** summary data of baclofen effect on $GABA_A$ current in the presence of PTX.

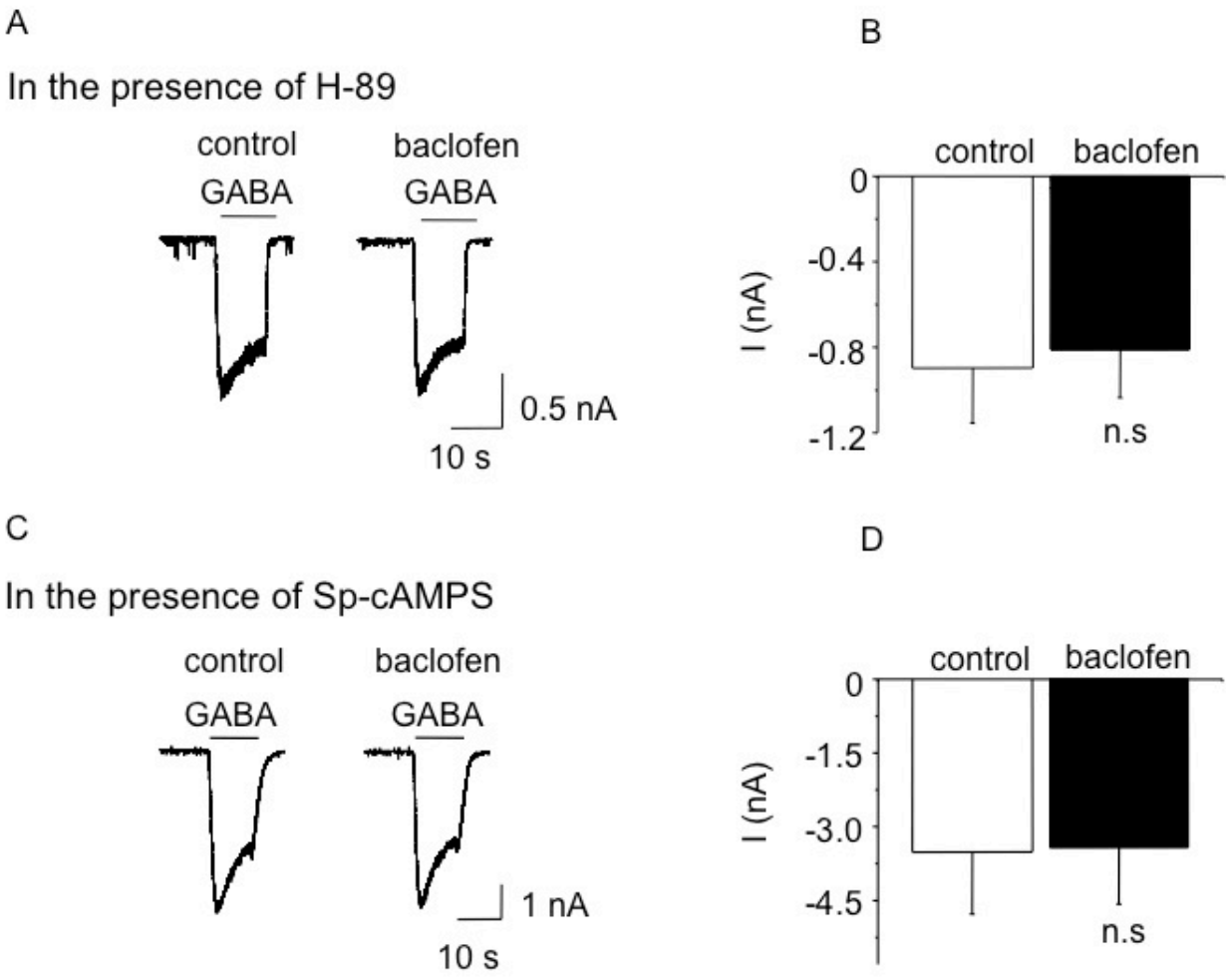


Fig 4.3 Pharmacological block and occlusion of PKA suppress $GABA_B$ modulation of $GABA_A$ current. **(A)** Representative $GABA_A$ current traces in DGGC (pretreated with 10 μM H-89 for 30 min) before and after application of baclofen (20 μM, 20 min); **(B)** Summary data of $GABA_A$ current before and after application of baclofen; **(C)** Representative $GABA_A$ current traces in DGGC (pretreated with 10 μM Sp-cAMPS for 20 min) before and after application of baclofen (20 μM, 20 min); **(D)** Summary data of $GABA_A$ current before and after application of baclofen.

Surprisingly, PKC was also implicated in this modulation (Fig 4.5): PKC activator PDBU occluded and PKC inhibitor rottlerin blocked the baclofen effects (PDBU: 87 ± 7% of control, n = 5, p = 0.1; rottlerin: 102 ± 7% of control, n = 6, p = 0.8). To further examine other signal molecules involved in this process, we tested whether specific $G_{\beta\gamma}$ inhibitor gallein can block the effect of baclofen. Fig 4.4 shows that, gallein blocked the baclofen

effect (91 ± 5% of control, n = 5, p = 0.2), indicating that $G_{\beta\gamma}$ subunits were involved in this modulation. PLC inhibitor U73122 also blocked the baclofen effects (95 ± 4% of control, n = 5, p = 0.4), and its inactive analogy U73344 did not block the baclofen effect (164 ± 14%, n = 4, p < 0.05), indicating that PLC were involved in this modulation (Fig 4.6). Consistent with blockade of the baclofen effect by rottlerin, which is a Ca^{2+} - independent PKC inhibitor, we found that loading of the Ca^{2+} chelator BAPTA (10 mM) in the pipette solution did not block the baclofen effects (190 ± 20% of control, n = 4, p < 0.05) (Fig 4.6). Thus, these results indicated the $GABA_B$ modulation of $GABA_A$ currents required both PKA and Ca^{2+} independent PKC signaling pathways. These results were also confirmed by live cell images (as described in Chapter 3): Pretreatment of both PKA inhibitor H-89 and PKC inhibitor rottlerin blocked the baclofen effects on increased surface expression of delta subunits (H-89: 92 ± 4% of control, n = 5, p = 0.1; rottlerin: 96.6 ± 6% of control, n = 5, p = 0.3) (Fig 4.7)

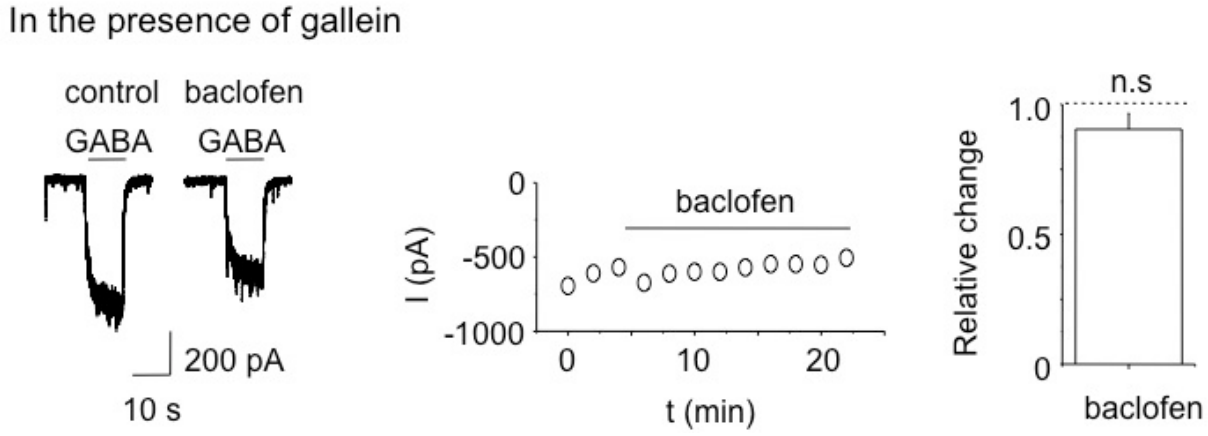


Fig 4.4 $G_{\beta\gamma}$ inhibitor gallein blocked the effect of baclofen on $GABA_A$ current. **(A)** Represetative $GABA_A$ current traces in DGGC (pretreated with 100 μM galleon for 30 min) before and after application of baclofen (20 μM, 20 min); **(B)** Time course of

experiment for cell in (A); **(C)** Summary data of relative change in $GABA_A$ current after application of baclofen.

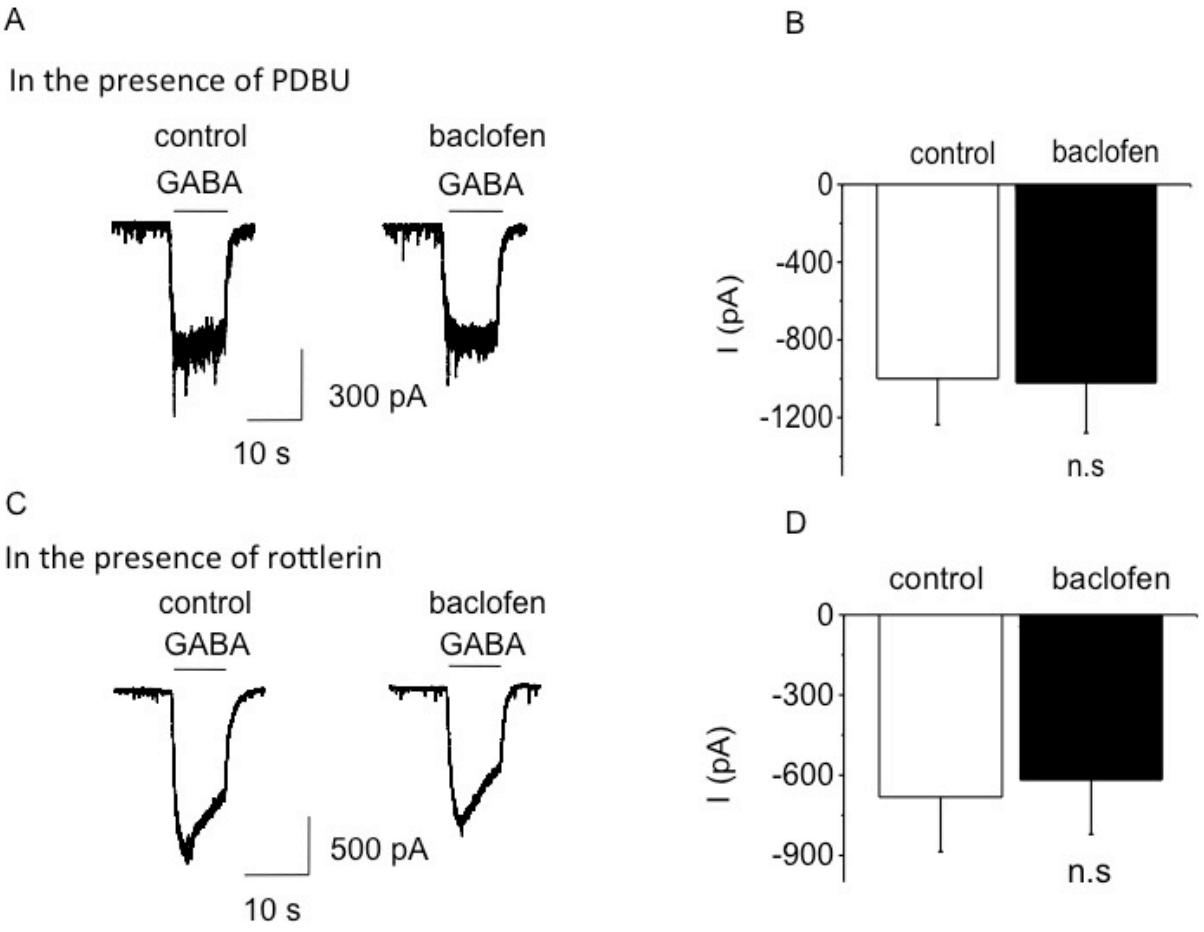


Fig 4.5 PKC was involved in $GABA_B$ modulation of $GABA_A$ current. **(A)** Representative traces of $GABA_A$ currents in DGGC (pretreated with 10 μM PDBU for 30 min) before and after application of baclofen (20 μM, 20 min); **(B)** Summary data of $GABA_A$ current before and after baclofen; **(C)** Representative traces of $GABA_A$ currents in DGGC (pretreated with 10 μM rottlerin for 30 min) before and after application of baclofen (20 μM, 20 min); **(D)** Summary data of $GABA_A$ current before and after baclofen.

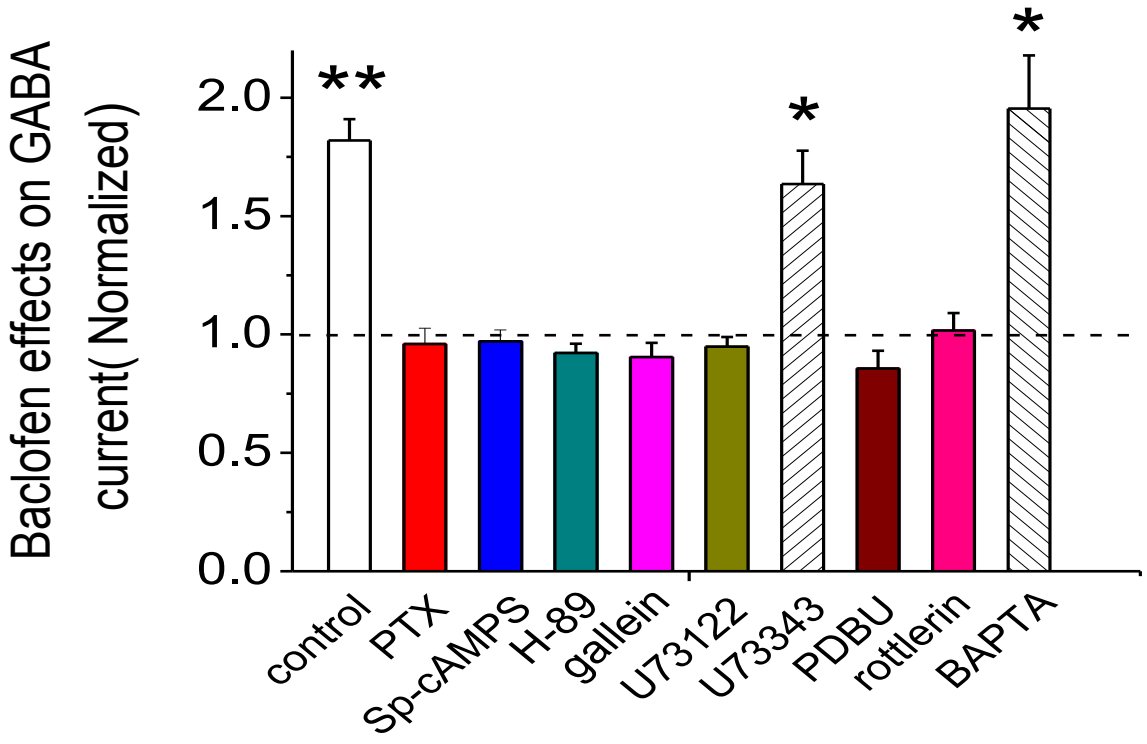


Fig 4.6 Summary of signaling pathways involved in $GABA_B$ modulation of $GABA_A$ current. Relative change of $GABA_A$ current during the application of solutions containing $G_{i/o}$ specific inhibitor (pertussis toxin, PTX, 2 μg/ml, for 2 hours), PKA activator and inhibitor (Sp-cAMP, 10 μM; H-89, 10 μM; 30 min pretreatment), PKC activator and inhibitor (PDBU, 10 μM; rottlerin, 10 μM; 30 min pretreatment), $G_{\beta\gamma}$ specific inhibitor (gallein, 100 μM; 30 min pretreatment), PLC inhibitor (U73122, 10 μM; 30 min pretreatment), U73122 inactive analog (U73343, 10 μM; 30 min pretreatment), Ca^{2+} chelator (BAPTA, 10 mM in pipette solution).

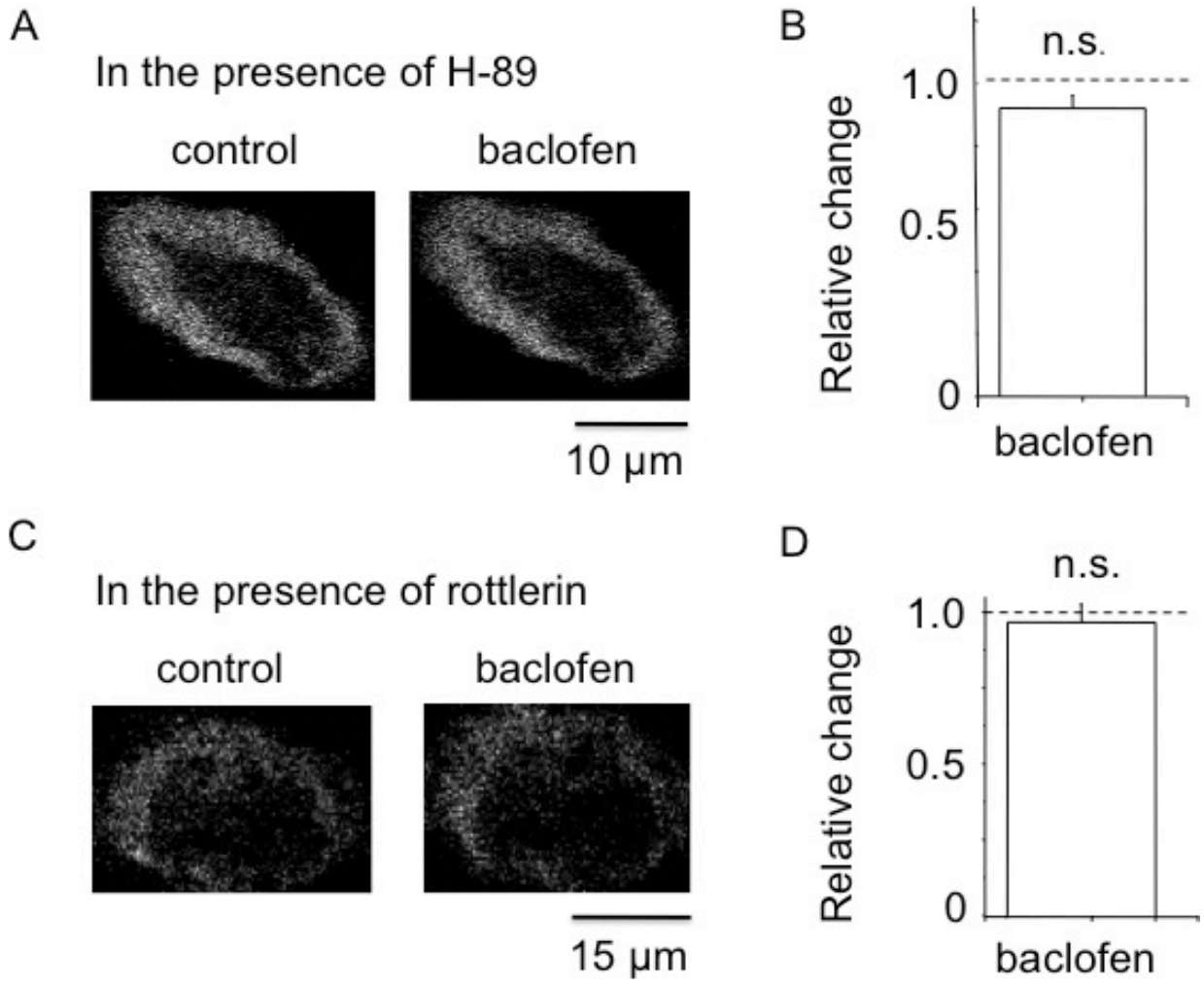
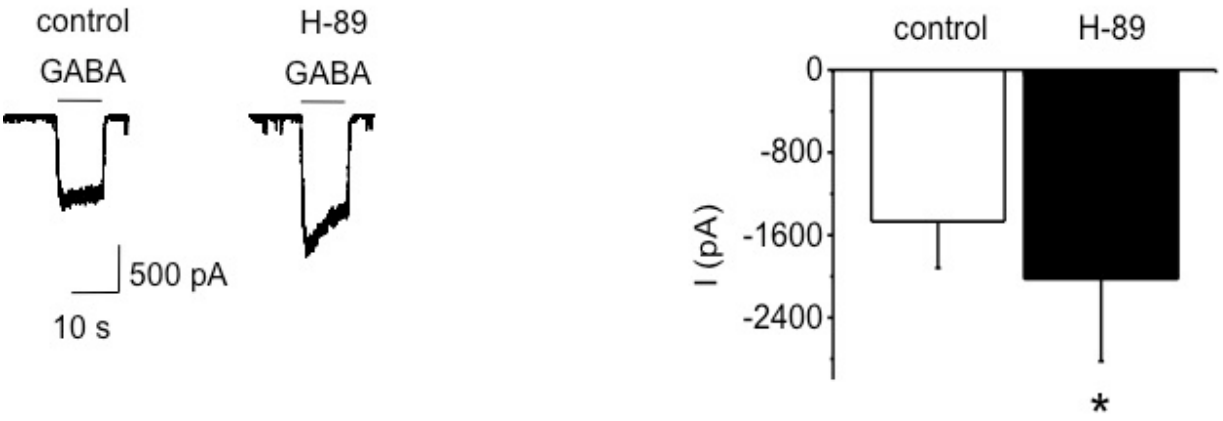


Fig 4.7 PKA and PKC were involved in $GABA_B$ modulation of delta subunit surface expression. **(A)** Representative pictures of live-cell imaging of SEP-delta subunits in cultured hippocampal neurons (pretreatment of 10 μM H-89 for 30 min) before and after application of baclofen (20 μM, 20 min); **(B)** Summary data of relative change in fluorescence intensity after application of baclofen; **(C)** Representative pictures of live cell imaging of SEP-delta subunits in cultured hippocampal neurons (pretreatment of 10 μM rottlerin for 30 min) before and after application of baclofen (20 μM, 20 min); **(D)** Summary data of relative change in fluorescence intensity after application of baclofen.

4.3.2 PKA and PKC differentially regulate tonic current

From the results above, we knew that PKA and PKC were involved in $GABA_B$ receptor activation, but the individual functional role of them remains unknown. Thus, we tested

their functional role individually. We found that PKA inhibitor H-89 increased $GABA_A$ current and tonic $GABA_A$ current ($GABA_A$ current: from −1468 ± 600 pA to −2022 ± 800 pA , n = 4, p < 0.05; tonic current: from −14 ± 5 pA to −24 ± 8 pA, n = 4, p < 0.05) (Fig 4.8;4.9); PKC inhibitor rottlerin reduced $GABA_A$ current and tonic GABA current evoked by SKF ($GABA_A$ current: from −1238 ± 440 pA to −899 ± 351 pA, n = 6, p < 0.05; tonic current: from −47.6 ± 15 pA to −25.8 ± 8 pA, n = 5, p < 0.05) (Fig 4.10; 4.11). Thus, these results indicated that PKA and PKC signaling pathways regulated extrasynaptic $GABA_A$ receptors in opposite directions.



(A) (B)

Fig 4.8 Tonic PKA activation reduced $GABA_A$ current. **(A)** Representative traces of $GABA_A$ currents before and after application of H-89 (10 μM, 20 min); **(B)** Summary data of $GABA_A$ current before and after H-89.

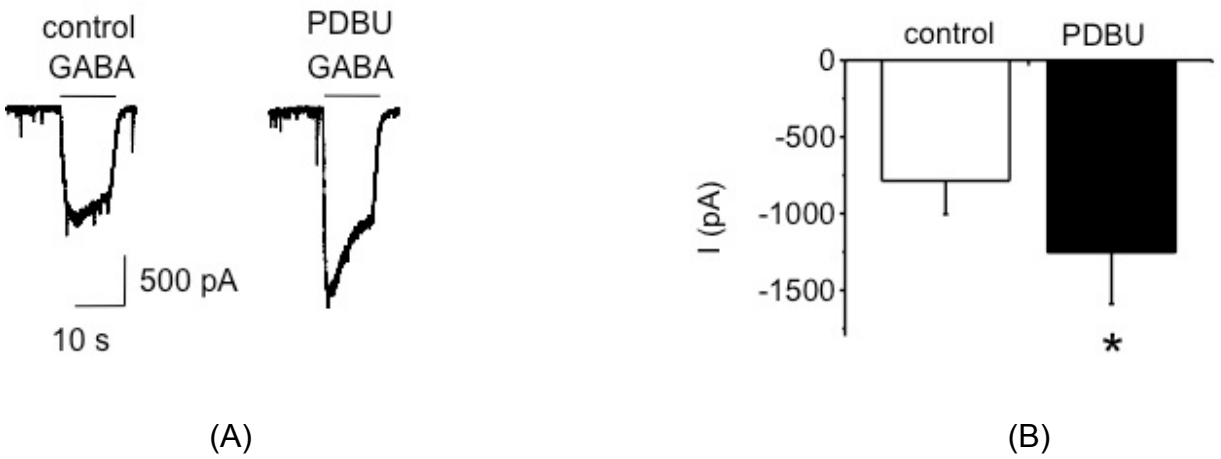


Fig 4.9 Tonic PKC activation increased $GABA_A$ current. **(A)** Representative traces of $GABA_A$ currents before and after application of PDBU (10 μM, 20 min); **(B)** Summary data of $GABA_A$ current before and after PDBU.

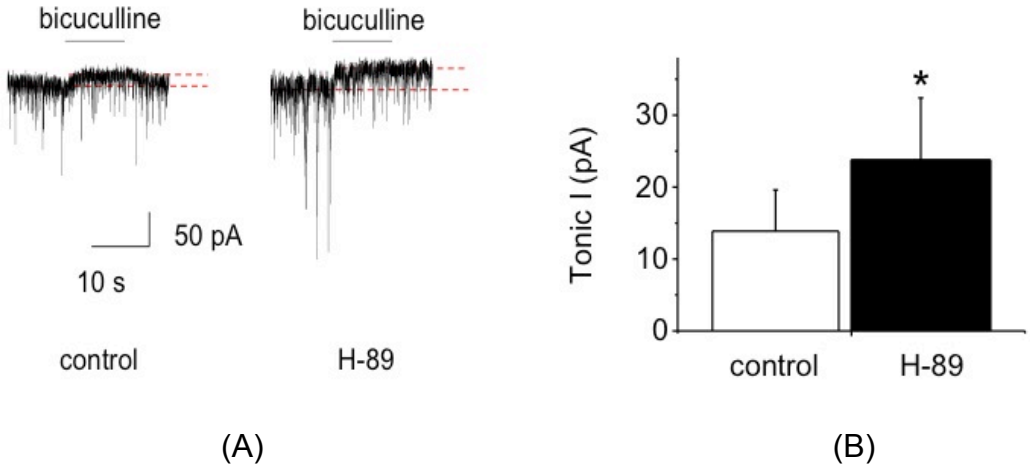


Fig 4.10 PKA activation reduced tonic GABA current. **(A)** Representative traces of tonic currents before and after application of H-89 (10 μM, 20 min); **(B)** Summary data of tonic current before and after H-89.

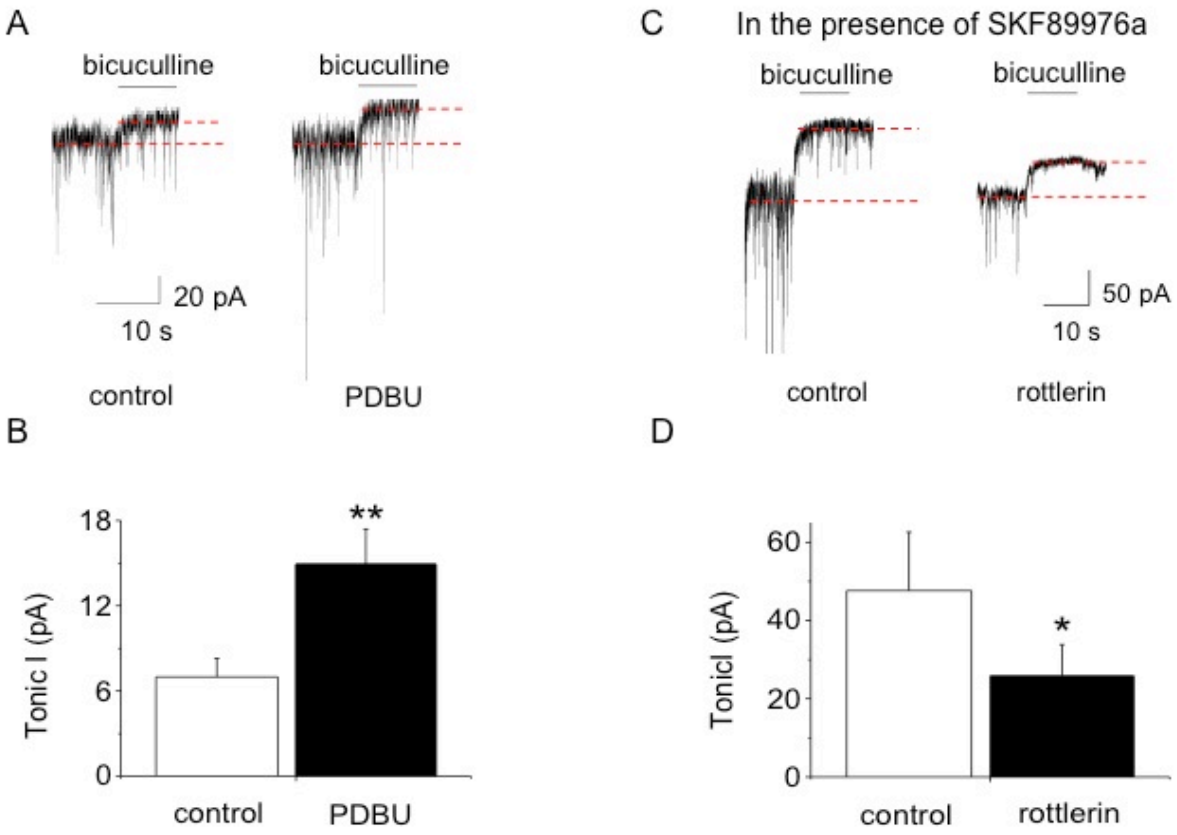


Fig 4.11 PKC activation increased tonic GABA current. **(A)** Representative traces of tonic currents before and after application of PDBU (10 μM, 20 min); **(B)** Summary data of tonic current before and after PDBU; **(C)** Representative traces of tonic currents before and after application of rottlerin (10 μM, 20 min); **(D)** Summary data of tonic current before and after rottlerin.

4.3.3 PKA and PKC differentially regulate membrane trafficking of delta subunits of GABA

We found that the effects of PKA and PKC on functional current changes were associated with their effects on the surface expression of delta subunits: live cell imaging of SEP-delta subunits showed that PKA inhibitor H-89 increased surface expression of delta subunits (153 ± 8% of control, n = 5, $p < 0.01$) (Fig 4.12 AB); PKC

inhibitor rottlerin reduced the surface expression of delta subunits (56 ± 6% of control, n = 6, $p < 0.05$) (Fig 4.12 CD). Thus, these results indicated that PKA and PKC regulated delta subunits surface expression and functional current in opposite directions.

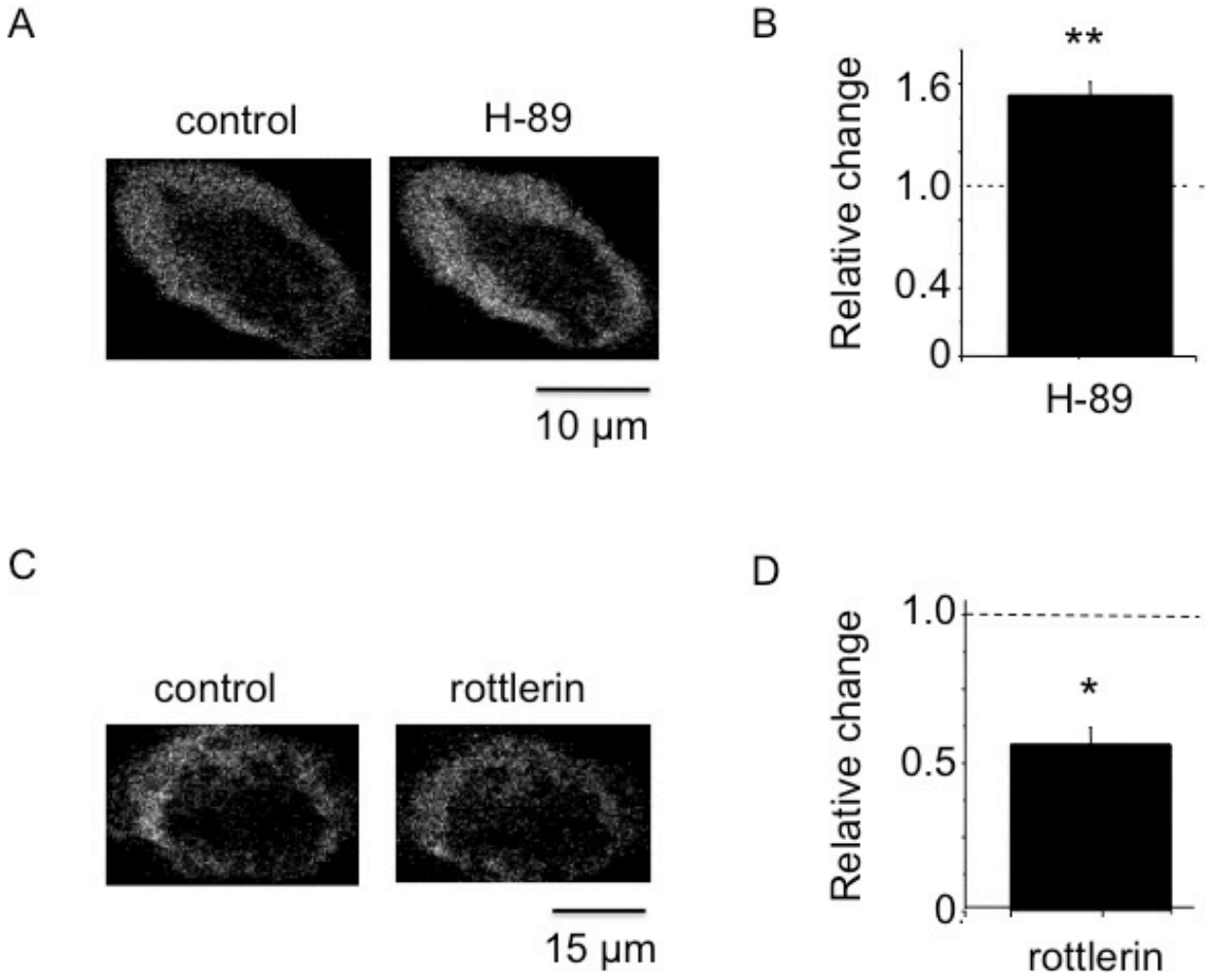


Fig 4.12 PKA activation reduced surface expression of delta subunits and PKC activation increased surface expression of delta subunits. **(A)** Representative pictures from live-cell imaging of SEP-delta subunits of cultured hippocampal neurons before and after H-89 treatment (10 μM, 20 min); **(B)** Summary data of relative change in fluorescence intensity after application of H-89; **(C)** Representative pictures from live-cell imaging of SEP-delta subunits of cultured hippocampal neurons before and after rottlerin (10 μM, 20 min); **(D)** Summary data of relative change in fluorescence intensity after application of rottlerin.

4.4 Discussion

In this study, we identified a new mechanism that regulates the trafficking of $GABA_A$ receptor. Specially, PKA activation reduced the surface expression of delta subunits of $GABA_A$ receptors, whereas PKC activation increased the surface expression of delta subunits of $GABA_A$ receptors (Fig 4.13). Based on these findings, we propose that PKA is responsible for pulling out the delta subunit-containing $GABA_A$ receptors from plasma membrane, whereas PKC is responsible for pushing the delta subunit-containing $GABA_A$ receptors onto plasma membrane. The kinases' effects on receptor level correlated positively with their effects on the tonic GABA current. We proposed that this new trafficking mechanism for extrasynaptic $GABA_A$ receptors would dynamically tune the regulation of tonic inhibition.

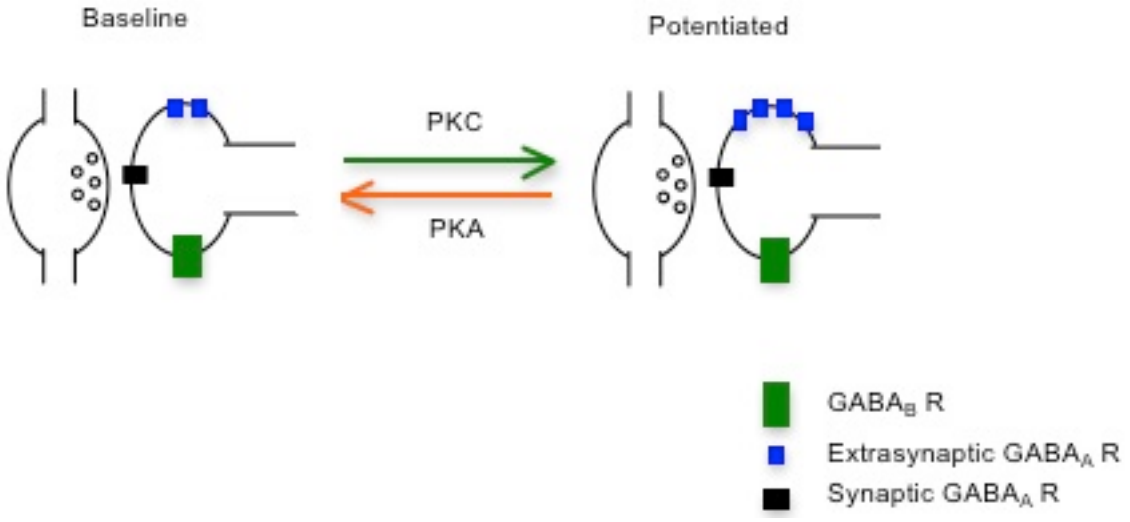


Fig 4.13 Model for the mechanisms of potentiation of $GABA_A$ receptor function by postsynaptic $GABA_B$ receptor activation. We propose that under certain conditions: for example, intense network activity increases spillover of GABA, which further activates $GABA_B$ receptors. Then, the activation of $GABA_B$ receptors increases surface expression of delta-containing extrasynaptic $GABA_A$ receptors, without change on

expression of synaptic $GABA_A$ receptors. This modulation requires two signaling pathways mediated by PKA and PKC, and they work in opposite direction to control the trafficking of extrasynaptic $GABA_A$ receptors.

4.4.1 Signaling pathways involved in $GABA_B$ receptor modulation of $GABA_A$ current

We reported here that PKA and PKC were both involved in $GABA_B$ receptor activation, which is complementary to the classical studies that $GABA_B$ receptor activation is linked to only $G_{i/o}$ /c-AMP/PKA pathway. Although in some preparations, it has been found that $GABA_B$ receptor activation involved PKC (Dutar and Nicoll, 1988; Mizuta et al., 2011), these PKC effects are more favorable to the presynaptic actions. Our postsynaptic effect of PKC may refine the $GABA_B$ receptor downstream signaling networks under certain conditions. For example, in epileptic seizure or when neurons undergo intense activity, the spillover of GABA from synaptic transmission significantly increases the concentration of ambient GABA, resulting in the activation of both $GABA_A$ and $GABA_B$ receptors.

We also found that neither PKA inhibition nor PKC activation alone was sufficient to produce the baclofen effect, indicating that $GABA_B$ modulation required both signaling pathways. We proposed that at resting condition, $GABA_B$ receptors may only need PKA signaling pathway to carry out signal processing, but under certain conditions, especially when neurons undergo intense activity, neurons are required to recruit an additional signaling pathway (e.g. PKC) to process more complicated signals. How these two signaling pathways work together remains unknown, but it is likely that these

two pathways may cross talk with each other through inhibiting AC by PKC (Lai et al., 1997).

Classically, the PLC-PKC signaling pathway is linked to Gq-coupled receptors (Taylor et al., 1991). In this study, we found that the baclofen-induced response was blocked by PTX, indicating that this signaling pathway was mediated by $G_{i/o}$. Further experiment showed that the baclofen effect was blocked by gallein, suggesting that $G_{\beta\gamma}$ dissociated from $G_{i/o}$ were involved in baclofen modulation. This is possible, because activation of PLC-beta by G $_{\text{beta gamma}}$ has been reported (Dickenson and Hill, 1998). However, there is another possibility that $GABA_B$ receptors induce cross talk with other receptors that mediate the Gq signaling pathway, to generate PLC and PKC. This is possible, as some groups have reported (Quitterer et al, 1999). However, we think this is less likely, because in this study, the PLC and PKC inhibitors all block the baclofen response, indicating that PLC and PKC are more likely from the $GABA_B$ receptor activation.

Also, in this study, we found that $GABA_B$ modulation of $GABA_A$ receptors is Ca^{2+} independent, and this was consistent with the result from rottlerin, a specific inhibitor of Ca^{2+} independent PKC, that blocked the baclofen effect. We proposed that PKC delta may be involved in $GABA_B$ modulation of $GABA_A$ receptors based on the following evidences: 1) Rottlerin is a highly selective inhibitor of PKC-delta (Gschwendt et al., 1994); 2) Choi et al., (2008) showed that PKC delta co-localized with delta subunits of $GABA_A$ receptors in hippocampus, and this anatomical evidence implies their functional interaction; 3) more importantly, poster from Dr. Messing (SFN 2012) showed the

phosphorylation of delta subunits of $GABA_A$ receptors by PKC delta.

4.4.2 The regulation of delta subunits surface expression by PKA and PKC

Our study showed that PKC activation increased the surface expression of delta subunits and this is consistent with some groups' findings: for example, Joshi and Kapur (2009) showed that PDBU increased the surface expression of delta subunits in cultured hippocampal neurons, and Kuver et al., (2009) found that rottlerin reduced the surface expression of delta subunits in cultured hippocampal neurons. But we also noticed that one group found that PKC activation reduced the surface expression of delta subunits in HEK293 cells (Bright and Smart, 2013). However, this study was done in non-neuron cell types, which makes it difficult to compare our data with their data. We also found that activation of PKA reduced the surface expression of delta subunits. This is consistent with the finding of Tang et al., (2010) that in HEK 293 cells, PKA inhibited the expression of $\alpha_4\beta_3\delta$ receptors.

In this study, the effects of kinases on receptor expression were positively correlated with $GABA_A$ current changes：Inhibition of PKA and activation of PKC both increased $GABA_A$ current and delta subunit surface expression. Kinases' effects on $GABA_A$ receptor current are diverse, depending on a lot of factors such as, receptor component, cell type, brain region, and even different drug agent tested (e.g. target at different isoform of kinases). For example, PKA has opposite effects on beta1- and beta3-containing $GABA_A$ receptors (McDonald et al., 1998); PKC increases the amplitude of

mIPSC in DG, but has no effect on CA1 pyramidal cells; PKA decreases the amplitude of mIPSC, but has no effect on DG (Poisbeau, 1998).

Another interesting question is how do PKA and PKC signaling pathways regulate delta subunits-containing $GABA_A$ receptors. It is likely that PKA and PKC can directly phosphorylate delta subunits (through communication with Dr. Messing in SFN; Kittler et al., 2005), but this idea is argued against by Abramian et al (2010) 's finding that PKC did not phosphorylate delta subunits. Another possibly is that these kinases modulate the surface expression of delta subunits through some other intermediated proteins that regulate delta subunit trafficking (e.g. gephyrin). It would be very interesting to test these ideas because it would not only elucidate out how kinases regulate the receptors, but also might provide insight into how PKA and PKC pathways work together.

4.4.3 Functional implication

The study of delta subunits of $GABA_A$ receptor trafficking is important to the understanding of inhibitory synaptic plasticity, which was proposed to be an important mechanism for learning and memory (Nicoll and Roche, 2013). We propose that the regulation of trafficking of delta subunits will shape the inhibitory signaling dynamically.

The hippocampus is an important structure for learning and memory in the mammal brain. DG acts as an information-processing gate for the hippocampus by filtering out excessive excitation input from the cortex. To achieve this goal, DG neurons have to

use some strategies to increase inhibition, such as increasing their tonic inhibitory signal to balance the activity of excitable input. One way to increase tonic inhibition is to increase tonic receptor expression (e.g. delta subunit of $GABA_A$ receptors). Thus, we think that understanding the regulation of trafficking of delta subunits is not only important for our understanding of the mechanisms for tonic inhibition, but also important for our understanding of the mechanisms of learning and memory. In conclusion, we propose that dynamic tuning of the surface expression of delta subunits of $GABA_A$ by $GABA_B$ receptors would further refine the regulation of tonic inhibition.

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VITA

I was born in a non-science family and did not fall in love with science until sophomore year, when my physiology course teacher Dr. Cui showed us a classical neurophysiology experiment: using a metal wire to stimulate the nerve of neuron-muscle junction preparation from frog, and resulting in the contraction of muscles. How amazing the phenomenon: one tiny nerve can control so precisely the movement of muscles. Intrigued by this, I began to pay attention to physiology, more precisely, to neurophysiology. Then I did my M.S study in Sun Yat-sen University, where I got electrophysiology training with focus on how neurotoxins affect biophysical properties of voltage gated sodium channels. Thanks to my advisor Dr. Xiang ’s recommendation, I got opportunity to go to the US for further training. Then I spent around 4 years in Spain laboratory at University of Washington, Seattle. During these four years, my scientific training in neurophysiology was extensively enriched and this also made me determined to choose science as a career path.

In spare time, I love playing pingpong, which began almost 15 years ago. During this time, I joined the professional teams in my hometown city and all colleges attended. I really enjoy playing pingpong, not only for exercising the body, but also making friends, releasing pressure from science. This is very important especially when I came to the US, where I had few friends. Now, I am a father of a three-year-old boy, I teach my son to play pingpong and watch him grown up; maybe some day, he will be a better pingpong player than me.