Activity-mediated plasticity of GABA equilibrium potential in the CA1 region of the rat hippocampus

by

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ABSTRACT

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian central nervous system (CNS). $GABA_A$ receptor mediated inhibitory postsynaptic currents (IPSCs) can affect both excitatory and inhibitory synapses, and thus, regulate CNS network activity. Amplitudes and the direction of IPSCs are subject to changes in the GABA equilibrium potential (E_{GABA}). Changes in E_{GABA} can affect various types of activity-dependent plasticity of the IPSC. Interestingly, E_{GABA} is set at a more positive level in neonatal than that in adult central neurons, rendering GABA excitatory in neonates and inhibitory in adults. Therefore, mechanisms underlying activity-mediated as well as age-dependent plasticity of E_{GABA} in rat hippocampus were examined in the current study.

Since E_{GABA} is mainly determined by the levels of intracellular Cl^- concentration ($[Cl^-]_i$) in central neurons, the activities of two cation-Cl^- cotransporters (K^+-Cl^- cotransporter, KCC2 and Na^+-K^+-Cl^- cotransporter, NKCC1) contribute to changes in E_{GABA}. Accordingly, factors which influence KCC2 or NKCC1 activity can induce shifts in E_{GABA}. In this thesis, the involvement of $GABA_B$ receptors, metabotropic glutamate receptors (mGluRs), G-proteins and postsynaptic Ca^{2+} in the regulation of KCC2 or NKCC1 activity, and thus in E_{GABA} in immature and juvenile hippocampal CA1 neurons were examined.

Whole-cell patch recordings were made from hippocampal CA1 pyramidal neurons (from 9-12 or 3-5 day old rats), in a slice preparation. Glutamatergic excitatory postsynaptic currents were blocked with dl-2-Amino-5-phosphonovaleric acid (APV) and 6,7-dinitroquinoxaline-2,3-dione (DNQX). Western blot and immunohistochemistry methods were also used to monitor changes in receptor distribution and localization. The results indicate that shifts in E_{GABA} are associated with several types of activity-mediated plasticity of IPSCs via changes in the activity of KCC2 or NKCC1 in hippocampal neurons. Interestingly, one type of specific, and

behaviorally relavant, stimulation (theta burst stimulation, TBS) is able to induce a two-direction-shift in E_{GABA} in juvenile and neonatal hippocampal neurons. $GABA_B$ receptors and G-porteins are involved in TBS-induced shifts in E_{GABA} in juvenile hippocampal neurons while both postsynaptic Ca^{2+} and mGluRs appear to contribute to TBS-induced shifst in E_{GABA} in both juvenile and neonatal neurons. However, the exact signal transduction pathways involving those above-mentioned factors awaits further investigation.

PREFACE

The following papers and abstracts were published by Bo Yang, the Ph.D. candidate with other co-authors.

1. J-Y. Xu, **B. Yang**, Sastry BR. 2009. The involvement of $GABA_C$ receptors in paired-pulse depression of inhibitory postsynaptic currents in rat hippocampal CA1 pyramidal neurons. *Exp Neurol.*, 216(1):243-246.

The contribution of Bo Yang to experiments reported in this paper is about 50%; he did most of the analyses of the data, and made the figures. The contribution of J-Y. Xu to the experiments is 50%. The contribution of all authors in writing the manuscript is equal. Sastry's contribution is in designing the experiments and interpreting the results.

2. **B. Yang**, R. Tadavarty, J-Y. Xu, B.R.Sastry. 2010. Activity-mediated plasticity of GABA equilibrium potential in rat hippocampal CA1 neurons. *Exp Neurol.*, 221(1):157-165.

The contribution of Bo Yang to experiments reported in this paper is about 75%; he did most of the analyses of the data and made the figures. The contribution of authors in writing the manuscript is as follows: Bo Yang 70%, Tadavarty 10% and Sastry 20%. B. Yang's contribution to design of experiments and interpretation of results is about 40%; Tadavarty's is about 10%; Xu's is 10% and Sastry's is 40%.

3. **B. Yang**, R. Tadavarty, J-Y. Xu, B.Sastry. Activity-mediated changes in the equilibrium potential for γ-aminobutyric acid A receptor mediated postsynaptic currents in rat hippocampal CA1 neurons (*Society for Neuroscience 2007 conference abstract).*

The contribution of Bo Yang to experiments reported in this abstract is about 75%; he did most of the analyses of the data and made the figures. He also wrote the abstract.

4. B. Yang, P. Rajput, U. Kumar, B. R. Sastry, 2010. Metabotropic glutamate receptors regulate the GABA equilibrium potential in neonatal and juvenile rat hippocampal CA1 neurons *(Society for Neuroscience 2010 conference abstract).*

The contribution of Bo Yang to experiments reported in this abstract is about 80%; he did most of the analyses of the data and made the figures. He also wrote the abstract.

The animal experiment protocols used in this thesis were approved by the UBC Animal Care Committee (approval #s: A07-0536 and A10-0381).

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AC	Adenylate cyclase
ACPD	1-Aminocyclopentane-*trans*-1,3-dicarboxylic acid
ACSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methylioxazolate-4-propionic acid
AMPA-R	AMPA-gated glutamate receptor
ANOVA	Analysis of variance
APV	Dl-2-amino-5-phosphonopentanoic acid
ATP	Adenosine trisphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BDNF	Brain-derived neurotrophic factor
CA	Carbonic anhydrase
Ca^{2+}	Calcium
CA1	Cornu ammonis area 1
CA3	Cornu ammonis area 3
CACA	c*is*-4-Aminocrotonic acid
CaMKII	Calcium/calmodulin-dependent protein kinase II
CaN	Calcineurin
CCC	Cation chloride cotransporter
CGP35348	(3-Aminopropyl) (diethoxymethyl) phosphinic acid
CGP55845	(2*S*)-3-[[(1*S*)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl]

	(phenylmethyl)phosphinic acid hydrochloride
Cl^-	Chloride
$[Cl^-]_i$	Intracellular chloride concentration
CNS	Central nervous system
CPCCOEt	7-(Hydroxyimino)cyclopropa[*b*]chromen-1a-carboxylate-ethyl ester
DCG-IV	(2S,1'R, 2'R,3'R)-2-(2,3-dicarboxycyclopropyl) glycine
DG	Dentate gyrus
DHPG	3,5-dihydroxyphenylglycine
DIOA	(R(+)-[(2-nbutyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy] acetic acid
DMSO	Dimethyl sulfoxide
DNQX	6,7-Dinitroquinoxaline-2,3-dione
E_{Cl^-}	Equilibrium potential of Cl^-
E_{GABA}	Equilibrium potential of $GABA_A$-mediated currents
EDTA	Ethylenediaminetetraacetic acid
EGLU	(2S)-a-Ethylglutamic acid
EPSC, EPSP	Excitatory postsynaptic current, potential
GABA	γ-aminobutyric acid
$GABA_AR$	GABA-gated receptor channel (type A)
$GABA_BR$	GABA-gated receptor channel (type B)
$GABA_CR$	GABA-gated receptor channel (type C)

GABARAP	$GABA_A$-receptor-associated protein
GDP	GABAergic depolarizing potential
GDP-β-S	Guanosine 5'-[β-thio]diphosphate trillithium
GPCR	G-protein coupled receptor
GTP-γ-S	Guanosine 5'-O-[gamma-thio]triphosphate
HCO_3^-	Bicarbonate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFS	High frequency stimulation
IPSC, IPSP	Inhibitory postsynaptic current, potential
ISI	Interstimulus interval
KCC	Potassium chloride cotransporter
KCC2	Potassium chloride cotransporter 2
L-AP4	2-amino-4-phosphonobutyrate
LTD	Long-term depression
LTP	Long-term potentiation
LY367385	(S)-(+)-a-Amino-4-carboxy-2-methylbenzeneacetic acid
MAP-1B	Microtubule-associated protein
Mg^{2+}	Magnesium
$[Mg^{2+}]_i$	Intracellular magnesium concentration
mGluR	Metabotropic glutamate receptor
mM	Milimolar
MPEP	2-methyl-6-(phenylethynyl)pyridine hydrochloride

ms	Milisecond
mV	Milivolt
NGS	Normal goat serum
NKCC1	Sodium potassium chloride cotransporter 1
nM	Nanomolar
NMDA	N-methyl-d-aspartate
NMDA-R	NMDA-gated glutamate receptor
OA	Okadaic acid
ODNs	Oligodeoxynucleotides
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PI	Phosphoinositide hydrolysis
PKA	Protein kinase A
PKC	Protein kinase C
PSC	Postsynaptic current
PSD	Postsynaptic density
PTD	Post-tetanic depression
PTP	Post tetanic potentiation
PP1	Protein phosphatase 1
PPD	Paired-pulse depression
REM	Rapid eye movement
SDS	Sodium dodecyl sulphate

(S)-MCPG	(S)-α-Methyl-4-carboxyphenylglycine
TPMPA	(1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid
µM	Micromolar
µm	Micrometer
VGCC	Voltage-gated calcium channels

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DEDICATION

To my parents

Chapter 1. Introduction

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS). Changes in GABA-ergic synaptic transmission can have a powerful modulatory influence on neuronal network activity by balancing the excitation and inhibition in the CNS. In hippocampal CA1 pyramidal neurons, plasticity of $GABA_A$-mediated inhibitory postsynaptic currents (IPSCs) has been extensively studied in order to shed light on various mechanisms involved. Since the amplitude and the direction of IPSCs are subject to changes in the equilibrium potential of the $GABA_A$ receptor mediated current (E_{GABA}), an understanding of the mechanisms involved in changes in E_{GABA} is necessary, and may have important implications for those studies on the plasticity of the IPSC as well.

Activity-mediated plasticity of E_{GABA} was first reported by our laboratory in 2000 (Ouardouz and Sastry 2000). E_{GABA} undergoes both activity-mediated (Fiumelli et al. 2005; Ouardouz and Sastry 2005; Ouardouz et al. 2006; Xu and Sastry 2007; Yang et al. 2010) and age-dependent (Ben-Ari 2002; Cherubini et al. 1991; Rivera et al. 1999) plasticity in rat hippocampus. Since the conductance mediated by $GABA_A$ receptors in the soma of hippocampal neuron is largely due to Cl^- ions under physiological conditions (Kaila 1994), E_{GABA} is mainly determined by the transmembrane Cl^- gradient (Jedlicka and Backus 2006; Yang et al. 2010). Thus, two major cation-chloride cotransporters (CCCs), Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) and K^+-Cl^- cotransporters (KCC2), play critical roles in modulating E_{GABA} in the soma of central neurons. Considering that KCC2 and NKCC1 undergo developmental expression in the CNS (Lu et al. 1999; Rivera et al. 1999), whether activity-mediated plasticity of E_{GABA} exists in immature neurons via modulation of KCC2 and/or NKCC1 activity awaits further investigation. In addition to these two types of CCCs (KCC2 and NKCC1), activation of $GABA_B$ receptors, G-

proteins were examined in juvenile rat hippocampal neurons as well. Moreover, metabotropic glutamate receptors (mGluRs) have been suggested to be involved in various physiological cellular processes including synaptic plasticity (Hollmann and Heinemann 1994). Given that mGluRs are co-localized with KCC2 or NKCC1 in the vicinity of excitatory synapses in the hippocampus and cerebral cortex (Lujan et al. 1996; Schomberg et al. 2001), it is worth investigating whether mGluRs participate in the regulation of activity-mediated plasticity of E_{GABA} via changes in KCC2 or NKCC1 activity. In this thesis, experiments were carried out on rat hippocampal CA1 neurons, to address the issues mentioned above.

The involvement of $GABA_B$ receptors and mGluRs in modulating E_{GABA} is interesting since shifts in E_{GABA} have been reported to be associated with physiological and pathophysiological phenomena like spike-timing plasticity, learning and memory, pain, trauma, addiction (Jedlicka and Backus 2006). Therefore, therapeutic strategies involving drugs affecting these receptors could prove useful in treating some CNS disorders.

Chapter 2. Review of the literature

2.1 GABA-ergic inhibition in rat hippocampus

As a predominant inhibitory neurotransmitter, GABA plays an important role in regulating the excitability of neuronal networks in mammalian CNS. In the hippocampus, the net flow of information through the CA1 region is modulated by GABA-ergic interneurons through feedback or feed-forward inhibition (Freund and Buzsaki 1996). Feed-forward inhibition inhibits excitatory signals onto dendrites and restricts the effects of depolarizing inputs to target cells via efficiently modulating the kinetics of EPSPs (Alger and Nicoll 1982; Buzsaki and Eidelberg 1982). Feedback inhibition, on the other hand, regulates the spread of excitation within neural network via facilitating synchronization of principal cell discharges and regeneration of hippocampal oscillations (Buzsaki et al. 1992; Fisahn et al. 1998). Feed-forward inhibition plays a key role in the control of archicortical EEG activity, epilepsy and long-term alteration of cellular excitability (Buzsaki 1984). The combination of the two types of regulatory mechanisms may serve to time action potentials and provide stable, effective external control over network excitability (Pouille and Scanziani 2001).

Most of the major inputs to the hippocampal formation dually innervate both interneurons and principal cells and hence the excitability of the principal cells depends upon the relative strengths of the inputs to these two cell types (Buzsaki 1984). At least five different types of interneurons have been identified to generate inhibition in the CA1 region (Nurse and Lacaille 1997), targeting different domains on the somata and dendrites of the pyramidal cells. Moreover, the short latency response of interneurons enables them to fire earlier than principal cells and to exert feed-forward inhibition (Buzsaki 1984). Synchronization of neuronal firing and behavior-related large scale network oscillations (such as θ, γ oscillations) are generated by

inhibitory inputs close to the soma, controlling the output from pyramidal cells (Klausberger et al. 2004; Nimmrich et al. 2005). Hence, any disruption of this fine balance through changes in GABA-ergic transmission may have a significant impact on the net neuronal excitability, plasticity and network behavior of central neurons.

2.2 Effects of changes in E_{GABA} on GABA-ergic inhibition in rat hippocampus

GABA-ergic inhibition plays a critical role in balancing the excitation and inhibition in the neural network of the CNS. In addition to the feed-forward and feedback inhibition mentioned above, changes in E_{GABA} can influence GABA-ergic inhibition in the CNS and thus affect cell excitation, synaptic plasticity and network behavior. However, compared with extensive studies on mechanisms underlying feed-forward and feedback inhibition and activity-mediated plasticity of IPSC amplitudes in the hippocampus, mechanisms involved in the shifts in E_{GABA} have not been widely examined until recently. There is a large body of evidence in literature suggesting that E_{GABA} undergoes both activity-dependent and age-related plasticity in rat hippocampus (Fiumelli et al. 2005; Ouardouz and Sastry 2005; Ouardouz and Sastry 2000; Ouardouz et al. 2006; Xu and Sastry 2007; Yang et al. 2010). However, the exact mechanisms underlying shifts in E_{GABA} under various physiological or pathological conditions still await further investigation. Since imbalance between inhibition and excitation can lead to serious pathological disorders in the CNS, studies examining the mechanisms involved in shifts in E_{GABA} are crucial in achieving a more comprehensive understanding of neurological disorders in which GABA-ergic transmission is affected.

2.3 Regulation of E_{GABA} in rat hippocampus

Even though $GABA_A$ receptor channels are selectively permeable to both Cl^- and HCO_3^- ions, the relatively low bicarbonate permeability has only a modest influence on the E_{GABA} (Bormann et al. 1987; Jedlicka and Backus 2006; Yang et al. 2010), setting E_{GABA} slightly positive to the chloride equilibrium potential (E_{Cl}^-) (Kaila and Voipio 1987). Moreover, the contribution of HCO_3^- to E_{GABA} usually occurs during sustained high frequency stimulation in dendrites (Staley and Proctor 1999). Under physiological conditions, the curve for E_{GABA} vs. intracellular Cl^- concentration ($[Cl^-]_i$) is rather steep, and also even small changes in $[Cl^-]_i$ can have profound effects on the E_{GABA} (Jarolimek et al. 1999; Staley and Smith 2001). Therefore, maintaining the homeostasis of $[Cl^-]_i$ is important for maintaining normal inhibitory function (Thompson 1994) in the CNS. Theoretically, any factor which affects $[Cl^-]_{i,}$ either directly or indirectly, may lead to changes in E_{GABA} in central neurons. Among those factors, functions of GABA receptors, mGluRs, G proteins, KCC2 and NKCC1 on the regulation of E_{GABA} will be reviewed in this section.

2.3.1 GABA receptors

GABA receptors play critical roles in maintaining the excitation-inhibition balance of the CNS, and are involved in many pathological processes such as epilepsy, pain and anxiety. To date, three pharmacologically and molecularly distinct GABA receptors, $GABA_A$, $GABA_B$ and $GABA_C$, are well recognized in the CNS. $GABA_A$ and $GABA_C$ receptor belong to ionotropic receptors, both of which are coupled to channels permeable to Cl^- (Jackel et al. 1994) even though the conductances of such coupled Cl^- channels vary (Johnston 1996). Apart from this similarity in receptor category, $GABA_A$ and $GABA_C$ receptors differ from each other in many

aspects: $GABA_A$ receptors are present in all CNS regions while $GABA_C$ receptors are highly enriched in vertebrate retina although their distribution in CNS has been reported in recent years (Alakuijala et al. 2005; Enz 2001; Rozzo et al. 2002; Zhang et al. 2001); $GABA_A$ receptors are assembled by various sub-units (α, β, γ, δ, ε, π and θ) (Mehta and Ticku 1999), which endows $GABA_A$ receptor with unique characteristics in terms of agonist affinity, rate of desensitization, single channel kinetics and ionic conductance (Sigel et al. 1990; Verdoorn et al. 1990), while $GABA_C$ receptors are composed of only two of the sub-units (rho1-3) (Alakuijala et al. 2005), which offers $GABA_C$ receptor specific features such as different agonist and antagonist binding, lack of desensitization, relatively low channel conductance (Johnston 1996); $GABA_C$ receptors are linked to the cytoskeleton via the colocalization with microtubule-associated protein (MAP-1B) whereas $GABA_A$ receptors are intracellularly anchored by colocalizing with $GABA_A$-receptor-associated protein (GABARAP) instead; $GABA_A$ receptors are activated by GABA in a more extended conformation whereas $GABA_C$ receptors are activated by GABA in a partially folded conformation (Johnston 1996); $GABA_A$ receptors are modulated allosterically by neuroactive steroids, barbiturates and benzodiazepines (Johnston 1996; Xu and Sastry 2005), however, $GABA_C$ receptors are mostly insensitive to bicuculline, allosteric modulators (like benzodiazepines) and other specific agonists of $GABA_A$ receptors but sensitive to 4-aminocrotonic acid (CACA, selective $GABA_C$ agonist) (Johnston 1996) and (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA, $GABA_C$ antagonist). Two remarkable and physiologically significant features of $GABA_C$ receptors are their weak desensitization (even at high concentration of agonist) and high agonist sensitivity (Bormann and Feigenspan 1995; Feigenspan and Bormann 1998; Johnston 1996; Lu and Huang 1998; Polenzani et al. 1991). Therefore, the robust sustained responses with $GABA_C$ receptors make it more suited for mediating long-lasting inhibition (such as lateral inhibition in the vertebrate retina) than $GABA_A$

receptor (Bormann and Feigenspan 1995).

Even though $GABA_C$ receptors were first discovered and characterized in the retina of vertebrates, there is ample evidence that $GABA_C$ receptors exist in some other brain regions (such as hippocampus, cerebellum, superior colliculus, etc.) (Enz 2001; Rozzo et al. 2002; Zhang et al. 2001). However, compared with the wealth of information on the functions of $GABA_C$ receptors in the retina, much less is known about the functional role of these receptors in the hippocampus. No conclusive evidence about functional hippocampal $GABA_C$ receptors in the hippocampus has been presented until recently. It has been reported that both $GABA_C$ receptor subunit mRNA and protein are expressed in the CA1 region of the adult rat hippocampus (Alakuijala et al. 2005; Didelon et al. 2002; Ogurusu et al. 1999; Wegelius et al. 1998). Since $GABA_C$ receptors have been shown to inhibit transmitter release at bipolar-cell terminals (Lukasiewicz and Werblin 1994; Pan and Lipton 1995), it is tempting to examine whether this action of $GABA_C$ receptors also occurs in hippocampal neurons. One notion put forward by Alakuijala et al. (Alakuijala et al. 2005) was that $GABA_C$ receptors are extrasynaptic and activated via spillover of synaptically released GABA upon strong stimulation. However, whether $GABA_C$ receptors can be activated under low frequency stimulations or involved in activity-mediated changes in E_{GABA} is not known.

$GABA_B$ receptors are G-protein coupled metabotropic receptors and consist of two distinct subunits: GBR1 and GBR2 (Jones et al. 1998). Receptor autoradiography of native $GABA_B$ receptors and immunohistochemistry studies of GBR1 and GBR2 proteins demonstrate comparable distributions in mammalian brain (Bowery 1989; Sloviter et al. 1999). Intriguingly, $GABA_B$ receptor expression appears to be developmentally regulated: expression of $GABA_B$ receptors is barely detected in neonatal neurons while it appears to be robust in mature neurons (Correa et al. 2004; Harris and Teyler 1983). Therefore, the modulatory function of $GABA_B$

receptors on GABA-ergic inhibition is usually examined in juvenile or adult animals. $GABA_B$ receptors are localized in both presynaptic and postsynaptic membranes of vertebrate central neurons (Couve et al. 2000; Mott and Lewis 1994). Activation of $GABA_B$ receptors causes an increase in K^+ conductance that underlies the slow inhibitory postsynaptic current (IPSC) and/or decrease in Ca^{2+} conductance which accounts for a decrease in either GABA or glutamate release at inhibitory synapses, or excitatory synapses, respectively. Thus, $GABA_B$ receptors are capable of modulating synaptic transmission by either inhibition of transmitter release or hyperpolarizing postsynaptic cells (Kaupmann et al. 1997; Thompson et al. 1993). Moreover, $GABA_B$ receptors can be activated during theta activity (Isaacson et al. 1993) and are suggested to be involved in synaptic plasticity (Davies et al. 1990; Wagner and Alger 1995). However, whether $GABA_B$ receptors are involved in activity-mediated plasticity of E_{GABA} is currently unknown.

2.3.2 Cation-chloride cotransporters

Cation-chloride cotransporters (CCCs) represent a family of transport proteins which participate in modifying a neuron's electrophysiological phenotype during development, synaptic plasticity and diseases in the CNS (Blaesse et al. 2009). A cross-talk among CCCs and trophic factors is critical for short-term and long-term modification of neuronal properties. Among nine members of the CCC family, two isoforms (KCC2 and NKCC1) have attracted attention due to their unique expression in neurons and specific functions in modulating neurotransmission. Both NKCC1 and KCC2 exert their actions by modulating $[Cl^-]_i$ in central neurons: NKCC1, a Na^+-K^+-$2Cl^-$ cotransporter, is responsible for excitatory GABA-ergic activity in immature brain and in adult sensory neurons by raising $[Cl^-]_i$; KCC2, a neuronal-

specific isoform of the K^+-Cl^- cotransporter, accounts for inhibitory GABA responses in mature central nervous system neurons by lowering $[Cl^-]_i$ (Delpire 2000). Changes in $[Cl^-]_i$ brought by the actions of these two chloride cotransporters cause subsequent shifts in E_{GABA}: KCC2 extrudes Cl^- out of central neurons (Deisz and Lux 1982; Jarolimek et al. 1999; Misgeld et al. 1986; Thompson et al. 1988; Zhu et al. 2005), leading to a negative shift (hyperpolarizing) in E_{GABA} (Jarolimek et al. 1999; Kakazu et al. 1999; Payne 1997; Rivera et al. 1999) which renders GABA inhibitory in those neurons; NKCC1 accumulates Cl^- into neurons, resulting in a positive shift (depolarizing) in E_{GABA} which is responsible for GABA excitatory response (Plotkin et al. 1997; Zhu et al. 2005).

2.3.2.1 K^+/Cl^- cotransporter 2

To date, four members of K^+/Cl^- cotransporter (KCC) family have been identified: KCC1, KCC2, KCC3 and KCC4. All KCC members are capable of moving K^+ and Cl^- across the cell membrane in a 1:1 stoichiometric ratio in the same direction in an electroneutral fashion (i.e. their activities do not affect membrane potential) (Gamba 2005). KCC1 is widely expressed throughout the brain where it fulfills the housekeeping roles in volume maintenance and regulation in neurons (Payne et al. 1996); KCC3 is expressed in large cortical pyramidal cells (Pearson et al. 2001) whereas KCC4 is only found in cranial nerves (Karadsheh et al. 2004). Unlike the other three members of KCC family, KCC2 has been widely studied in recent years. A range of experimental techniques have been used to demonstrate the wide expression of KCC2 in central neurons. For example, expression of KCC2 has been detected in cortex (Bayatti et al. 2008), retina (Vardi et al. 2000), hypothalamus (Belenky et al. 2008), temporal lobe (Huberfeld et al. 2007), spinal cord (Vinay and Jean-Xavier 2008) with techniques such as western blots,

immunohistochemistry, *in situ* hybridization. Interestingly, KCC2 is found to be highly expressed in several brain regions that are highly associated with epileptic seizures such as the inferior colliculus (Reid et al. 2001) and dentate gyrus (Pathak et al. 2007), which suggests a correlation of KCC2 and epilepsy. Moreover, there is ample evidence that KCC2 is exclusively expressed in mature neurons (Payne et al. 1996; Rivera et al. 1999) while other K^+-Cl^- cotransporters (KCC1, KCC3 and KCC4) have been found in the nervous system with a much more limited expression in neurons (Gillen et al. 1996; Kanaka et al. 2001; Payne et al. 1996; Pearson et al. 2001; Rivera et al. 1999).

KCC2 is involved in many physiological processes including cell volume regulation and neuronal communication (Gamba 2005). As one type of CCCs, KCC2 has drawn attention in this field since it was considered to be responsible for generating hyperpolarizing GABA response in rat hippocampus (Rivera, 1999). Interestingly, among those four cotransporters, only KCC2 shows substantial basal transport activity (Payne 1997; Song et al. 2002; Strange et al. 2000) whereas others need swelling activation (Mercado et al. 2000; Mount et al. 1999), indicating that KCC2 mainly regulates Cl^- homeostasis rather than volume control in central neurons. It has been well established that KCC2 plays an important role in maintaining the low $[Cl^-]_i$ in neurons and the hyperpolarizing postsynaptic inhibition in central neurons (Karadsheh et al. 2004; Payne et al. 1996; Rivera et al. 1999). KCC2 knockout mice died immediately after birth because of respiratory failure caused by anomalous excitatory actions of GABA and glycine (Hubner et al. 2001). Moreover, mice with only 5-10% of KCC2 display spontaneous, generalized seizures in both cerebral cortex and hippocampus (Woo et al. 2002). Another special feature of KCC2 is its developmental expression during the maturation of central neurons: expression of KCC2 is extremely low in neonatal neurons (postnatal 3-6 days) while gradually increases by the end of the second postnatal week (Payne et al. 2003; Payne et al. 1996; Rivera et al. 1999). KCC2

mRNA expression also seems to increase with the maturation of individual neurons and reach a high level in adults (Wang et al. 2002). Therefore, most of studies on KCC2 are carried out in juvenile or adult central neurons, where the expression of KCC2 is easily detected and functional activity of this protein is well examined. Developmental up-regulation of KCC2 expression has been suggested to be responsible for the GABA-ergic functional switch from excitatory to inhibitory in the CNS (DeFazio et al. 2000; Rivera et al. 1999). Increased activity of KCC2 renders GABA hyperpolarizing response in adult neurons (Deisz and Lux 1982; Jarolimek et al. 1999; Misgeld et al. 1986; Rivera et al. 1999; Thompson et al. 1988) while a knock-down of KCC2 reverses a negative shift in E_{GABA} (Rivera et al. 1999). Hubner et al (Hubner et al. 2001) reported that E_{GABA} in the spinal cord motor neurons of KCC2 knock-out mice was set at a more depolarized (-33 mV) than that in wild-type neurons (-52 mV), indicating that KCC2 is responsible for the negative shift in E_{GABA} during the maturation of neurons. Although, levels of KCC2 are thought to increase with age and stabilize in adult neurons (Luhmann and Prince 1991; Zhang et al. 1991), it is possible that KCC2 and E_{GABA} are both dynamically regulated at all age groups (Yang et al. 2010). The third characteristic of KCC2 is that the lifetime of membrane-associated KCC2 is very short (tens of minutes), which makes KCC2 ideally suited for mediating GABA-ergic ionic plasticity (Khazipov et al. 2004; Rivera et al. 2004; Wardle and Poo 2003).

Different mechanisms underlying the modulation of KCC2 activity and resultant shifts in E_{GABA} have been put forward in recent years. GABA itself has been proposed to control the developmental expression of KCC2 (Ganguly et al. 2001). However, this statement has been challenged by several studies: Ludwig et al. reported that up-regulation of KCC2 even occurs after a complete blockade of $GABA_A$-mediated transmission in dissociated and organotypic hippocampal cultures (Ludwig et al. 2003); another electrophysiological study conducted by

Misgeld and co-workers (Titz et al. 2003) showed that GABA is not required for the developmental up-regulation of KCC2 which leads to the switch of GABA-ergic response from depolarizing to hyperpolarizing in cultured midbrain neurons. Activity-dependent regulation of KCC2 have been reported as well (Ouardouz and Sastry 2005; Woodin et al. 2003; Xu and Sastry 2007). Moreover, postsynaptic Ca^{2+} (Fiumelli et al. 2005) and protein phosphorylation (Kelsch et al. 2001; Lee et al. 2007)/dephosphorylation (Wake et al. 2007) have been proposed to contribute to regulation of KCC2 in various preparations. Interestingly, brain-derived neurotrophic factor (BDNF) has been suggested to play a role in regulating postsynaptic transmission by down-regulating KCC2 expression in cultured hippocampal neurons (Rivera et al. 2002; Wardle and Poo 2003). Therefore, the exact mechanisms underlying the regulation of KCC2 activity, and hence shifts in $E_{GABA,}$ are not well understood.

In the exploration of the functions of KCC2 in central neurons, several agents (including drugs) have been used in past decades. However, an ideal specific KCC2 inhibitor is still not available (Rivera et al. 2005). Furosemide (a loop diuretic) and DIOA (R(+)-[(2-nbutyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy] acetic acid) are widely used even though they have effects on a variety of enzymes and receptors. Furosemide is generally used at a concentration of 100 μM or above, to block KCC2 activity (Blaesse et al. 2009; Thompson and Gahwiler 1989a). At this concentration, although the drug antagonizes KCC2 activity, it may also have a weak inhibitory effect on NKCC1 activity. Antisense oligodeoxynucleotides (ODNs) against KCC2 mRNA were first used in culture medium by Rivera et al. in one study in an attempt to observe the knock-out effect of KCC2 on E_{GABA} in mature cultured hippocampal pyramidal neurons (Rivera et al. 1999). Antisense and sense KCC2 ODNs with the same sequence were also applied intracellularly in acute hippocampal slices in one of our previous studies (Ouardouz et al. 2006; Yang et al. 2010). However, owing to the variations in laboratory

conditions (temperature, preparations and experimental models) and relatively complicated operation of antisense technique, KCC2 specific inhibitors are still the most widely used. In a recent high-throughput screening study, a number of molecules were identified but these are not commercially available yet (Delpire et al. 2009).

Antibodies raised against the intracellular C-terminus (Williams et al. 1999) or N-terminus (Hubner et al. 2001) allow one to study the location of KCC2 using immunocytochemical techniques. In rat hippocampus, KCC2 was highly expressed in parvalbumin-immunoreactive GABA-ergic cells in the CA1 and CA3 subfields (Gulyas et al. 2001). Interestingly, in addition to a close association with extrasynaptic $GABA_A$ receptor, KCC2 is also highly expressed in the vicinity of excitatory synapses (Gulyas et al. 2001). The tight functional correlation between activity of KCC2 and activation of mGluRs has been reported recently in CA3 region of hippocampus (Banke and Gegelashvili 2008) as well. Therefore, KCC2 appears to play a critical role in modulating the balance between excitation and inhibition in the CNS.

2.3.2.2 Na^+-K^+-Cl^- cotransporters

Isoforms of NKCC cotransporters (NKCC1 and NKCC2) are widely distributed among animal cells. NKCC1 can be found in most neurons or glial cells (Blaesse et al. 2009) and it is often referred to as the "housekeeping" isoform, whereas NKCC2 is mainly found in the mammalian kidney where it is involved in the reabsorption of Na^+, K^+ and Cl^- (Flatman 2002). Since NKCC1 moves 1 Na^+, 1 K^+ and 2Cl^- ions across cell membrane in an electroneutral fashion, activation of NKCC1 neither generates currents, nor is affected by changes in membrane potentials (Geck et al. 1980; Haas and Forbush 1998; Russell 2000). However, activation of this cotransporter leads to an increase in cell volume due to the movement of water

together with transported ions into the cells. Therefore, NKCC1 plays critical roles in both ion homeostasis and volume control in cells (Flatman 2002). In central neurons, its primary function is to accumulate Cl^- into cells and thus leads to relatively higher $[Cl^-]_i$ (Achilles et al. 2007; Yamada et al. 2004), which accounts for GABA excitatory responses in immature neurons (Plotkin et al. 1997). NKCC1 also undergoes the developmental expression in central neurons: the expression of NKCC1 is dominant in immature neurons while it is barely detected in matured neurons (Clayton et al. 1998), which contributes to relatively high $[Cl^-]_i$ and resultant depolarized level of E_{GABA} in immature neurons. The developmental expression of NKCC1, together with the gradual increase in the expression of KCC2 during the maturation of the CNS, contributes to setting E_{GABA} at a more depolarized level in neonates than that in juvenile or adult neurons. GABA depolarizes and excites neuronal membranes in neonatal neurons (Cherubini et al. 1991; Luhmann and Prince 1991) and this depolarizing effect is of functional importance during neuronal maturation and differentiation (Schomberg et al. 2001). Thus, NKCC1 plays an important role in the determination of postsynaptic responses to GABA by regulating E_{GABA} in neonates.

A key characteristic of NKCCs is their high sensitivity to inhibition by bumetanide and other loop diuretics. NKCC2 is more sensitive to bumetanide than NKCC1 (Russell 2000). However, bumetanide, at lower concentration (20 μM), has been shown to selectively inhibit NKCC1 activity and hence has been widely used to examine the involvement of NKCC1 in various biological processes in neurons.

Reduced oxygen tension, increased intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) and decreases in $[Cl^-]_i$ have been suggested to contribute to an increase in NKCC1 activity (Flatman 1988; Haas et al. 1995; Muzyamba et al. 1999). Interestingly, although this transporter does not consume ATP, its function is inhibited in cells whose ATP has been reduced (Flatman 1991),

suggesting an important role for phosphorylation in regulating this transporter (Lytle and Forbush 1992). Despite the important role of NKCC1 in the accumulation of Cl^- in neurons (Alvarez-Leefmans et al. 1988; Ballanyi and Grafe 1985; Hara et al. 1992; Misgeld et al. 1986), little is known about the modulation of this cotransporter in central neurons. It has been reported that this cotransporter is phosphorylated on serine and threonine, but not tyrosine, residues in the cytoplasmic amino and carboxyl-termini (Flatman 2002; Lytle 1998; Lytle and Forbush 1992). Recent studies suggest that activation of ionotropic glutamate receptors and/or group I mGluRs stimulate NKCC1 activity via phosphorylation in cortical neurons (Schomberg et al. 2001; Sun and Murali 1999; 1998). However, whether this modulation mechanism also exists in hippocampal CA1 neurons is unknown. Moreover, whether or not other factors contribute to the regulation of NKCC1 activity in neonates awaits further investigation.

2.3.3 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) represent a large family of G-protein coupled receptors and are involved in various cellular processes, including synaptic plasticity in the CNS, via modulating various voltage- and ligand-gated ion channels (Hollmann and Heinemann 1994). At least eight mGluR subtypes have been cloned to date and those receptors can be divided into three groups (group I, II and III mGluRs) based on their pharmacology and second-messenger coupling and sequence homology (Anwyl 1999; Ferraguti and Shigemoto 2006; Pin and Duvoisin 1995). Group I mGluRs include mGluR1 and mGluR5, which are primarily coupled to phosphoinositide hydrolysis (PI) and selectively activated by 3,5-dihydroxyphenylglycine (DHPG) (Gereau and Conn 1995). Both group II mGluRs (mGluR2 and mGluR3) and group III mGluRs (mGlu4, mGluR6-8) are negatively coupled to adenylate cyclase (AC) and the widely

used group II or group III mGluRs agonists are (2S,1'R, 2'R,3'R)-2-(2,3-dicarboxycyclopropyl) glycine (DCG-IV) (Hayashi et al. 1993) or 2-amino-4-phosphonobutyrate (L-AP4) (Suzdak et al. 1994), respectively.

mGluRs are located both pre- and postsynaptically in the CNS and activation of mGluRs leads to a variety of physiological effects: direct excitatory effects on CA1 pyramidal cells (Desai and Conn 1991; Liu et al. 1993) and interneurons (Desai and Conn 1991; Liu et al. 1993; Pacelli and Kelso 1991); reduction in both excitatory (Baskys and Malenka 1991; Desai et al. 1994; Desai et al. 1992) and inhibitory synaptic transmission (Desai et al. 1994; Desai et al. 1992; Liu et al. 1993); induction of hippocampal long-term potentiation (LTP) (Bashir et al. 1993; Ben-Ari and Aniksztejn 1995). The conventional view has been that the depression of excitatory transmission is mediated by group III mGluRs owing to the observed sensitivity of mGluR autoreceptors to L-AP4 (Baskys and Malenka 1991; Evans et al. 1982). In contrast with many other mGluR subtypes acting as presynaptic autoreceptors in the hippocampus, the group I mGluRs are mainly expressed on postsynaptic membranes (Ferraguti and Shigemoto 2006; Lujan et al. 1996; Shigemoto et al. 1997) even though presynaptic effects of group I mGluRs are also observed in hippocampal CA1 neurons (Gereau and Conn 1995). Activation of group I mGluRs typically increases NMDA receptor activity (Skeberdis et al. 2001), stimulates the release of Ca^{2+} from IP_3-sensitive stores (Abe et al. 1992; Masu et al. 1991; Miller et al. 1996) and/or triggers PKC-dependent signaling pathways (Banke and Gegelashvili 2008). Group II and III mGluRs are mainly involved in the presynaptic inhibition of transmitter release (Endoh 2004).

It has been well established that glutamatergic activity modulates GABA-ergic transmission by modifying presynaptic GABA release (Mitchell and Silver 2000; Nishimaki et al. 2007; Semyanov and Kullmann 2000). Whether activation of mGluRs is involved in the modulation of GABA-ergic transmission through other mechanisms (such as regulating E_{GABA})

is unknown. However, there are several lines of evidence suggesting that mGluR modulates KCC2 activity in hippocampal CA3 neurons (Banke and Gegelashvili 2008) and stimulates NKCC1 activity in cortical neurons via phosphorylation of NKCC1 through protein-kinase A or protein-kinase C (Schomberg et al. 2001). Therefore, it was decided to investigate whether mGluRs are capable of regulating E_{GABA} via modulating KCC2 or NKCC1 activity in rat hippocampal CA1 neurons.

2.3.4 G proteins

G proteins represent a family of membrane-bound regulatory proteins which modulate the activity of ion channels and other effector systems. G proteins can be divided into hetrotrimeric G proteins (large G proteins), small GTPases, and other unconventional G proteins (Ding et al. 2008). Hetrotrimeric G proteins are activated by G protein-coupled receptors and made up of α, β, γ subunits while small GTPases are usually monomeric (α subunit).

G-proteins are present in extraordinarily high concentration in the mammalian CNS (Sternweis and Robishaw 1984) and are associated with many types of brain neurotransmitter receptors. Two hydrolysis-resistant guanine nucleotides, Guanosine 5'-[β-thio]diphosphate trillithium (GDP-β-S) and guanosine 5'-O-[gamma-thio]triphosphate (GTP-γ-S), are commonly used to study the involvement of G proteins in numerous physiologic systems. GDP-β-S blocks GTP-dependent activation of G proteins (Holz et al. 1986) while GTP-γ-S activates G proteins (Andrade et al. 1986). Since many metabotropic receptors such as $GABA_B$ receptors and mGluRs exert their actions via coupling to G proteins, it is worth examining whether activation of G proteins is involved in the regulation of E_{GABA} in rat hippocampus as well. To our knowledge, there has been no report on G protein involvement in this aspect in literature so far.

2.4 Activity-mediated plasticity of $GABA_A$-mediated IPSCs in rat hippocampus

GABA-ergic transmission is very labile under physiological conditions: its efficiency can vary noticeably depending on the preceding activity of surrounding neurons (Storozhuk et al. 2005). GABA-ergic synaptic transmission can be either enhanced or depressed by the preceding activity in neurons, and these changes may span temporal ranges from milliseconds to hours, days and presumably even longer (Citri and Malenka 2008). Therefore, those modifications (activity-dependent plasticity) play a critical role in both normal functioning of the neuronal networks and pathological processes in the CNS. The activity-dependent plasticity of IPSCs includes paired-pulse depression (PPD), post-tetanic potentiation (PTP), long-term potentiation (LTP) and frequency-dependent depression (FDD), etc. In contrast to the extensive studies on the plasticity of excitatory synapses over past decades, plasticity of inhibitory synapses has not received much attention until recent years.

2.4.1 Post-tetanic potentiation of $GABA_A$-mediated IPSCs

Various forms of short-term synaptic plasticity, lasting from milliseconds to several minutes, have been found in both invertebrates and mammals (Zucker and Regehr 2002). These short-term synaptic plasticities are responsible for short-term adaptations to sensory inputs, transient behavioral changes and short-lasting forms of memory. Most forms of short-term plasticity in GABA-ergic synapses are triggered by short bursts of stimulations which lead to a transient accumulation of Ca^{2+} in presynaptic nerve terminals (Citri and Malenka 2008). This increase in presynaptic Ca^{2+} in turn raises the probability of neurotransmitter release by direct modulation of the exocytosis of synaptic vesicles. Among numerous forms of short-term

synaptic plasticity in the mammalian CNS, post-tetanic potentiation (PTP) has drawn considerable attention in the past decade. PTP refers to an increase in synaptic strength for a few minutes following high frequency stimulation of the presynaptic fibers (Waziri et al. 1969). PTP in excitatory synapses has been extensively studied for decades (Waziri et al. 1969; Zucker 1989). However, this type of modification of GABA-ergic synaptic transmission has not received much attention until recently. PTP of the IPSC can be evoked by stimulation of the presynaptic GABA-ergic neurons at a frequency ranging from 5 to 80 Hz. The greatest increase in the PSC amplitude was about 50% and the duration was about 1 min (Jensen et al. 1999). Presynaptic mechanisms including changes in the probability of transmitter release before tetanization, extracellular Ca^{2+} levels (Jensen et al. 1999) and activity of L-type calcium channels (Storozhuk et al. 2002) have been suggested to contribute to this phenomenon in cultured hippocampal neurons. Interestingly, the opposite phenomenon − post-tetanic depression (PTD) can also be induced under this stimulation protocol (Storozhuk et al. 2002), which is exclusively induced by postsynaptic mechanisms (McCarren and Alger 1985). In addition, several lines of evidence suggest that acute E_{GABA} shift induced by Cl^- accumulation is involved in short-term "ionic plasticity" of GABA-ergic transmission (Rivera et al. 2005). Therefore, even though Storozhuk et al. attributed PTP to be a presynaptic phenomenon, the postsynaptic factors such as shifts in E_{GABA} may also take part in, and/or is associated with, this process.

2.4.2 Paired-pulse depression of $GABA_A$-mediated IPSCs

Paired pulse depression (PPD) is another type of short-term activity-mediated plasticity of IPSCs in rat hippocampus. PPD refers to a drop in the amplitude of the second postsynaptic response (PSC) as compared with that of the first response when a presynaptic unit is stimulated

by a pair of stimuli. This is the simplest form of a decrease in the efficiency of synaptic transmission resulting from the preceding activity. PPD of the IPSC has been extensively studied in the hippocampus (Davies and Collingridge 1993; Davies et al. 1990; Olpe et al. 1994; Wilcox and Dichter 1994). In contrast to the PPD in glutamatergic synapses, which is usually induced only at very short interstimulus intervals (ISI, about 10 ms), PPD in GABA-ergic synapses is often observed at longer ISI (50-2000ms) (Storozhuk et al. 2002; Xu et al. 2009). Both pre- and postsynaptic mechanisms have been proposed to explain the dynamics and mechanisms of PPD: transient decreases in transmitter release (Korn et al. 1984); decrease in the probability of release of neurotransmitter-containing vesicles (Dobrunz and Stevens 1997); a drop in the driving force of Cl^- ions (McCarren and Alger 1985); desensitization of $GABA_A$ receptors (Alger 1991); modulation of the conductance of those receptor coupled channels (Alger 1991; Delfs and Dichter 1983). Intriguingly, experimental proof exists for the hypothesis that autoinhibition induced by presynaptic $GABA_B$ receptor is responsible for the PPD in GABA-ergic synapses (Davies et al. 1990; Davies et al. 1991; Deisz and Prince 1989; Otis and Mody 1992). Nonetheless, this statement was challenged by the findings from another study. When a single presynaptic neuron, rather than numerous GABA-ergic presynaptic units, is subjected to test stimulation, activation of $GABA_B$ receptors seems not to be involved in PPD in both cultured hippocampal neurons and hippocampus slices (Wilcox and Dichter 1994). Moreover, concentrations of $GABA_B$ antagonists used in those studies are higher than those required to block postsynaptic $GABA_B$ responses (Davies and Collingridge 1993; Olpe et al. 1994). Therefore, the exact cellular mechanism underlying PPD of the IPSC in hippocampal neurons remains controversial.

Although $GABA_C$ receptors are present in the hippocampus, their functional significance is not well understood. Whether activation of $GABA_C$ receptor is involved in PPD of the IPSC in

rat hippocampal neuron is unknown.

2.4.3 Long-term GABA-ergic synaptic plasticity in rat hippocampus

GABA receptors impart a powerful regulatory influence on the excitability of the principal cells (Freund and Buzsaki 1996; Stelzer 1992; Wigstrom and Gustafsson 1983). Evidence in literature suggests that the decreased $GABA_A$ receptor activity, or blockade of $GABA_A$ receptors facilitates the induction of long-term potentiation (LTP) of the excitatory postsynaptic potential (EPSP) (Hess and Donoghue 1996). On the contrary, Costa et al. (Costa and Grybko 2005) found that in Ts65Dn mice, in which $GABA_A$ receptor-mediated inhibition is enhanced, LTP of the EPSP is difficult to induce unless GABA-ergic inhibition is pharmacologically blocked. Therefore, any changes in GABA-ergic transmission, including those changes in E_{GABA}, would have significant implications on the maintenance of the equilibrium between excitation and inhibition in the CNS.

2.4.3.1 LTP of $GABA_A$-mediated IPSCs

LTP in the hippocampus is a remarkable example of synaptic plasticity. The first report on LTP of $GABA_A$ receptor-mediated synaptic transmission was from our laboratory (Morishita and Sastry 1991; Xie and Sastry 1991). Our previous studies suggested that high-frequency stimulation (HFS) induced LTP arose from a presynaptic site and activation of $GABA_B$ receptor, cyclic AMP/protein kinase A (PKA) and sulfhydryl-alkylation, but not postsynaptic Ca^{2+} or PKC, were involved in this process (Morishita and Sastry 1991; Perez and Lacaille 1995; Wang and Stelzer 1996). Further studies indicated that neither the activation of $GABA_A$ receptor during

tetanic stimulation nor a change in postsynaptic membrane potential was required for the induction of LTP (Shew et al. 2000).

2.4.3.2 LTD of $GABA_A$-mediated IPSCs in rat hippocampus

Since IPSCs originating from both feed-forward (Alger and Nicoll 1982) and feed-back activation of the hippocampal CA1 interneurons (Lacaille and Schwartzkroin 1988), upon the Schaffer-collateral stimulation are polysynaptic and thus the link between properties of IPSCs and efficacy of individual inhibitory synapses was obscured (Wang et al. 2003). Conventionally, the study of $GABA_A$-mediated IPSCs in CA1 pyramidal cells was conducted under blockade of excitatory glutamatergic transmission. Unfortunately, this blockade fails to account for the functions of NMDA receptors and the initiation of sustained changes in inhibitory synaptic strength. By applying double whole-cell patch-clamp recording technique, Wang et al. reported that the dephosphorylation by calcineurin (CaN) of $GABA_A$ receptor is involved in LTD of unitary IPSCs (Wang et al. 2003). However, it was unknown whether or not changes in E_{GABA} were involved in that study. Owing to the uncertainty of the induction of LTD of IPSCs under conventional whole-cell patch-clamp recording, changes of $E_{GABA,}$ in this specific long-term plasticity of IPSCs, were not examined in the current study.

2.4.4 Frequency-dependent depression of $GABA_A$-mediated IPSCs

Enormous attention has been given to the activity-dependent long-term modulation (such as LTP) of excitatory synaptic transmission over past decades. However, relatively little is known about the activity-dependent short-term modulation (such as repetitive stimulation induced depression) of inhibitory synaptic transmission. It was known that inhibition caused by

exogenous GABA faded during prolonged applications (Curtis et al. 1959). Later studies conducted by Andersen et al. suggested that amplitudes of IPSPs also decreased upon repetitive stimulation (Andersen and Lomo 1968). During repetitive stimulation (5-10 Hz), the amplitudes of the IPSP undergo marked depression in intracellular recordings (McCarren and Alger 1985; Thompson and Gahwiler 1989a; Wong and Watkins 1982). This frequency-dependent depression (FDD) is now a well-known phenomenon.

The amplitude of the synaptic current (IPSCs) depends on the driving force for Cl^- and the activated conductance (g_{IPSP}) (Thompson 1994). g_{IPSP} will in turn depend on the amount of transmitter released from presynaptic terminals and the sensitivity of postsynaptic receptors. Therefore, both presynaptic and postsynaptic factors may participate in the activity-dependent depression of the IPSC in rat hippocampus. Several factors have been suggested to contribute to this process: reduced transmitter release (Ben-Ari et al. 1981), desensitization of GABA receptors (Numann and Wong 1984; Wong and Watkins 1982), decrease of ionic conductance and changes in E_{GABA} (McCarren and Alger 1985); extrasynaptic factors such as changes in transmitter uptake or extracellular K^+ accumulation (McCarren and Alger 1985). Among those factors, two factors have drawn considerable attention of investigators in this field: desensitization of GABA receptors and shifts in E_{GABA}.

Desensitization of GABA receptors was initially put forward to account for the decrease in synaptic conductance upon repetitive stimulation (Ben-Ari et al. 1979). However, focal application of GABA to presumed synaptic receptors failed to demonstrate any significant postsynaptic GABA receptor desensitization following repetitive stimulation (McCarren and Alger 1985; Thompson 1994). Therefore, other presynaptic mechanisms might contribute to this use-dependent decrease in evoked synaptic conductance. Presynaptic $GABA_B$ receptors have

been suggested to contribute to a negative feedback of synaptically released GABA upon repetitive stimulation and subsequent decrease in GABA release, which may explain the activity-dependent decrease in the conductance during IPSPs (Deisz and Prince 1989). The best supporting evidence comes from paired-pulse experiments in which $GABA_B$ antagonists (CGP35348 and saclofen) were able to fully block paired pulse depression (PPD) (Davies et al. 1990; Davies et al. 1991). Interestingly, another $GABA_B$ antagonist, phaclofen, has been shown to reduce activity-dependent disinhibition following low frequency stimulation in rat dentate gyrus (Mott et al. 1990). However, whether this mechanism also exists in rat hippocampus is unknown.

Repetitive stimulation not only results in a decrease in g_{IPSP}, but also leads to significant shifts in IPSP reversal potential (E_{IPSP}) (Thompson 1994). The critical determinant of the IPSP/IPSC is not E_{IPSP} itself but the IPSP/IPSC driving force (V_m-E_{IPSP}). Even though a small change in E_{IPSP} may appear inconsequential, it is able to significantly change amplitudes of IPSPs/IPSCs. Interestingly, the amplitudes of the IPSP fluctuate even during low frequency stimulation (0.1-1Hz), which is also called "IPSCs draining". Whether shifts in E_{GABA} contribute to this specific phenomenon is unknown.

2.4.5 θ-burst stimulation induced plasticity of E_{GABA} in rat hippocampus

θ-rhythm, an approximately sinusoidal extracellularly recorded potential of 5-9 Hz can be recorded in the dentate gyrus (DG), and the CA1 field of the hippocampus, in various species during certain specific behaviors (Winson 1972). In rats, it occurs during voluntary movement (such as exploration) as well as in rapid eye movement (REM) sleep (Vanderwolf 1969). However, the exact mechanism of generation of θ-rhythm is unknown but presumably reflects

the action of synchronous postsynaptic potentials in the granule cells of DG and the CA1 pyramidal cells (Andersen 1980; Buzsaki 1986; Fox et al. 1983; Leung 1984). A recent model of hippocampal theta activity assumes that rhythmic somatic feed-forward inhibition from the septum and distal dendritic excitation from the entorhinal cortex arrives synchronously and the high amplitude field theta waves are a result of cooperative active sources and sinks (Buzsaki et al. 1983). Hippocampal projection cells have been reported to show a transmembrane potential in the theta frequency range (Leung and Yim 1986).

θ oscillations are prominent in rat hippocampus and are suggested to be critical in learning and memory function (Bland 1986). Theta-burst stimulation (TBS), which mimics θ oscillation firing patterns in hippocampal CA1 neurons (Nguyen and Kandel 1997), has been widely used to induce synaptic plasticity. During theta activity, rhythmically firing interneurons are known to produce GABA-mediated fluctuations of the membrane potential of CA1 pyramidal cells (Fox 1989; Leung and Yim 1986). Using paired intracellular recordings, Cobb et al. showed that rhythmic activation of presynaptic basket or axo-axonic interneurons at theta frequency simultaneously subsides the spontaneous firing of the pyramidal cells in CA1 (Cobb et al. 1995). Since GABA-ergic interneurons have extensive axonal arborizations, this synchronized inhibition may be imposed on a large population of principal neurons (Cobb et al. 1995; Dekker and Parker 1994), leading to various types of synaptic plasticity in hippocampus.

It has been reported that TBS is able to induce the LTP at both apical and basal dendritic synapses on hippocampal CA1 neurons (Capocchi et al. 1992). Interestingly, it has been suggested that theta rhythm in CA1 pyramidal neurons is caused by a rhythmic modulation of somatic IPSPs, which may result from direct excitation of inhibitory interneurons, or from inhibition of tonically firing inhibitory interneurons or suppression of transmitter release from inhibitory terminals (Leung and Yim 1986). Nevertheless, whether the "*vice versa*" scenario

(theta rhythm modulating somatic IPSP/IPSC in hippocampal CA1 pyramidal neurons) exists, is not known. Since chloride-mediated IPSPs have been suggested to play a critical role in the generation of intrasomatic theta rhythm (Leung and Yim 1986), it is also tempting to examine whether TBS is capable of modulating IPSP/IPSCs via changes in chloride conductance.

Compared to the extensive study on the induction of LTP of the EPSP by TBS of the input in hippocampal CA1 neurons (Staubli et al. 1998), little is known about the effects of TBS on the IPSP/IPSC in these cells. Our previous studies suggest that TBS is able to induce a negative shift in the E_{GABA} in juvenile rat hippocampal CA1 neurons via an up-regulation of KCC2 activity (Xu and Sastry 2007). Nevertheless, the trigger for, and the mechanisms involved in, up-regulation of KCC2 and plasticity of E_{GABA} are unknown. In addition, since TBS is able to influence activity of various receptors involved in LTP (Benson et al. 2000), it is tempting to speculate that TBS can affect activity of these receptors or transporters other than KCC2, to induce shifts in E_{GABA} as well.

2.5 Age-dependent plasticity of E_{GABA} in rat hippocampus

GABA response is known to be excitatory in neonates but inhibitory in juvenile and adult animals (Ben-Ari 2002; Ben-Ari et al. 1994; Rivera et al. 1999), which is caused by a developmental switch in the polarity of $GABA_A$ receptor-mediated inhibition from depolarizing in neonates to hyperpolarizing in juvenile (>7 day old) and adult animals (Cherubini et al. 1991; Zhang et al. 1991). Many other developmental transitions may also contribute to changes in the functional capabilities of inhibitory networks: changes in synaptic density accompanying synaptogenesis (Ben-Ari and Represa 1990; Blue and Parnavelas 1983); differences in both receptor subunit composition (Killisch et al. 1991) and receptor kinetics (Hutcheon et al. 2000); variations in IPSC kinetics (Hollrigel and Soltesz 1997; Otis and Mody 1992); developmental

shift of E_{GABA}. Among those factors, change in E_{GABA} has drawn a great deal of attention. It is well known that E_{GABA} is set at a more depolarized level in neonatal neurons than in adult neurons (Ben-Ari 2002; Rivera et al. 1999; Yamada et al. 2004; Zhang et al. 1991), which seems to be related to differential expression of KCC2 and NKCC1 during development and maturation of central neurons (Ben-Ari 2002; Lu et al. 1999). The dominant expression of NKCC1 leads to relatively higher $[Cl^-]_i$ in immature neurons which sets E_{GABA} at a depolarized level (Yamada et al. 2004), while developmental up-regulation of KCC2 expression in mature neurons accounts for lower $[Cl^-]_i$ that maintains E_{GABA} at a hyperpolarizing level (DeFazio et al. 2000; Rivera et al. 1999). The developmental switch of E_{GABA} can be found in DCN neurons as well (Ouardouz and Sastry 2005; Ouardouz and Sastry 2000). Therefore, the age-dependent plasticity of E_{GABA} may be a universal hallmark in the development of mammalian CNS.

Both LTP and LTD of inhibitory synapses have been described in different developing brain regions including the cerebral cortex (Komatsu and Iwakiri 1993) and hippocampus (McLean et al. 1996). Elevation of $[Ca^{2+}]_i$ and NMDA-gated calcium channels are involved in the induction and maintenance of long-term plasticity at developing inhibitory synapses (Caillard et al. 1999; Komatsu and Iwakiri 1993). Whether other factors such as changes in E_{GABA} are also involved in this long-term plasticity is unknown.

2.6 Aims of the study

In the development of new GABA-ergic drugs for the treatment of many neurological disorders (epilepsy, pain, addiction, etc.), the foremost goal for many pharmaceutical companies is to seek drugs that act on certain types of receptors or proteins (transporters). Even though shifts in E_{GABA} have been reported to be associated with the above-mentioned disorders whereby GABA-ergic inhibition is affected, the physiological and pathological implications of a plasticity

of E_{GABA} and consequent abnormality in neuronal network behavior in brain are poorly understood. A variety of factors including mGluRs, $GABA_B$ receptors, G proteins, postsynaptic Ca^{2+}, high frequency activity in inputs, etc., are known to affect synaptic plasticity. It is unclear if any of these is involved in the plasticity of E_{GABA}. To elucidate the mechanisms underlying the regulation of E_{GABA} under various conditions in rat hippocampus, a series of experiments were carried out in the juvenile and neonatal rat CA1 hippocampal neurons. The aims of my thesis work are listed as follows:

1) To examine whether activation of $GABA_B$ and $GABA_C$ receptors are involved in the modulation of E_{GABA} under physiological low frequency stimulation.

2) To investigate if changes in E_{GABA} are involved in paired-pulse depression or frequency-dependent depression of $GABA_A$-mediated IPSCs.

3) To examine the mechanisms underlying TBS-induced shifts in E_{GABA} in juvenile rat hippocampus.

4) To test whether shifts in E_{GABA} also occur following theta-burst stimulation in neonatal rat hippocampal neurons and examine the underlying mechanisms.

5) To examine the expression of KCC2 and NKCC1 under various conditions in both juvenile and neonatal rat hippocampus CA1 region by using western blot and immunohistochemistry methods.

Chapter 3. Materials and methods

3.1 Animals

Male Wistar rats (9-12 and 3-5 day old) were provided by Animal Care Center at the University of British Columbia (UBC). One animal was brought to our laboratory in a clean, warm, ventilated box 1-2 hours prior to each experiment, All experiments were performed in accordance to the approved guidelines of the Canadian Council on Animal Care and Animal Center at UBC (Certificate no's: A07-0536 and A10-0381). A total of 150 male Wistar rats were used in the current study and approximately 2 recordings per animal were obtained.

3.2 Hippocampal slice preparation

Hippocampal slices were prepared by procedures routinely used in our laboratory (Xie et al. 1995). Animals were anesthetized with halothane and decapitated using a guillotine. The rat head was quickly immersed in ice-cold artificial cerebrospinal fluid (ACSF) oxygenated with carbogen (95% O_2~5% CO_2). An incision was made, using a pair of surgical scissors, in the middle of the scalp and two additional cuts were made at the posterior portion of the skull in order to expose the brain. Then, the whole brain was scooped out by a modified spatula and transferred into ice-cold ACSF for 1-1.5 min to cool down. A slightly moistened (with ACSF) filter paper was used to obsorb excess solution on the surface of the brain. Brainstem-cerebellum was removed and the two hemispheres separated. One hemisphere was immersed in ice-cold ACSF as a backup and the other was glued to a metal platform with cyanoacrylate (LePage No.8). The mounted brain block was then placed into a bath chamber surrounded with ice-cold oxygenated ACSF. Transverse sections (400 μm) were made from one hemisphere. Slices with

this thickness 350-400 μm could yield relatively clear images under the microscope and the damage on the dendrites and axons of neurons was minimized as well. CA3 region was rountinely cut off to reduce the spontaneous activity in axons connected to these neurons. Typically, 4 slices could be obtained from one hemisphere in this preparation and only up to 2 slices per animal were used to collect data. The cut sections were immediately transferred to a petri dish filled with oxygenated cold ACSF. The cerebral cortex was dissected out from each slice and one small section containing intact hippocampus was transferred into an incumbation chamber containing oxygenated ACSF. Slices were allowed to equilibrate with normal ACSF at room temperature for at least 1-1.5 h before being transferred into the recording chamber.

3.3 Extracelluar and intracellular solutions

Extracellular superfusion ASCF contained (in mM): 120 NaCl, 3.1 KCl, 1.3 NaH_2PO4, 26 $NaHCO_3$, 2 $MgCl_2$, 2 $CaCl_2$ and 10 dextrose (saturated with 95% O_2~5% CO_2) and was maintained at a pH of 7.35-7.4. Intracelluar pipette solution contained (in mM): 135 K-gluconate, 10 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES, 10 KCl, 1 (1,2-bis(o-aminophenoxy)eth-ane-N,N,N',N'-tetraacetic acid) BAPTA, 5 Mg-ATP, 0.1 $CaCl_2$, 10 Na_2-phosphocreatine, 0.4 Na_3-GTP and creatine phosphokinase 50 U/ml; pH was adjusted to 7.20-7.3 with KOH.

3.4 Recording and electrical stimulation

3.4.1 Stimulation electrode and recording electrode

The stimulation electrode was attached to a Narishige micromanipulator (model: NMN-21)

and recording electrodes were connected to a Narishige Water Hydraulic micromanipulator (model: MHW-3). Stimulation electrode used in the present study was a bipolar concentric platinum electrode (SNEX-100, David Kopf Instruments) with a shaft length of 50 mm and the resistance was around 1 MΩ. The stimulation electrode was rountinely placed in the stratum radiatum of the CA1 region 300-400 μm from the planned recording site. Recording electrode was positioned on the soma of the recorded neurons (see Section 3.4.3).

3.4.2 Electrical Stimulations

Current was delivered from a Grass S88 stimulator (Grass Instruments). Control stimulation frequency was set at 0.05 Hz with square pulses (0.1-0.2 ms duration). The duration of the stimulation was usually set at 3-4 ms and strength ranged from 100-150 μA. This was done to allow enough room for potentiation or depression of corresponding responses following a conditioning stimulation.

3.4.3 Whole-cell patch clamp recording

Pyramidal neurons were visually recognized by their specific shape and location using the Zeiss microscope. The stimulating electrode was placed in the stratum radiatum, as mentioned above. Intracelluar pipette solution was filled into the recording electrode with caution in order not to bring dust inside. Before lowering the pipette into tha bathing solution, a slight pressure (about 10 cm of water) was applied on the pipette fluid through a 10 ml syringe in order to blow any contaminations in the bathing solutions away from the pipette tip. A small block pulse (5 mV, 30 ms) was delivered through the pipette every 2 s and the changes to the rectangular pulse

were monitored on an oscilloscope (model: Tektonix 5111A oscilloscope). A smooth movement of a clean pipette towards a healthy pyramidal cell will usually lead to a successful "patch" and thus, whole-cell configuration was formed. If the cell could not be patched successfuly in the first attempt, it was discarded. A new recording electrode was used to repeat the same procedure on another cell. Fast capacitative transients were offset using Axopatch 200A amplifier (Axon Instruments). Series resistance in the present study ranged between 20-30 MΩ and was carefully monitored throughout the recording. Recordings were accepted only if both IPSCs in control recordings were stable and the series resistance can be properly compensated to 75%.

PSCs were recorded from CA1 pyramidal cells in response to different frequency stimulations (see Section 3.4.4) while voltage-clamping the recorded neuron at - 60 mV (see Fig. 3-1). In all experiments, glutamatergic synaptic transmission was blocked with APV (50 μM) and DNQX (20 μM) so that the IPSCs could be properly (time constant of decay and the amplitude) quantified. All synaptic currents were recorded using an Axopatch 200A amplifier (Axon Instruments) connected to Digidata 1322A interface with the low pass filter set at 5 kHz. For each experiment, control recordings were made for at least 10-15 minutes prior to other experimental manipulations so as to ensure stability. Recordings were digitized and stored using Clampex 9.0 software (Axon Instruments) and off-line data analysis was performed with Clampfit 9 (Axon Instruments).

3.4.4 Electrical stimulations

Conditioning stimulations used in the current study were 0.1, 0.5, 1.0 Hz (for at least 30 min till the depression of IPSC amplitudes was seen), 100 Hz (for 0.5s, 20 pulses to induce PTP) and theta bursts stimulation (see Fig. 3-1). In all experiments, the influence of conditioning

stimulations on E_{GABA} amplitude and conductance of PSCs were examined 30 min post-tetanus, while evoking them at 0.05 Hz. When a conditioning stimulation was given during any experiments, "control" refers to the pre-conditioning response.

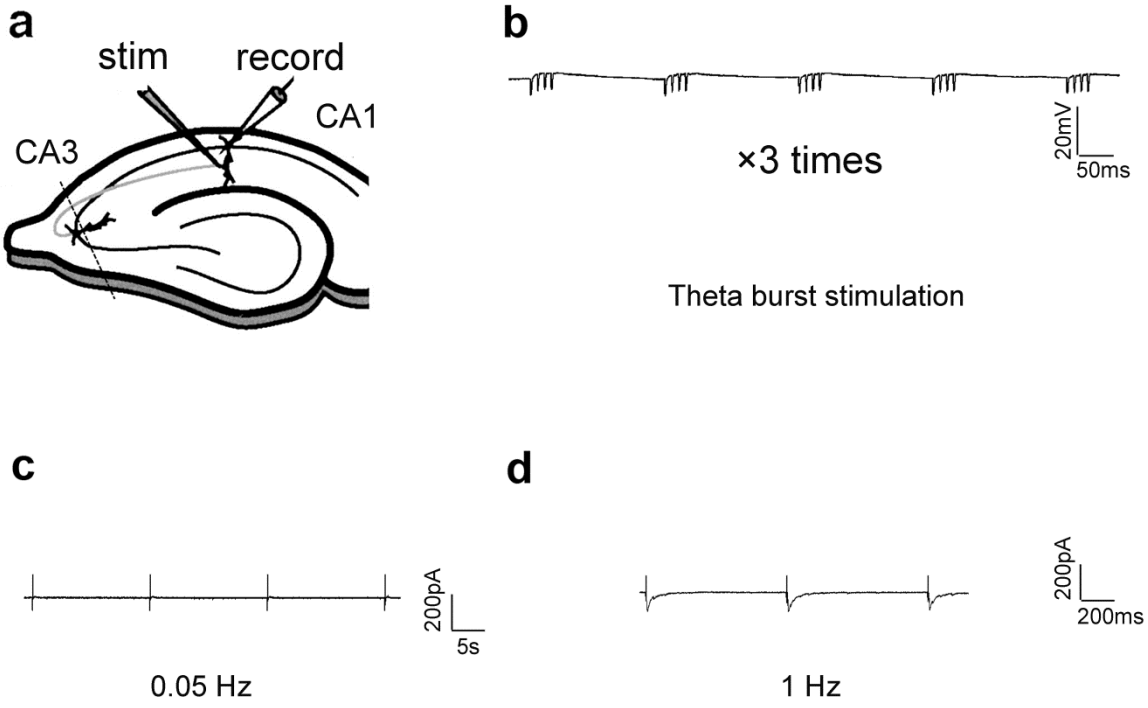

Figure 3-1 Schematic of the slice preparation and various conditioning stimulations used in this study

Panel A shows a schematic illustration about the placement of stimulation and recording electrodes within the hippocampal CA1 region. Whole cell patch clamp recordings were obtained from hippocampal CA1 pyramidal neurons. A surgical cut was made between CA3 and CA1 region to remove the influence of discharges generated by CA3 neurons. Three types of conditioning stimulations used were shown in panels B, C and D. Panel B shows the theta-burst stimulation protocol: 4 pulses at 100 Hz in each burst in a train consisting of 5 bursts with an inter-burst interval of 200 ms; the train repeated thrice at 30 s intervals. Theta burst stimulation was applied while holding the CA1 neuron under current clamp. Panels

C and D represent two recordings obtained during 0.05 and 1 Hz stimulations, respectively; note the different time scales in C & D.

3.4.5 Perforated patch clamp recording

To examine if the cell dialysis with the traditional whole cell patch clamp significantly contributes to changes in amplitudes of the IPSC, perforated patch clamp technique (Kyrozis and Reichling 1995; Yip et al. 1996) were used in one series of experiments in the present study. Electrodes (glass capillaries) with a resistance ~5 MΩ were tip filled with intracellular solution and then backfilled with the same pipette solution used in whole cell patch clamp recordings but containing 50 μg/ml gramicidin D, which was diluted from a stock solution of 50 mg/ml in dimethyl sulfoxide (DMSO). The extracellular solution is the same as used in whole cell patch clamp recordings. APV and DNQX were continually perfused (1.5-2 ml/min) throughout recordings.

The advantage of this technique is that it can greatly reduce cell dialysis, which can happen in the classical whole-cell recording method, by not being permeable to Cl^- with the gramicidin perforated pores (Kyrozis and Reichling 1995). Even though perforated patch recording has been used in some other studies in literature due to its preservation of the intracellular milieu, this technique may not be suitable for my studies on plasticity of E_{GABA} requiring quantitatively stable recordings over prolonged periods of time. It takes much longer time to initiate and stabilize the recording using perforated patch clamp technique compared to the whole-cell clamp method. It takes about 20 min to establish a complete perforated patch configuration. Another 10 min is required for the capacitive transient to be stable. Our studies on changes in E_{GABA} require stable recordings over about 1 hr. With the perforated patch method, the initial stabilization time adds to this experimental time and the health of slices can deteriorate

with the added time. Second, the critical factor in perforated patch recording is timing, which is determined by the amount of normal intracellular solution in the pipette tip. Too much or too less normal intracellular solution filled in the pipette tip will result in unsuccessful perforated patch recordings. Unfortunately, the amount of normal solution is not easy to control by filling the solution into the recording pipette through a tiny tube by hand. Third, due to possible long-lasting actions of antifungals (such as amphotericin B or nystatin) included in recording pipette, the perforated patch recording is not stable compared to whole-cell recording, especially during longer observation periods making quantitative assessment of IPSC amplitudes, needed in our studies, difficult. The recording can be noisy due to a partial loss of the "seal" caused by persistent effects of the antifugals. Finally, it is not possible to intracellularly load agents into the recorded neuron, which is required in several series of experiments in the current study. For these reasons and because of the reliable and stable recordings of IPSCs, when stimulated at 0.05 Hz, using the whole-cell patch recording method, the latter technique was used in our studies.

3.5 Antisense technique

The sequences of KCC2 antisense, sense ODN_S and NKCC1 antisense, sense and scrambled ODNs, phosphorothionated at all positions and purified by high-performance liquid chromatography, were as follows: KCC2 sense, 5'-TGACGGCAATCCCAAGGAGA-3'; KCC2 antisense, 5'-TCTCCTTGGGATTGCCGTCA-3'; NKCC1 sense, 5'-GTCATCACAAGA-AAAGTCACCTGGTACCAAGGATGT-3'; NKCC1 antisense, 5'-ACATCCTTGGTACCA-GGTGACTTTTCTTGTGATGAC-3'); scrambled NKCC1, 5'-TCCATCATATCTGGGATGA-AAGACGAA-GCCCTAAGA -3'). The sequences of KCC2 ODNs were the same as those used in our previous studies (Ouardouz and Sastry 2005) and identical to those tested in a study conducted by Rivera et al. (Rivera et al. 1999). The ODNs were diluted in a weak buffer such as

TE buffer (10 mM Tris, pH 7.5 - 8.0, 1 mM ethylenediaminetetraccetic acid (EDTA)) to reach a stock concentration at 1 μM and reserved in deep freezer at - 80 ℃. Specific intracellular pipette solution containing each ODN targeting KCC2 or NKCC1were made and the final concentration was 100 nM. In our experiments, intracellular pipette solution was directly loaded into the recording electrode before obtaining a whole-cell configuration. After the rupture of the cell membrane, ODNs slowly diffused into the cell plasma. Twenty min following this equilibration procedure between intracellular pipette and cell content, control IPSCs were monitored for 10 min to check for the stability of recordings. Thus, the same procedure as whole cell patch clamp recording was followed.

3.6 Calculation of E_{GABA}

E_{GABA} was calculated by recording the synaptic current during 500 ms voltage pulses applied on the holding potential (-100 or -90 mV to -40 or -30 mV). Steady-state currents were measured close to the end of the 500 ms pulse and were subtracted from the peak IPSC amplitude. The corrected IPSC amplitude was plotted against the holding potential. The reversal potential (E_{GABA}) was then extrapolated from a linear regression of the IPSC amplitude vs. the membrane potential (see Fig. 3-2).

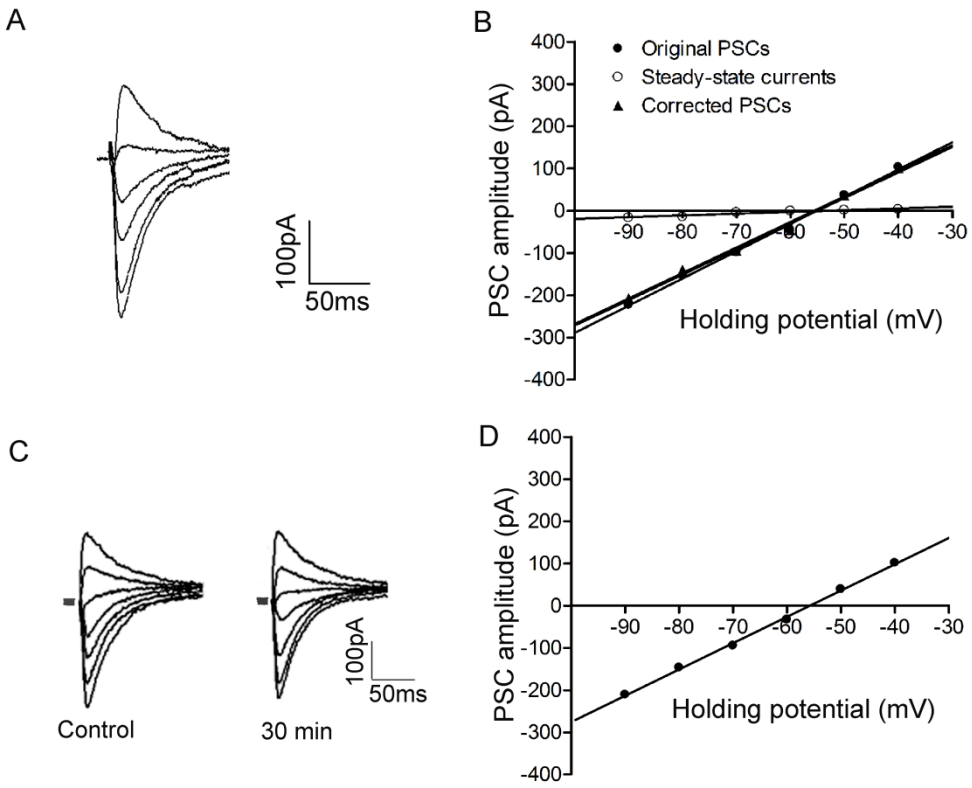

Figure 3-2 Method used for calculation of E_{GABA}

In A, a series of inhibitory postsynaptic synaptic currents (IPSCs) were elicited in a CA1 neuron while holding it at various membrane potentials from -100 or -90 to -40 or -30 mV in the presence of APV and DNQX in the superfusing medium. Steady-state currents were measured close to the end of the 500 ms pulse; the peak IPSC amplitude was measured and, as shown in B, plotted against different holding potentials. C shows that steady-state currents were stable during one recording. In D, the steady-state currents were subtracted from the peak IPSC amplitude and the corrected IPSC amplitude plotted against the holding potentials. The reversal potential (E_{GABA}) is extrapolated from a linear regression of the I-V plot in D. In subsequent figures in the thesis, only the corrected I-V plots are illustrated.

3.7 Western blot analysis

To determine the expression of KCC2 and NKCC1 in both neonatal and juvenile rat

hippocampus, western blot analysis was performed in tissue lysate prepared from 3-5 and 9-12 day old rat hippocampus (CA1 region) as described previously (Rajput et al. 2009). Briefly, hippocampal slices were collected after electrophysiological experiments and homogenized in homogenizing buffer containing (62.5mM Tris-HCl, 50mM dithiothretiol [DTT], 10% glycerol, 2% SDS). Protein concentration in the tissue was estimated using the Bradford protein assay. 20 µg of protein prepared in Laemmli sample buffer was subjected to 7% SDS-PAGE and transferred to nitrocellulose membrane in transfer buffer (20mM Tris, 192mM glycine and 20% methanol). Membrane was blocked with 5% non-fat dried skim milk at room temperature for 1 h and further incubated overnight at 4 ℃ in presence of either NKCC1 or KCC2 primary antibody (at 1:1000 dilutions in 5% bovine serum albumin). The next day morning, the membrane was incubated with peroxidase conjugated goat anti-rabbit secondary antibody at room temperature for 1 h. Bands were detected using a chemiluminescence detection system and photographed on Alpha Innotech FluorChem 8800 (Alpha Innotech Co., USA) gel box imager. β-actin was used as the housekeeping protein for loading control. The bands were quantified using densitometric analysis and protein expression was calculated as the ratio of band of interest to the density of β-actin.

3.8 Immunohistochemistry

Immunohistochemical studies were performed on same slices used for electrophysiological experiments according to the procedure described previously (Rajput et al. 2009). Briefly, slices were immediately collected from electrophysiological experiments and preserved in 4% paraformaldehyde. Post-fixation, the slices were washed with distill water and cryoprotected in 20% sucrose and 40% sucrose solution. Following a wash with cold water, all the sections were

incubated in 1% H_2O_2 for 20 min and 0.2% Triton X-100 for 15 min, then washed 3 times in Tris-Buffered Saline (20 mM) post-incubation in between each treatment. The sections were then blocked with 5% normal goat serum (NGS) for 1h at room temperature. The sections were then incubated with polyclonal KCC2 antibody at 1:800 dilution (or polyclonal NKCC1 antibody at 1:1000 dilution), overnight at 4°C in a humid atmosphere. Following three subsequent washes in Tris-buffered saline, sections were incubated with goat anti-rabbit Alexa-594 secondary antibody for 1h at room temperature. Finally, the sections were mounted on slides, viewed and photographed using Leica IBRE fluorescence microscope (Leica Microsystems, Germany) equipped with a Cool Snap camera.

3.9 Chemicals

Chemicals were stored in concentrated stock solutions and diluted directly using normal-ACSF to their final concentrations. Chemicals used in the present study were shown as below: APV, Baclofen, (2*S*)-3-[[(1*S*)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl]-(phenyl-methyl)phosphinic acid hydrochloride (CGP55845), DNQX, (2S)-a-Ethylglutamic acid (EGLU) and (S)-(+)-a-Amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) were purchased from Tocris (UK) and Bicuculline methiodide, BAPTA, Gramicidin, TPMPA were bought from Sigma (USA). (RS)-a-Methyl-4-carboxyphenylglycine (MCPG) and 2-Methyl-6-(phenylethylnyl)pyridine hydrochloride (MPEP) were from Ascent Scientific (UK). Chemicals (Fisher Scientific, USA) used in the recording medium and sucrose were obtained from the Chemical Store in UBC. KCC2 sense, antisense ODNs, NKCC1 sense, scrambled and antisense ODNs were custom ordered from Invitrogen Life Technologies (Canada). The primary anti-rabbit KCC2 antibody and NKCC1 antibody were purchased from Sigma (USA) and ProteinTech Group Inc. (USA), respectively. Goat anti-rabbit Alexa-594 secondary antibody was

purchased from Invitrogen (Canada).

3.10 Statistical analysis

One cell per slice and up to two cells from one animal, were used for data analysis. Data were analyzed expressed as mean ± SEM. Statistical analysis of the data was performed using a paired Student's t-test or one-way ANOVA. Unless specifically mentioned otherwise in individual sets of experiments, paired Student's t-test was employed in general. The level of significance (p value) was arbitrarily chosen to be <0.05 for both electrophysiological data and western blot analysis.

Chapter 4. Results

4.1 Changes in E_{GABA} are associated with frequency dependent depression of IPSCs in rat hippocampal CA1 neurons

It has been reported that owing to dialysis of the intracellular content into recorded neurons, amplitudes of IPSCs are gradually reduced during low frequency stimulations under the whole-cell patch configuration (Sun et al. 2000). Different frequencies of stimulations have been used to mimic various activities in rat hippocampus in literature (Bolshakov and Siegelbaum 1994; Dudek and Bear 1992; Fiumelli et al. 2005; Fiumelli and Woodin 2007; Kaila et al. 1997; Wang et al. 2006; Woodin et al. 2003). However, in order to find a proper frequency of stimulation to record $GABA_A$-mediated IPSCs under control condition, multiple frequency stimulations was applied in current study: 0.05, 0.1, 0.5 and 1 Hz. E_{GABA} was measured at two time points (before conditioning stimulation and 30 min after conditioning stimulation) at 0.05 Hz. Our data suggest that when stimulated at 0.05 Hz, the magnitude of IPSCs was not changed significantly (see Fig. 4-1A) while it could either be decreased (Fig. 4-1B & C) or even reversed in direction (Fig. 4-1D) under 1 Hz stimulation. The changes in PSC amplitudes and corresponding E_{GABA} during conditioning stimulations were shown in Table 4-1 as well. In our study, under 0.05 Hz stimulation, IPSC amplitudes can be maintained stable over 1 hour (Ouardouz and Sastry 2005; Xu and Sastry 2007; Yang et al. 2010). Therefore, 0.05 Hz stimulation was chosen as the control stimulation in current study.

The possibility that shift in E_{GABA} is related to the number of stimulation pulses was also examined in current study. At 1 Hz stimulation, 300 - 600 pulses were inadequate to significantly shift E_{GABA} ($E_{GABA\text{-}control}$: -58.4±0.8 mV, after 300 pulses: -59.2±0.6 mV, after 600 pulses: -59.3±0.8 mV, n=6, P>0.05), whereas 900 - 1800 pulses induced a further shift (after 900

pulses: -61.2±0.8 mV, after 1800 pulses: -65.5±0.6 mV, n=6, P<0.05). In the case of 0.05 Hz, E_{GABA} was not significantly shifted with increased number of pulses (see Fig. 4-2). Therefore, conditioning stimulation induced shifts in E_{GABA} seem to be associated with the frequency of stimulations but not number of pulses within the conditioning stimulations.

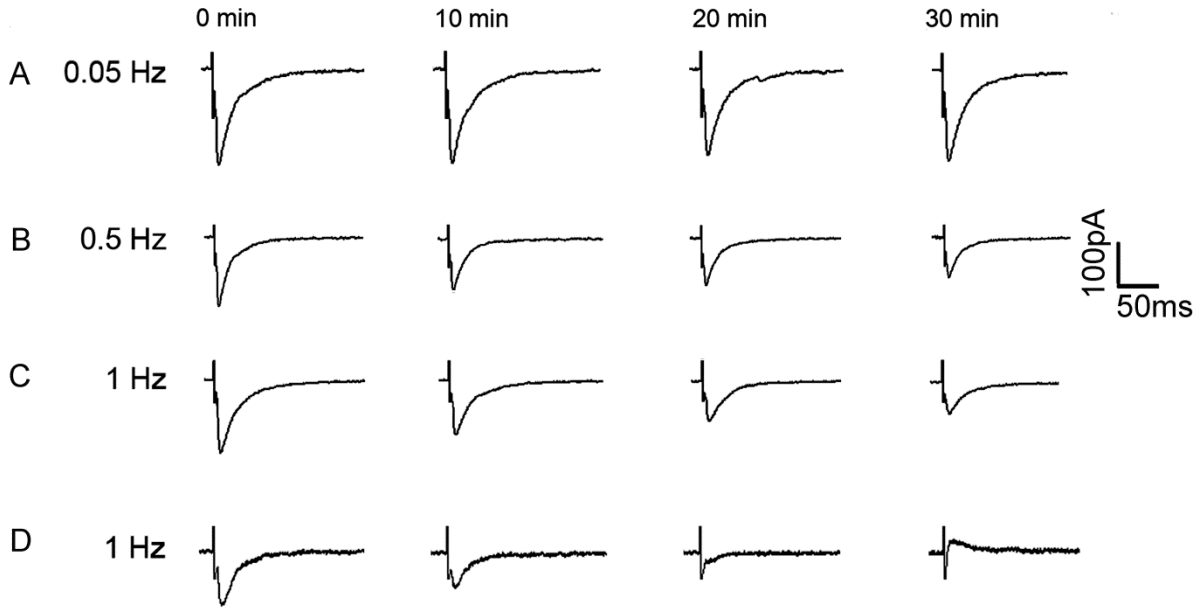

Figure 4-1 The effect of frequency of stimulation on the PSC amplitude

Recordings from four different CA1 pyramidal neurons of rat hippocampus (9-12 day old) are illustrated in A-D. Stimulation in the stratum radiatum was given at 0.05, 0.5 and 1 Hz. The PSC amplitude did not change significantly with 0.05 Hz stimulation (Panel A) but decreased with 0.5 Hz or 1 Hz stimulation (Panels B and C). The direction of PSC, in some cases, was reversed with 1 Hz stimulation (Panel D), pointing to a shift in the reversal potential to the other side of the holding potential. Slices were superfused with APV and DNQX throughout to block the EPSCs. The holding potentials for traces in A-D were -64, -64, -60 and -60 mV, respectively.

Table 4-1 Changes in PSC amplitude, conductance and E_{GABA} during conditioning stimulations in rat hippocampal neurons

Groups	PSC amplitude (as a% of control at 30 min post-conditioning)	PSC conductance (nS)		E_{GABA} (mV)	
		Control (before tetanus)	30 min after tetanus	Control (before tetanus)	30 min after tetanus
0.1 Hz (n=6)	70.2 ±1.3	5.5 ±0.4	4.5 ±0.2	-58.6 ±0.9	-62.0 ±1.0 *
0.5 Hz (n=6)	48.9 ±5.8	5.6 ±0.3	5.0 ±0.3	-57.7 ±0.9	-63.8 ±0.7 *
1 Hz (n=6)	41.9 ±1.4	5.5 ±0.2	5.2 ±0.2	-58.4 ±0.8	-65.6 ±0.6 *

Note: numbers inside brackets refer to *n* in individual studies and * indicates P< 0.05.

To investigate the mechanisms underlying frequency-dependent depression of $GABA_A$ receptor mediated IPSCs, the KCC2 inhibitor furosemide, at a concentration of 500 μM, was applied in another study. The PSC amplitude was stable when stimulated at 0.05 Hz, with no significant change in either the conductance (*g*) of PSC ($g_{psc\text{-}control}$ 6.2 ±0.3 nS, $g_{psc\text{-}30min}$: 6.0 ±0.3 nS, n=6; p>0.05; see Fig. 4-2 B) or E_{GABA} ($E_{GABA\text{-}control}$: -58.2 ±1.9 mV, $E_{GABA\text{-}30min}$: -58.6 ±1.1 mV, n=6; p>0.05; see Fig. 4-2). Bath application of furosemide did not affect either g_{psc} ($g_{psc\text{-}control}$: 6.2 ±0.3 nS, $g_{psc\text{-}30min}$: 5.8 ±0.3 nS, n=6; p>0.05) or E_{GABA} ($E_{GABA\text{-}control}$: -60.1 ±2.1 mV, $E_{GABA\text{-}30min}$: -61.5 ±3.1 mV, n=6; p>0.05; see Fig. 4-2).

In the presence of furosemide, the changes in PSC amplitude and E_{GABA} during 1 Hz conditioning, were significantly suppressed (p<0.05, n=5; see Fig.4 and Table 4-2). Furosemide, at a concentration of 100 μM or above, may have a weak effect on NKCC1 activity (Blaesse et al. 2009). To verify whether NKCC1 is involved in the shift in E_{GABA}, bumetanide was applied in another study. Our data suggest that bumetanide, when applied at 20 μM or 100 μM, did not

have any effects on the changes in PSC amplitudes and E_{GABA}, following 1Hz conditioning when compared with control ($p>0.05$, n=6; see Table 2). Moreover, shifts in E_{GABA} following 1Hz stimulation were parallel with no associated change in conductance in both drugs ($p>0.05$, n=6; see Table 4-2).

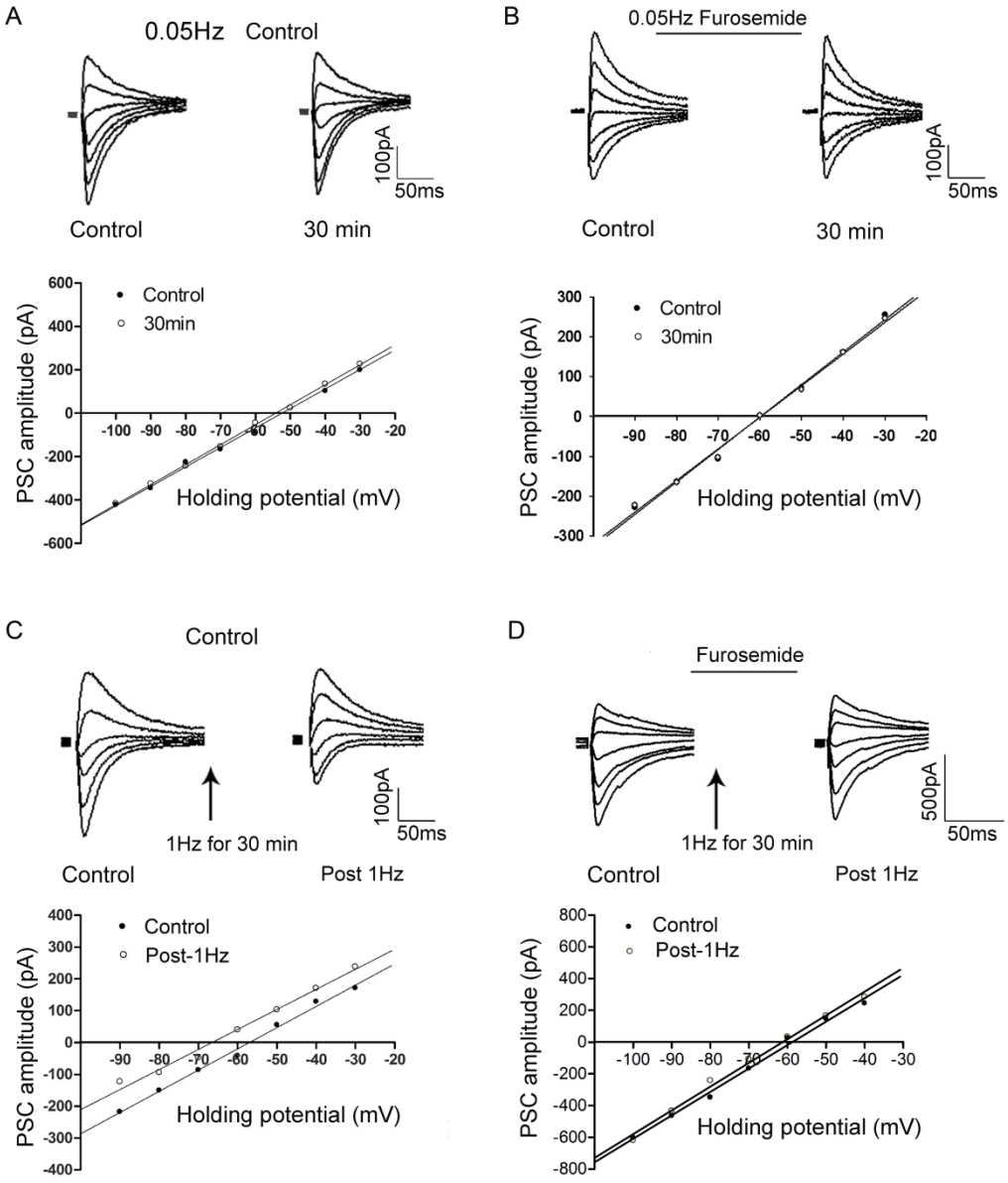

Figure 4-2 Furosemide blocks the depression of the PSC amplitude and the shift in E_{GABA} induced by 1 Hz

Panels A and B show PSC records and I-V plots during 0.05 Hz stimulation, while panels C and D show records taken at 0.05 Hz following a 1 Hz conditioning for 30 min. In A & C, PSCs evoked at different holding potentials (-90 mV to -40 or -30 mV, in 10 mV steps) were shown; PSCs on the left were taken at the beginning of the experiment (control) and those on the right were taken after a 30 min conditioning. In B & D, records were shown as in A & C except that furosemide (500 μM) was present in the superfusing medium. I-V plots on the bottom, in A & B, show that the E_{GABA} was stable after 30 min when stimulated at 0.05 Hz but not after stimulating at 1 Hz (panel C). The I-V plots in D show that furosemide blocks the shift in E_{GABA} induced by 1 Hz. Note that the I-V plots in C are parallel, suggesting that the conductance was not changed and that any apparent change in the amplitude of the PSC is a reflection of the underlying change in E_{GABA}. Data in A, B, C and D were obtained from four different CA1 neurons and the holding potential for each cell was -60 mV. Slices were taken from 9-12 day old rats. Note that, in this and subsequent figures, the representative PSC records and I/V plots illustrate data from individual neurons; quantitative data compiled from several neurons in each study, were shown in the text of the thesis.

Table 4-2 Effects of furosemide and bumetanide on 1 Hz conditioning induced changes in PSC amplitude, conductance and E_{GABA}

Groups	PSC amplitude (as a% of control at 30 min post-conditioning)	PSC conductance (nS)		E_{GABA} (mV)	
		Control (before tetanus)	30 min after tetanus	Control (before tetanus)	30 min after tetanus
Control (n=6)	41.9±1.4	6.4±0.3	6.0±0.3	-58.4±0.8	-65.6±0.6
Furosemide (500 μM) (n=6)	75.2±2.1	6.0±0.1	5.9±0.3	-58.5±1.7	-62.0±2.7*
Bumetanide (20 μM) (n=6)	44.5±1.4	6.1±0.2	5.9±0.1	-58.4±0.8	-65.6±0.6
Bumetanide (100 μM) (n=6)	46.0±1.3	6.0±0.3	5.5±0.1	-59.3±0.4	-65..2±1.1

Note: numbers inside brackets refer to *n* in each individual study and * indicates P< 0.05.

In recent years, substantial advances in antisense technique have been reported in literature. In order to further investigate whether KCC2 is responsible for the negative shift in E_{GABA} under 1 Hz stimulation, antisense KCC2 or sense KCC2 ODNs were used in one series of experiments. In cells loaded with the KCC2 antisense ODNs, the shift in E_{GABA}, following a 0.5 or 1 Hz conditioning stimulation, was not significantly changed ($P>0.05$, see Fig. 4-3 and Table 4-3). However, in cells loaded with KCC2 sense ODNs, both 0.5 and 1 Hz induced a significant shift in E_{GABA} as observed in no drug controls while the PSC conductance was unaffected (see Fig. 4-3 and Table 4-3). These results indicate that changes in KCC2 activity is involved in 0.5 or 1 Hz stimulation-induced negative shift in the E_{GABA}. Therefore, at least, part of the apparent change in the PSC amplitude is secondary to changes in E_{GABA}.

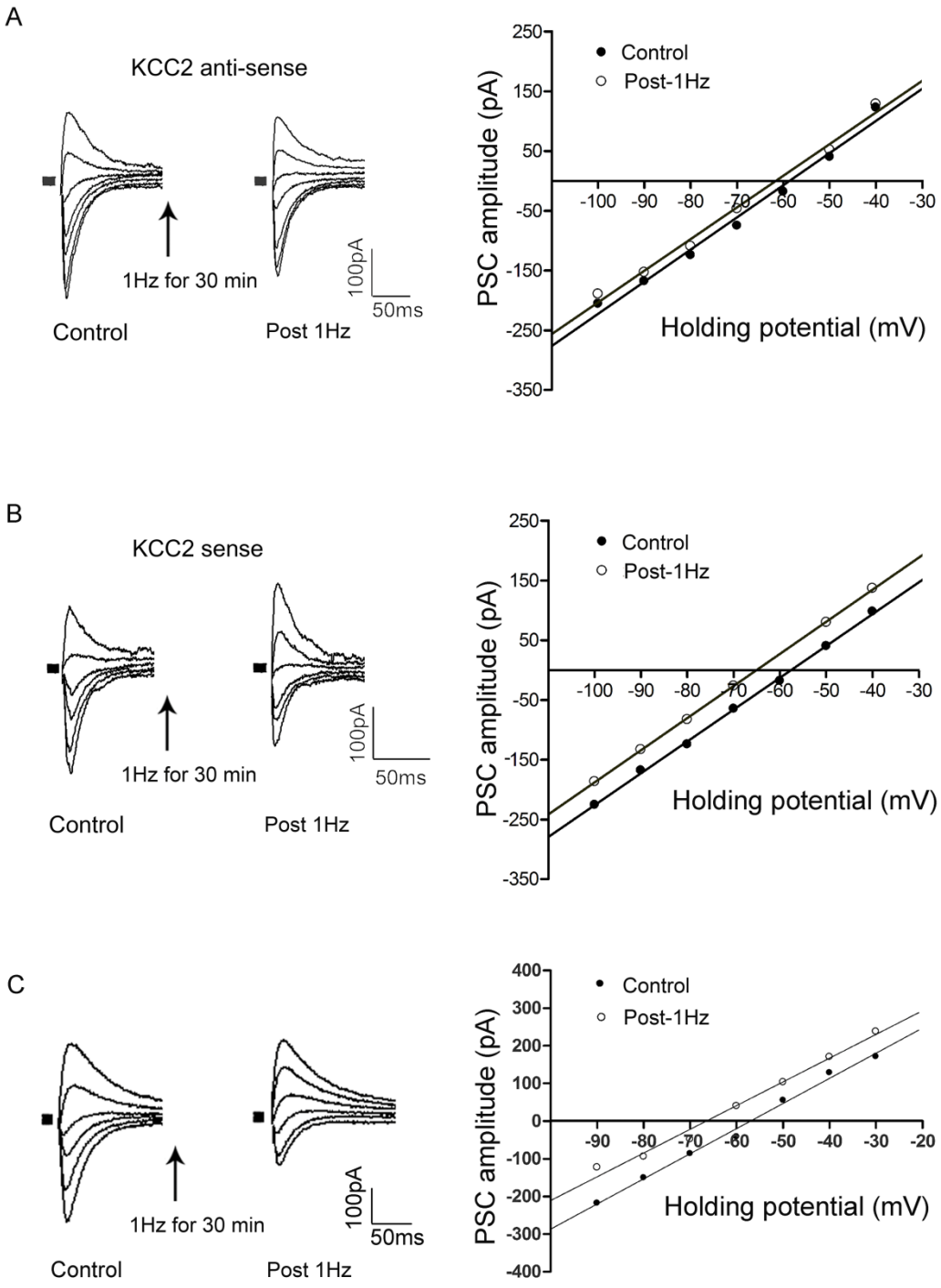

Figure 4-3 KCC2 antisense ODNs block negative shifts in E_{GABA} induced by 1 Hz

KCC2 sense and antisense ODNs were added into the recording patch pipette and were allowed to equilibrate with cell contents for 20 min before recordings were initiated. The records show PSCs evoked at different holding potentials. I-V plots in each case represent PSC amplitudes at different holding

potentials, before and 30 min after the conditioning stimulation. In A and B, the left records are controls and the right ones are taken following a 1 Hz conditioning for 30 min. The effects of KCC2 antisense and sense ODNs were shown in A and B, respectively. In C, E_{GABA} was shifted in the negative direction at 30 min following 1 Hz stimulation in a "control" (no ODNs loaded) neuron. Data in A, B and C were obtained from three different CA1 neurons and the control holding potential for each individual cell was -60 mV. Note that the KCC2 antisense, but not sense ODNs, significantly decreased the shifts in E_{GABA} induced by 1 Hz. Slices were taken from 9-12 day old rats.

Table 4-3 Changes in PSC amplitude, conductance and E_{GABA} during 0.5 and 1 Hz stimulations in KCC2 antisense or sense ODNs loaded hippocampal CA1 neurons

		PSC conductance (nS)		E_{GABA} (mV)	
		Control (before tetanus)	30 min after tetanus	Control (before tetanus)	30 min after tetanus
KCC2 antisense ODNs	0.5Hz (6)	5.9 ±0.3	5.3 ±0.3	-59.6 ±1.3	-61.6 ±1.2
	1Hz (6)	5.6 ±0.4	5.0 ±0.3	-61.1 ±1.2	-63.3 ±1.2
KCC2 sense ODNs	0.5Hz (6)	5.6 ±0.3	5.0 ±0.3	-59.3 ±0.5	-68.3 ±0.9*
	1Hz (6)	5.5 ±0.2	5.2 ±0.2	-58.6 ±0.8	-67.7 ±1.0*

Note: numbers inside brackets refer to *n* in individual study and * indicates $P< 0.05$.

4.2 Changes in E_{GABA} are associated with PTP but not PPD of the IPSC

Following a pair of stimuli of the input, PPD of the IPSC can be induced in hippocampal CA1 neurons when the ISI was between 100 and 1000 ms (see Fig. 4-4). The suppression reached the maximum when ISI was around 200 ms (paired-pulse ratio, PPR: 78.03 ± 2.8, n = 6, $P<0.05$). However, when the equilibrium potential for the first IPSC (E_{IPSC1}) and second IPSC (E_{IPSC2}) were compared, no significant difference was observed (E_{IPSC1}: -58.7 ±0.7 mV, E_{IPSC2}: -

59.3±0.8 mV, n=6; p>0.05) at 200 ms ISI. To further investigate the mechanisms involved the PPD of IPSC in rat hippocampal neurons, both $GABA_B$ antagonist (CGP55845, 2 μM) and $GABA_C$ antagonist (TPMPA, 10 μM) were applied and the effects of these two agents on PPD were compared. CGP55845 did not have a consistent effect on PPD (PPR at 200 ms ISI: 81.5 ± 2.0; n = 6, p>0.05, see Fig. 4-4) while TPMPA, on the other hand, reversed the PPD at higher ISIs beyond 200 ms (PPR at 200 ms ISI: 104.1 ± 1.9, n = 6, p<0.05, see Fig. 4-4) and even reversed PPD into facilitation at lower inter-pulse intervals (100-500 ms, see Fig. 4-4). The effect of TPMPA was reversible and was abolished 10 min after the washout of this agent (PPR at 200 ms ISI, 12 min post-TPMPA: 81.9 ± 1.2, n=6). 15 min after the washout of CGP55845, PPR was 82.9 ± 2.0, which is comparable to control (PPR: 78.0 ± 2.8, P>0.05). Those data suggest that PPD of the IPSC induced in this condition was caused by the activation of $GABA_C$ receptor but not that of $GABA_B$ receptors and changes in E_{GABA} seem not to be involved in this process.

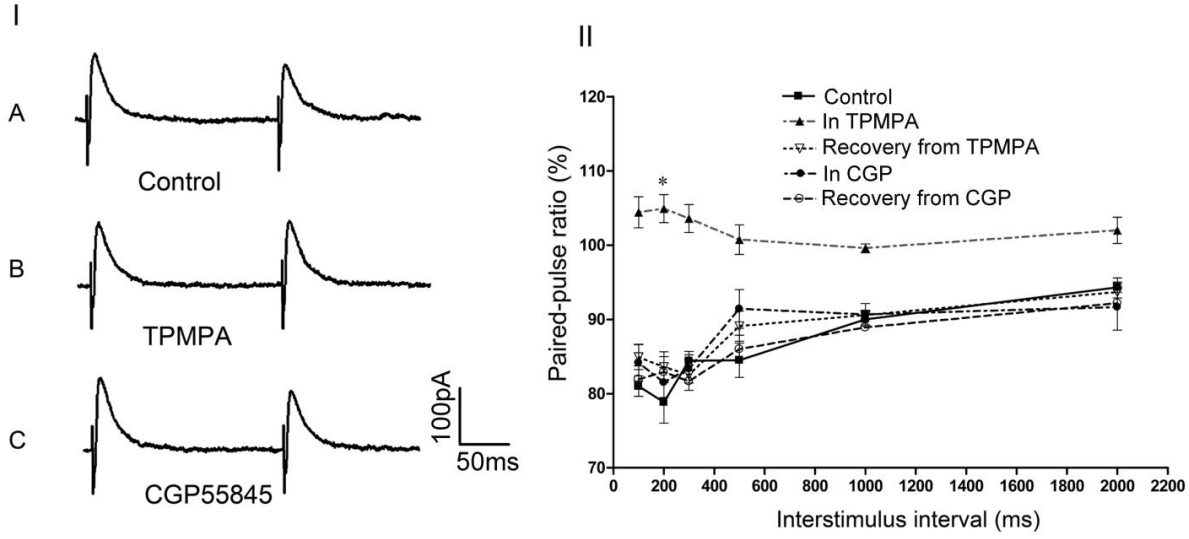

Figure 4-4 TPMPA reverses PPD of the IPSC in rat hippocampal neurons

In panel I, three records A, B and C show PPD induced at 200 ms ISI. A shows the control IPSC record in which the second IPSC was depressed to 85% of the first. The record in B was taken during the

application of TPMPA (10 μM); note the blockade of PPD by TPMPA. C shows PPD during the application of CGP 55845 (2 μM); the PPD ratio was comparable to that in control (see A). In panel II, the graph shows the time course of PPD and effects of TPMPA and CGP55845 on PPD of the IPSC induced in rat hippocampal CA1 neurons. Note a significant blockade of PPD by TPMPA but not by CGP55845 when ISI was 200 ms (n=6 for each group, $P<0.05$, one-way ANOVA). These results indicate that PPD is due to the activation of $GABA_C$ receptors.

PTP refers to a short-term (about 2 min) increase in the amplitude of postsynaptic responses after a tetanic stimulation. Following 100 Hz stimulation (0.5 s) of the input, PTP of the IPSC can be induced in hippocampal CA1 neurons during 0.05 Hz stimulation (see Fig. 4-5). In order to further investigate whether activity of two Cl^- cotransporters (KCC2 or NKCC1) change and subsequent shifts in E_{GABA} occur during the observation of PTP, we applied furosemide (500 μM, KCC2 inhibitor) and bumetanide (10 μM, NKCC1 inhibitor) into superfusion medium throughout the recordings. Intriguingly, in furosemide treated neurons, PTP was significantly higher (post-tetanus response as % of control: 163.8 ±14.3%; n=7; $p<0.05$) than in either the control (122.2 ± 6.8%; n=13; $p<0.05$) or bumetanide (125.7 ± 9.3%; n=6; $p<0.05$, see Fig. 4-5) treated hippocampal CA1 neurons. Since PTP of the IPSC induced under this condition only last for less than 2 min, we could not be able to check E_{GABA} in such a short period of time. Taken together, those data suggest that PTP of the IPSC was masked by shifts in E_{GABA} following HFS of the input of hippocampal CA1 neurons.

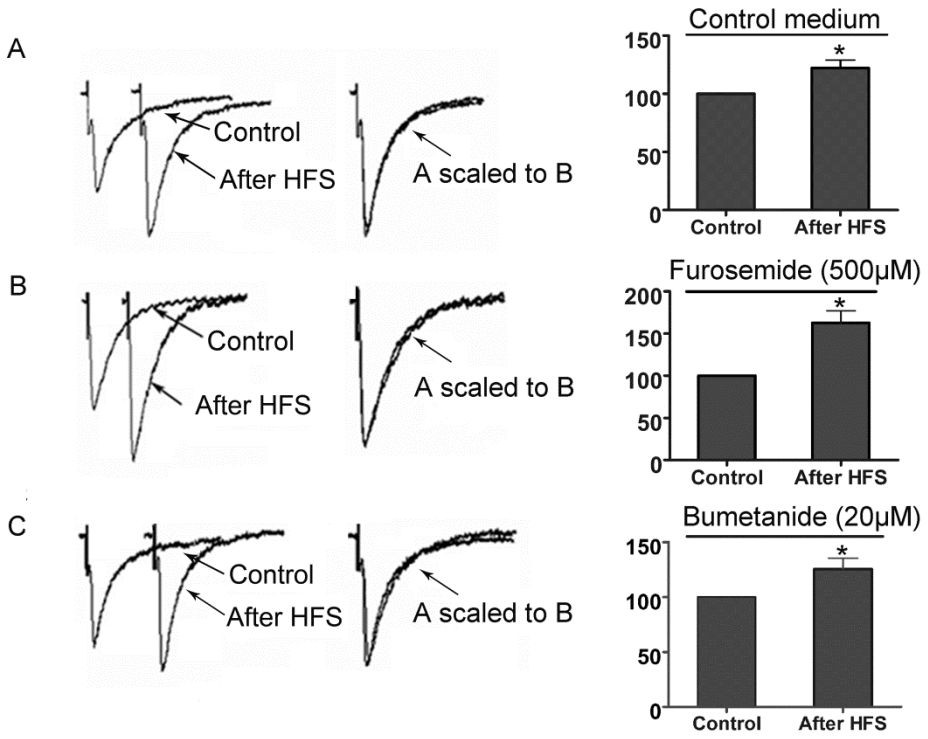

Figure 4-5 Effects of furosemide and bumetanide on high frequency stimulation-induced post-tetanic potentiation of the IPSC.

Panel A shows responses from control cells (not exposed to furosemide or bumetanide, n=13). Panels B and C represent records from furosemide (500 µM, n=7) and bumetanide (20 µM, n=6) treated cells, respectively. Note that furosemide (B), but not bumetanide (C), enhanced PTP when compared to control (A), suggesting that during PTP, there is a concomitant alteration in E_{GABA} which makes the PTP look smaller than it really is. PTP records were taken 2 min after the conditioning high frequency tetanus (HFS, 100 Hz, 20 pulses). Slices were taken from 9-12 day old rats.

4.3 Activation of $GABA_B$ or $GABA_C$ receptors does not influence E_{GABA} in rat hippocampal CA1 neurons under control stimulations

During one of above studies on the possible involvement of shifts in E_{GABA} in short-term plasticity of IPSCs (PTP and PPD), we found that $GABA_C$ receptors but not $GABA_B$ receptors seem to be involved in PPD of IPSCs under that condition. Even though $GABA_C$ receptor

antagonist TPMPA and $GABA_B$ receptor antagonist CGP55845 didn't show significant effects on E_{GABA} during PPD, it is not known whether those agents have effects on both amplitudes of IPSCs and E_{GABA} during control stimulation (0.05 Hz). Baclofen, a widely used agonist at $GABA_B$ receptors, activates both pre- and postsynaptic $GABA_B$ receptors. It is well known that baclofen decreases the amplitudes of $GABA_A$-mediated IPSCs, which is suggested to be mostly caused by activation of presynaptic $GABA_B$ receptors (Iyadomi et al. 2000; Mouginot et al. 1998). Whether postsynaptic factors, such as shifts in E_{GABA}, also contribute to this apparent decrease in amplitudes of IPSCs is not clear. Therefore, the involvement of $GABA_B$ receptor in the modulation of E_{GABA}, and thus amplitudes of IPSCs, were assessed in this control stimulation.

After application of baclofen (10 μM) for 2 min, the amplitudes of IPSCs were significantly decreased (IPSC amplitude as a % of pre-drug control: 54.5 ±4.1, n=6, $P<0.05$, see Fig. 4-6.) while E_{GABA} did not change significantly ($E_{GABA\text{-}control}$: -57.9 ±0.9 mV, $E_{GABA\text{-}30min}$: -58.9 ±0.9 mV, n=6; $p>0.05$). The co-application with CGP55845 (2 μM) reverses the decrease in fast IPSC amplitude (IPSC amplitude as a % of pre-drug control: 97.3 ±1.2, n = 6, $P>0.05$). E_{GABA} was not markedly changed after application of CGP55845 ($E_{GABA\text{-}control}$: -58.7 ±0.6 mV, $E_{GABA\text{-}30min}$: -59.9 ±0.5 mV, n=6; $p>0.05$) alone. These data suggest that change in E_{GABA} was not involved in modulation of amplitudes of fast IPSC by $GABA_B$ receptors during control stimulation (0.05 Hz).

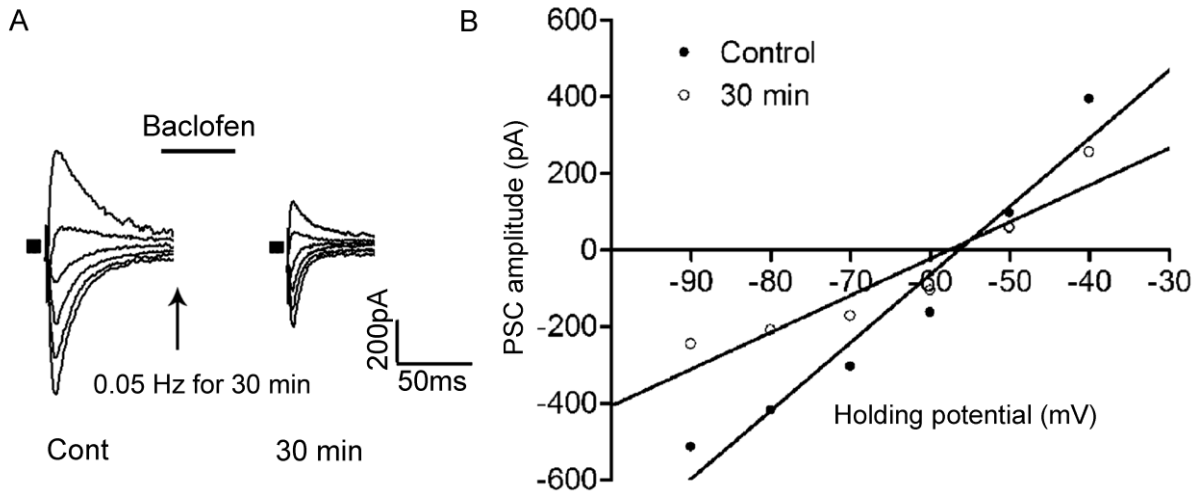

Figure 4-6 Baclofen decreases the amplitudes of IPSCs without changing E_{GABA}

In A, PSCs on the left were taken at the beginning of the experiment (control) and those on the right were taken 30 min after application of baclofen. The IPSC was evoked at 0.05 Hz. Bacolfen (10 μM), was applied for 5 min in the superfusion medium. In B, the I-V plots show that the E_{GABA} was not changed 30 min following application of baclofen. Note that the conductance was changed and outward rectification was seen when holding potentials range from -80 to -60 mV. This outward rectification could be observed in some hippocampal CA1 neurons when clamped at potentials close to resting membrance potential (Schwartzkroin and Kunkel 1982). Probably, this phenomenon becomes more evident due to the decrease in the amplitude of IPSCs caused by baclofen. Slices were taken from 9-12 day old rats.

It has been reported in literature that the activation of $GABA_C$ receptor leads to changes in IPSC amplitude in rat collicular slices when GABA was applied exogenously at a higher concentration (100 μM) (Kirischuk et al. 2003). However, the modulation effect of $GABA_C$ receptors on $GABA_A$-mediated IPSC in hippocampal CA1 neurons is unknown. In one set of experiment in current study, application of CACA (10 μM, $GABA_C$ agonist) for 5 min did not depress the amplitude of fast IPSC (IPSC amplitude as a % of pre-drug control: 99.9 ± 3.2, n = 6, $P>0.05$). Ten min following the washout of this agent, TPMPA, at a concentration of 10 or 20

μM, was applied in the superfusion medium for 5 min and did not suppress the fast IPSC either at both concentrations. In fact, the amplitudes of IPSCs were slightly increased by 10 μM TPMPA (IPSC amplitude as a % of pre-drug control: 103.5 ± 1.8, n = 6) and significantly potentiated by 20 μM TPMPA (IPSC amplitude as a % of pre-drug control: 114. 7 ± 2.9, n = 6, $p<0.05$, see Fig. 4-7). Moreover, E_{GABA} was not significantly changed by application of either CACA or TPMPA under control stimulation, indicating that change in E_{GABA} does not contribute to $GABA_C$ receptor mediated modulation of fast IPSC.

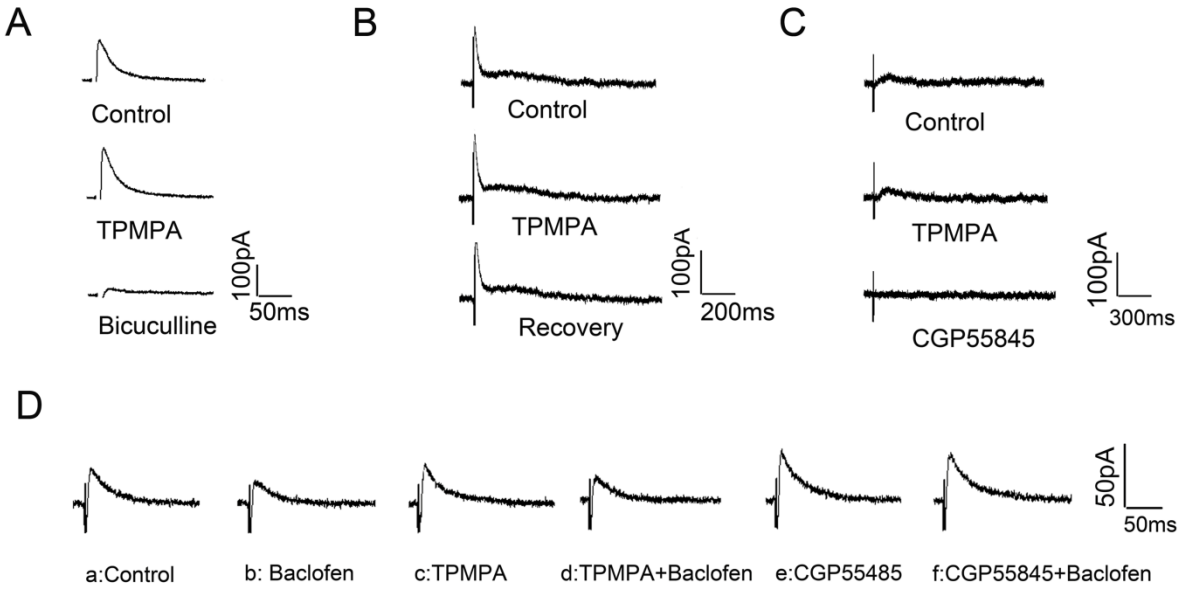

Figure 4-7 TPMPA does not block $GABA_A$ and $GABA_B$ responses in juvenile hippocampal CA1 neurons

In A, TPMPA (20 μM) slightly decreases the amplitude of the fast IPSC (the middle record) which was completely blocked by bicuculline (5 μM) (the bottom record), suggesting that the fast IPSC is mediated by $GABA_A$ receptor. In B, those three traces showed that TPMPA does not suppress either the fast or the slow IPSC in another hippocampal CA1 neuron. The three records (from top to bottom) were taken from the pre-drug control, during TPMPA application and 10 min after the washout of TPMPA, respectively. In C, CGP 55845 (1 μM), but not TPMPA, blocks the slow IPSC recorded in another CA1 pyramidal

neuron. The slice was superfused throughout with ACSF containing bicuculline to block the fast IPSCs. In D, CGP55845, but not TPMPA, antagonizes the depressant effect of baclofen (10 μM) on the fast IPSCs. The traces from left to right are: a) the no drug control, b) the effect of baclofen, c) the effect of TPMPA alone, d) the combination effect of baclofen and TPMPA, e) the effect of CGP55845 alone, f) the combination effect of CGP55845 and baclofen. For this experiment, Cs methyl sulfonate was included in in the recording patch-pipette in replace of K gluconate to block the postsynaptic effect (outward current) of baclofen. Taken together, those data indicate that TPMPA does not antagonize both $GABA_A$ and $GABA_B$ receptors. APV (50 μM) and DNQX (20 μM) were present in the superfusing medium throughout the experiments.

In addition to $GABA_B$ and $GABA_C$ receptors, the effect of $GABA_A$ receptor modulator diazepam was investigated as well. Diazepam (5 μM), as a $GABA_A$ receptor allosteric modulator, increased the amplitude of IPSC and prolonged the IPSC decay (see Fig. 4-8). However, E_{GABA} was not significantly changed ($E_{GABA\text{-}control}$: -58.3±0.7 mV, $E_{GABA\text{-}30min}$: -59.6±0.6 mV, n=6; $p>0.05$), suggesting the enhancement of IPSC amplitudes by diazepam is not due to changes in E_{GABA}.

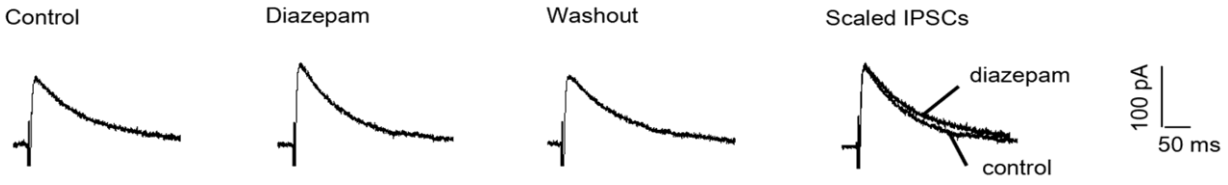

Figure 4-8 Effects of diazepam on IPSCs in hippocampal CA1 neurons

Individual records from a hippocampal CA1 neuron are shown. Note that diazepam (5 μM) enhanced the peak amplitude of the IPSC. The last record shows superimposition of scaled IPSCs in diazepam with the

no-drug control records. Note the prolongation of the decay of the IPSC by diazepam. Slices were taken from 9-12 day old rats.

4.4 TBS induces a negative shift in E_{GABA} in juvenile rat hippocampal neurons

Previous studies in our laboratory suggest that TBS in the stratum radiatum induced a negative shift in the E_{GABA} in hippocampal CA1 neurons (Xu and Sastry 2007). In literature, there are certain concerns about the application of whole cell patch clamp recording for documentation of E_{GABA}. Some investigators argued that the diffusion of intracellular pipette solution into the recorded neuron may cause a disturbance on intracellular Cl^- gradient and hence leading to errors in the calculation of any changes in E_{GABA}. However, a previous study from our laboratory on activity-mediated changes in E_{GABA} by sharp electrode recording did not yield significant differences from whole cell patch clamp recording (Ouardouz et al. 2006). Moreover, in order to further examine whether the shift in E_{GABA} observed following TBS of the input of in hippocampal neurons was just an "artifact" brought by whole cell patch recording, gramicidin perforated patch clamp was used as well in one series of experiment, in the current study. In gramicidin D (50 μg/ml) loaded hippocampal CA1 neurons, following TBS of the input, the E_{GABA} was shifted in the negative direction ($E_{GABA\text{-}control}$: -56.3±1.1, $E_{GABA\text{-}30min}$:-61.0±0.8 mV, n=6; $p<0.01$, see Fig. 4-9) while PSC conductance was not significantly changed ($g_{psc\text{-}control}$: 5.9±0.3 nS, $g_{psc\text{-}30min}$: 5.5±0.4 nS, n=6; $p<0.05$). In addition, E_{GABA} was not changed in the presence of gramicidin D ($E_{GABA\text{-}control}$: -56.3±1.1, $E_{GABA\text{-}before\ TBS}$:-56.6±1.0 mV, n=6; $p<0.01$, see Fig. 4-9).

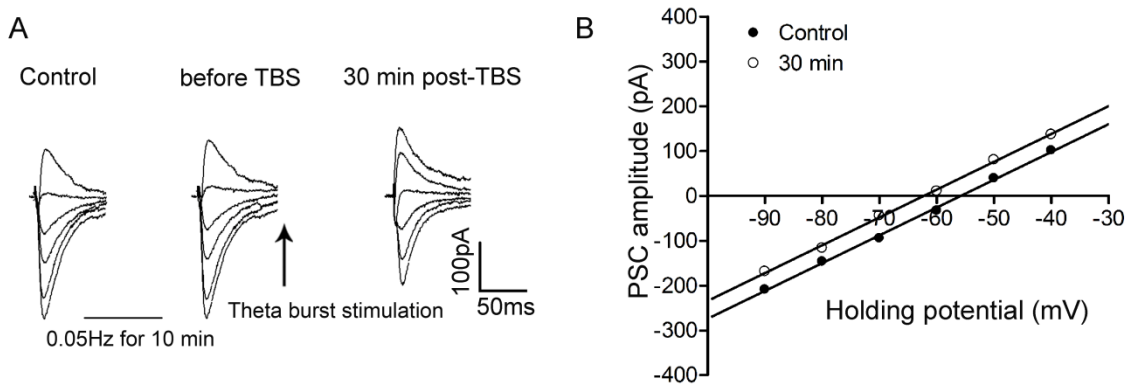

Figure 4-9 Theta burst stimulation induces a negative shift in E_{GABA} in perforated patch clamp recording.

The recording pipette tip was filled with gramicidin (50 nM). In A, the three PSC records were taken from the starting of recording, 1 min before and 30 min following TBS. Note that E_{GABA} was shifted in a negative direction 30 min following TBS. The slices were taken from 9-12 day old rats.

Another concern of some researchers on the study of changes in E_{GABA} is the possible involvement of HCO_3^-. It is worthwhile noting that certain stimulation protocols can lead to a breakdown of the Cl^-/HCO_3^- gradient across neurons. This may account for depolarizing $GABA_A$ receptor mediated responses, especially in dendrites, which possess a low ratio of volume-to-receptor density (Staley et al. 1995). Acetazolamide, a carbonic anhydrase inhibitor (Staley et al. 1995), was used to eliminate the effects of HCO_3^- on E_{GABA} in two series of experiments. However, the presence of acetazolamide (2 μM) did not alter the switch in the E_{GABA} caused by theta-bursts ($E_{GABA\text{-}control}$: -56.6±1.3 mV, $E_{GABA\text{-}30min}$: -62.7±1.5 mV, n=5; $p<0.05$) or 1Hz stimulation of the PSC ($E_{GABA\text{-}control}$: -56.9±1.7 mV, $E_{GABA\text{-}30min}$: -63.9±2 mV, n=5; $p<0.05$, see Fig. 4-10). Therefore, theta-bursts or 1 Hz conditioning induced shifts in E_{GABA} are not due to changes in transmembrane HCO_3^- gradient in recorded hippocampal neurons.

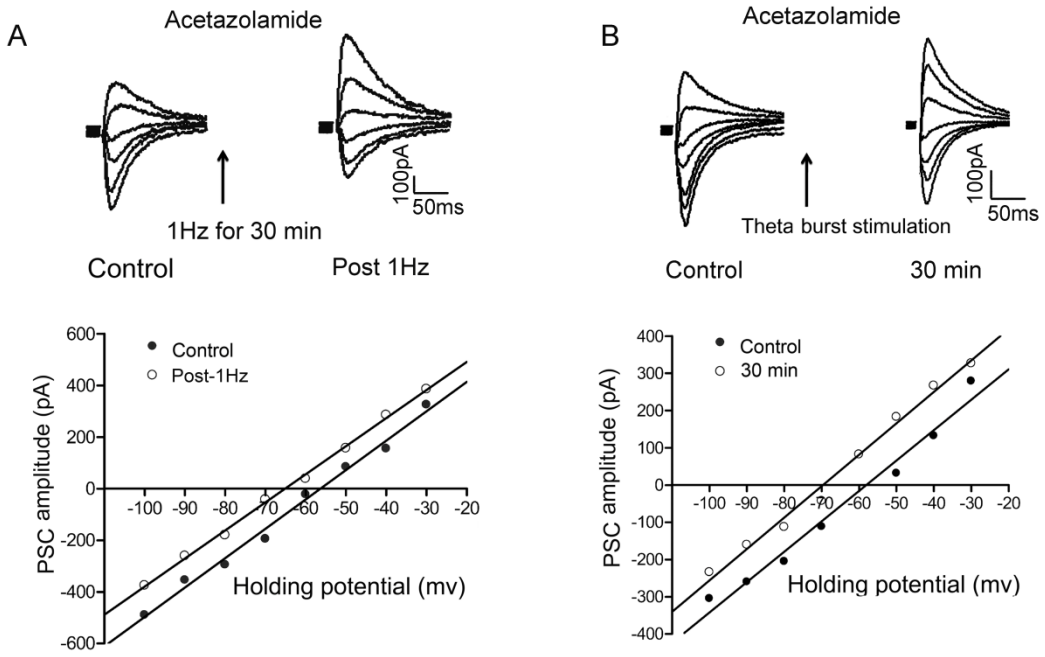

Figure 4-10 Effects of acetazolamide on 1 Hz stimulation- and TBS-induced shifts in E_{GABA}

Data from experiments involving 1 Hz stimulation are shown in panel A, and data from those involving TBS are shown in panel B. In each panel, the records on the left are controls and those on the right are taken 30 min following the conditioning stimulation. Acetazolamide (2 μM**,** a carbonic anhydrase inhibitor) was present throughout in all experiments. Note that acetazolamide did not affect the shifts in E_{GABA} induced by either conditioning stimulation, indicating that the shifts are not due to changes in bicarbonate. Data in A, B were obtained from two different CA1 neurons. The holding potential for each cell was -60 mV. The slices were taken from 9-12 day old rats.

In order to further investigate whether activation of $GABA_B$ receptors or G-protein is involved in TBS-induced shift in E_{GABA} in juvenile neurons, we applied CGP55845 (one potent $GABA_B$ receptor antagonist) and GDP-β-S (a G protein inhibitor) in this following study. TBS induced a negative shift in the E_{GABA} in control (no drug treatment) hippocampal CA1 neurons ($E_{GABA\text{-}control}$: -57.4±1.3 mV, $E_{GABA\text{-}30min}$: -65.2±2.1 mV, n=5; $p<0.05$), without changing the PSC conductance ($g_{psc\text{-}control}$: 6.0±0.4 nS, $g_{psc\text{-}30min}$: 6.0±0.5 nS, n=5; $p>0.05$; see Fig. 4-11A). The

control E_{GABA} was unaffected by 2 μM CGP55845 (pre-drug E_{GABA}: -59.3±1.2; E_{GABA} in drug: -58.2±1.6; n=6; p>0.05). However, CGP55845, when present in the superfusing medium, blocked the TBS-induced shifts in E_{GABA} ($E_{GABA\text{-}control}$: -59.3±1.2 mV, $E_{GABA\text{-}30min}$: -59.9±1.6 mV, n=6; p>0.05, see Fig. 4-11B); the PSC conductance was unaffected ($g_{psc\text{-}control}$: 6.1±0.4 nS, $g_{psc\text{-}30min}$: 6.0±0.3 nS, n=6; p>0.05). As a non-hydrolyzable GDP analog, GDP-β-S competitively inhibits G-protein activation by GTP and GTP analogs (Silk et al. 1989). Interestingly, if in the recording pipette, GTP was replaced with 100 μM GDP-β-S, theta-bursts failed to shift the E_{GABA} ($E_{GABA\text{-}control}$: -58.3±1.9, $E_{GABA\text{-}30min}$: -57.9±1.5 mV, n=7; p>0.05, see Fig. 4-11C.) without apparent changes in PSC conductance ($g_{psc\text{-}control}$: 6.4±0.3 nS, $g_{psc\text{-}30min}$: 6.2±0.2 nS, n=7; p>0.05). Taken together, TBS-induced negative shift in E_{GABA} in juvenile hippocampal neurons requires the activation of $GABA_B$ receptors and G-proteins.

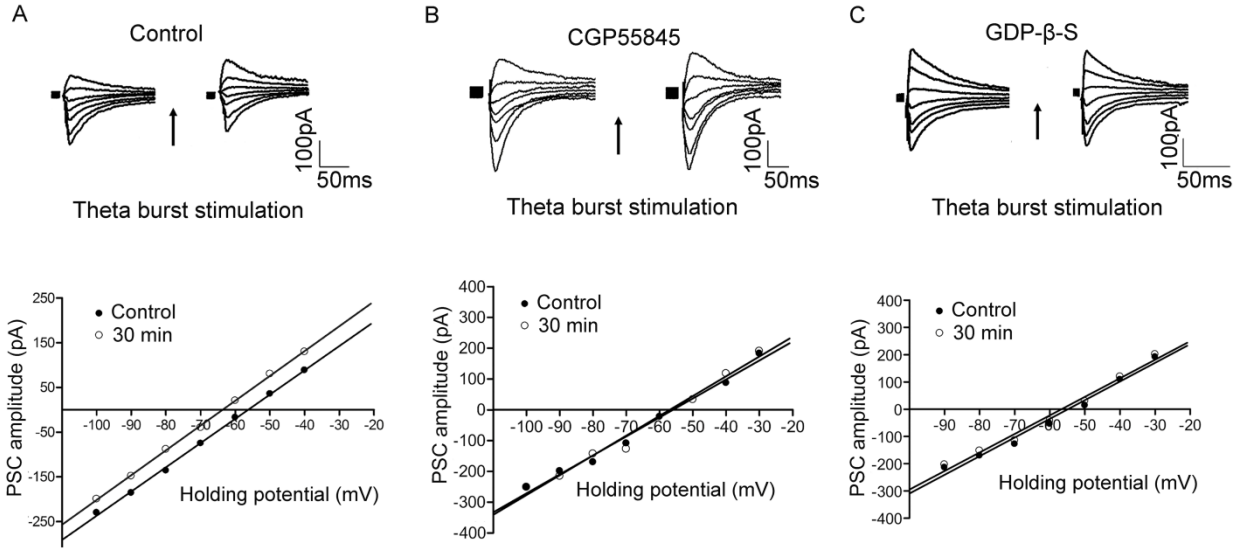

Figure 4-11 The involvement of $GABA_B$ receptors and G-proteins in TBS-induced shifts in E_{GABA}.

A shows no-drug control data; E_{GABA} was significantly shifted 30 min following theta burst stimulation; In B, a 10 min application of 2 μM CGP55845 ($GABA_B$ antagonist) blocked the induction of TBS-induced shift in E_{GABA}; In C, intracellularly loaded GDP-β-S (a G-protein inhibitor, 100 μM) reversed the

shifts in E_{GABA} caused by the theta burst stimulation. Data in A, B and C were obtained from three different CA1 neurons that were voltage-clamped at -60 mV. The slices were taken from 9-12 day old rats.

Our laboratory previous study (Xu and Sastry 2007) shows that furosemide blocks TBS-induced negative shift in E_{GABA} in juvenile rat hippocampal neurons. Since furosemide can also inhibit NKCC1 activity, the antisense technique was applied in this study to test whether change in KCC2 activity is responsible for TBS-induced shift in E_{GABA}. Sense and antisense ODNs were added to the recording patch pipette and were allowed to equilibrate with cell contents for 20 min before recordings were initiated. In cells loaded with the KCC2 antisense ODNs, theta-burst conditioning failed to induce a negative shift in E_{GABA} ($E_{GABA\text{-}control}$: -56.3±1.8 mV, $E_{GABA\text{-}30min}$: -58.4±1.9 mV, n=5; p>0.05; see Fig. 4-12); PSC conductance was unchanged ($g_{psc\text{-}control}$: 5.9±0.3 nS, $g_{psc\text{-}30min}$: 5.7±0.3 nS; n=5; p>0.05). However, if the recording pipettes contained KCC2 sense ODNs, TBS-induced shift in E_{GABA} was comparable to that in no-drug controls ($E_{GABA\text{-}control}$: -57.6±1.2 mV, $E_{GABA\text{-}30min}$: -64.0±1.8 mV, n=6; p<0.05, see Fig. 4-12), and the PSC conductance remained unchanged ($g_{psc\text{-}control}$: 5.9±0.2 nS, $g_{psc\text{-}30min}$: 5.4±0.2, n=6; p>0.05). KCC2 sense and antisense ODNs did not affect the resting properties of the neuron such as input resistance, membrane potential and spike-firing behavior. Those results provide more direct evidence that changes in KCC2 activity are responsible for TBS-induced negative shift in E_{GABA} in juvenile rat hippocampal neurons.

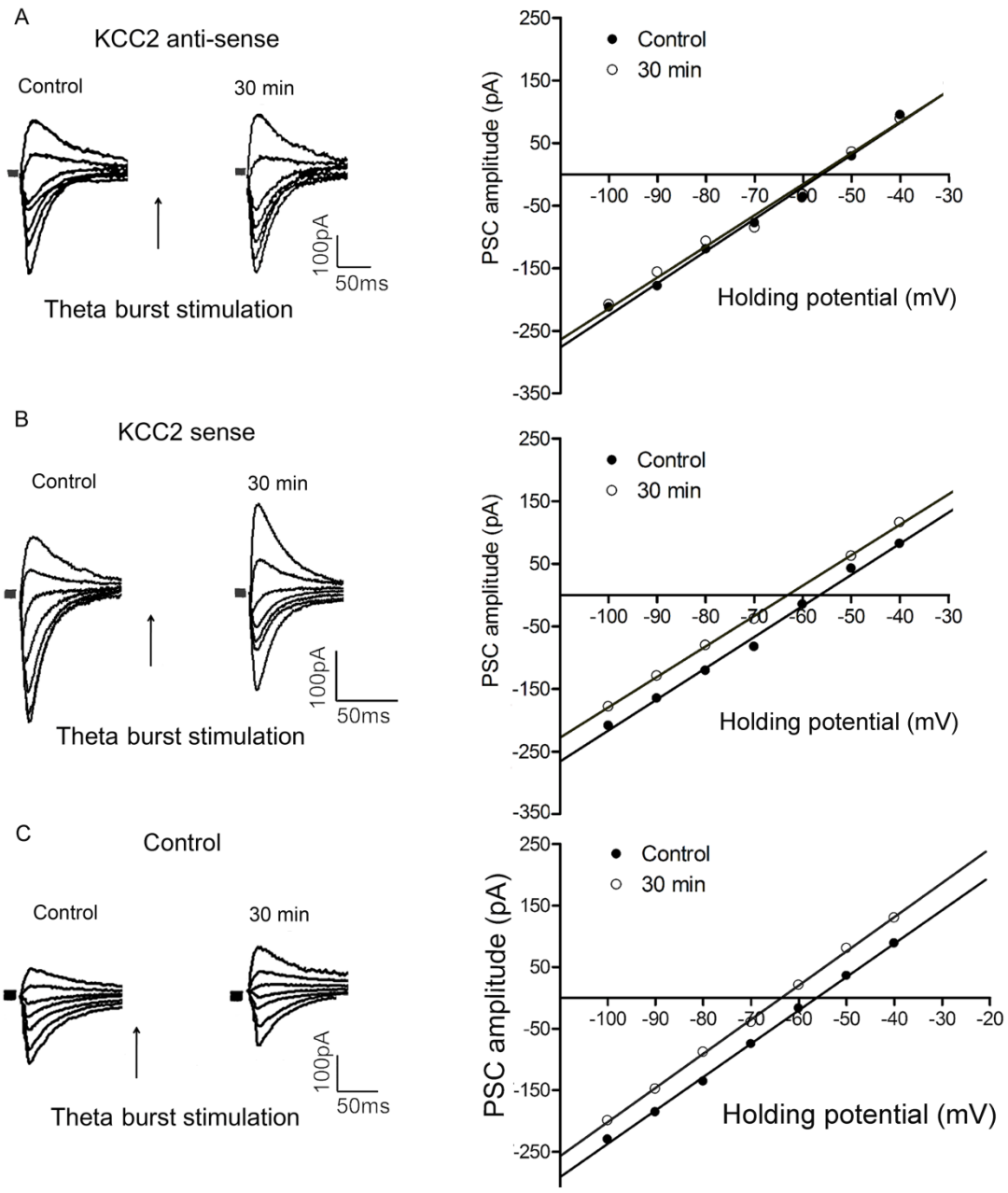

Figure 4-12 KCC2 antisense blocks the negative shifts in E_{GABA} induced by theta burst stimulation

Methods on the application of KCC2 sense and antisense ODNs are the same as shown in Fig. 4-3. In each panel, the left PSC records are controls and the right ones, are taken following TBS for 30 min. The effects of KCC2 antisense and sense were shown in A and B, respectively. Panel C shows the TBS-induced shift in E_{GABA} in one "control" (no ODNs loaded) neuron. Data in A, B and C were obtained from three different CA1 neurons and holding potential for each individual cell was -60 mV. Note that KCC2

antisense, but not sense ODNs, blocked the TBS-induced shift in E_{GABA}. Slices were taken from 9-12 day old rats.

To investigate the involvement of postsynaptic Ca^{2+} in the TBS-induced shifts in E_{GABA} in juvenile hippocampal CA1 neurons, high BAPTA (10 mM)-containing intracellular pipette was used in one study. Thirty min following TBS of the input, E_{GABA} was not markedly shifted ($E_{GABA\text{-}control}$: -58.7±0.6, $E_{GABA\text{-}30min}$: -59.9±0.5 mV, n=6; $p>0.05$). However, the PSC conductance was significantly changed ($g_{psc\text{-}control}$: 6.2±0.2 nS, $g_{psc\text{-}30min}$: 4.2±0.2 nS, n=6; $p<0.05$), indicating other factors (such as decrease in Cl^- permeability or decrease in Ca^{2+}-induced Cl^- conductance) may also contribute to TBS-induced shift in E_{GABA} in juvenile rat hippocampal neurons.

Phosphorylation and dephosphorylation of KCC2 has been suggested to be involved in the corresponding down-regulation or up-regulation of KCC2 in central neurons (Fiumelli et al. 2005). Compared to phosphorylation of KCC2 protein, little is known about the effects of dephosphorylation of KCC2 protein on its activity. To examine this possible involvement of dephosphorylation factors in the regulation of TBS-induced shifts in E_{GABA} in juvenile neurons, okadaic acid, a PP1 and PP2A inhibitor, was used to pre-treat slices one hour before recording. Thirty min following TBS of the input, E_{GABA} was markedly shifted ($E_{GABA\text{-}control}$: -56.0±1.5, $E_{GABA\text{-}30min}$: -62.2±0.9 mV, n=6; $p<0.05$) in hippocampal CA1 neuron which is comparable to no drug control condition, indicating dephosphorylation of KCC2 seems not to be involved in this phenomenon.

4.5 TBS induces a positive shift in E_{GABA} in neonatal rat hippocampal neurons

In recording neonatal neurons, control E_{GABA} was measured by evoking the PSC at the same frequency (0.05 Hz) as used in juvenile hippocampal neurons. Thirty min after TBS of the input, E_{GABA} was shifted in a positive direction in immature neurons (from -53.5±0.5 mV to -49.1±1.0 mV; n=6, P<0.05, see Fig. 4-13 C) in contrast with a shift in a negative direction in juvenile neurons as described previously (Xu and Sastry 2007; Yang et al. 2010). In order to further investigate the possible involvement of NKCC1 in this process, antisense technique was applied in present study. Interestingly, in cells loaded with the NKCC1 antisense ODNs, the positive shift in E_{GABA}, quantitated 30 min following TBS conditioning stimulation, was not significantly changed ($E_{GABA\text{-}control}$: -52.9±0.7 mV, $E_{GABA\text{-}30min}$: -53.9±0.8 mV, n=6; p>0.05) and the PSC conductance was not changed either ($g_{psc\text{-}control}$: 4.9±0.6 pS, $g_{psc\text{-}30min}$: 4.2±0.5 pS, n=6, P>0.05). However, in cells loaded with NKCC1 sense ODNs, TBS-induced positive shift in E_{GABA} was still observed ($E_{GABA\text{-}control}$: -54.1±0.5 mV, $E_{GABA\text{-}30min}$: -49.9±0.4 mV, n=6; p<0.05, see Fig. 4-13). Sense or antisense ODNs did not affect the resting properties of the neurons. These data suggest that NKCC1 activity is involved in TBS-induced depolarizing shift in E_{GABA} in neonatal hippocampal neurons.

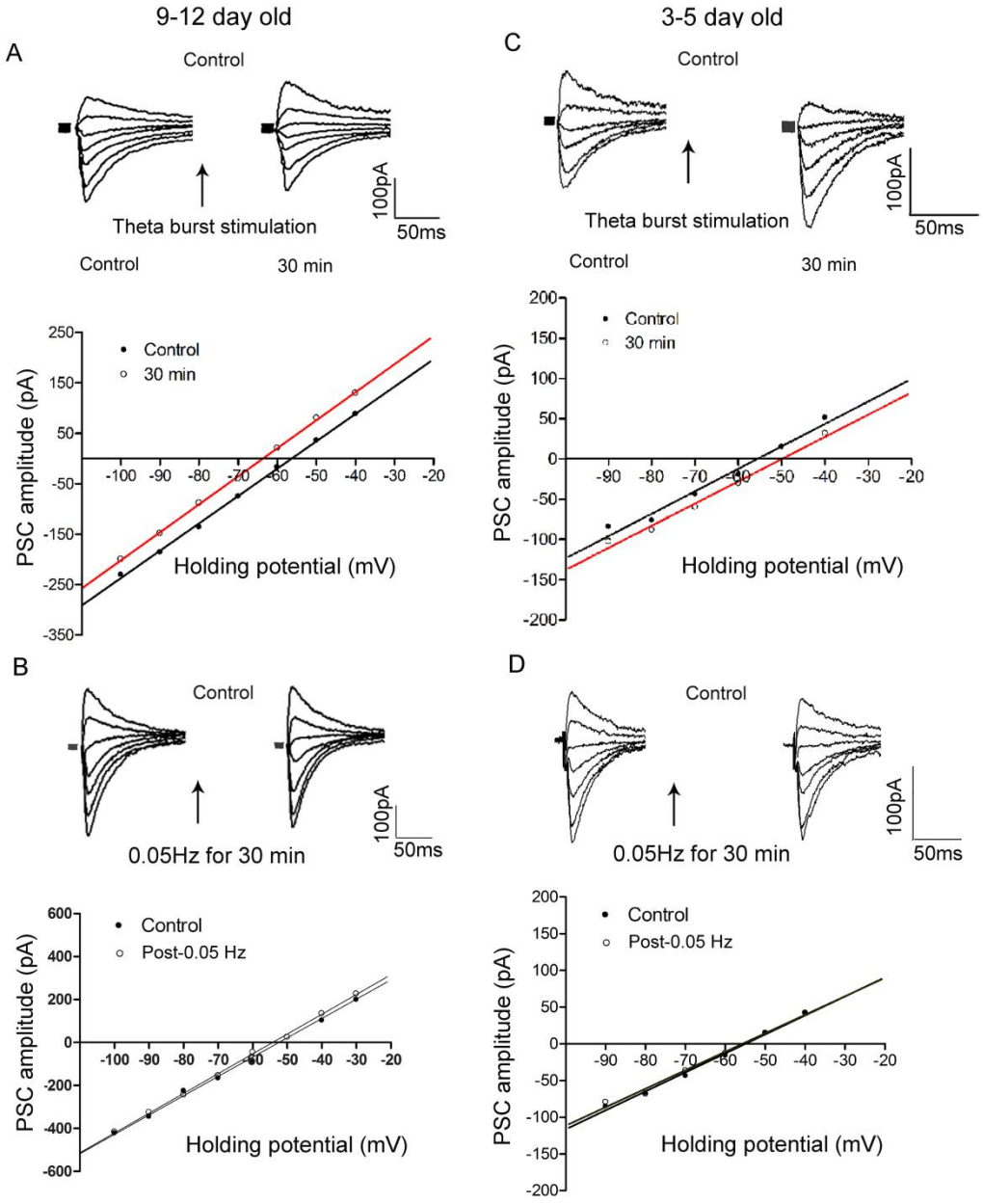

Figure 4-13 TBS induces two opposite shifts in E_{GABA} in juvenile and neonatal hippocampal neurons

In all panels (A, B, C & D), the left records are controls and the right ones are taken 30 min following TBS conditioning. Panels A and B represent data obtained from 9-12 day old rat hippocampal neurons while panels C and D show data collected from 3-5 day old rats. Note that TBS induces a negative shift in E_{GABA} (panel A) and a positive shift in E_{GABA} (panel C). However, 30 min following a 0.05 Hz stimulation did not change E_{GABA} significantly in both juvenile and neonatal hippocampal neuron (panels B & D).

Data in A, B, C and D were obtained from four different CA1 neurons and holding potential for each individual cell was -60 mV.

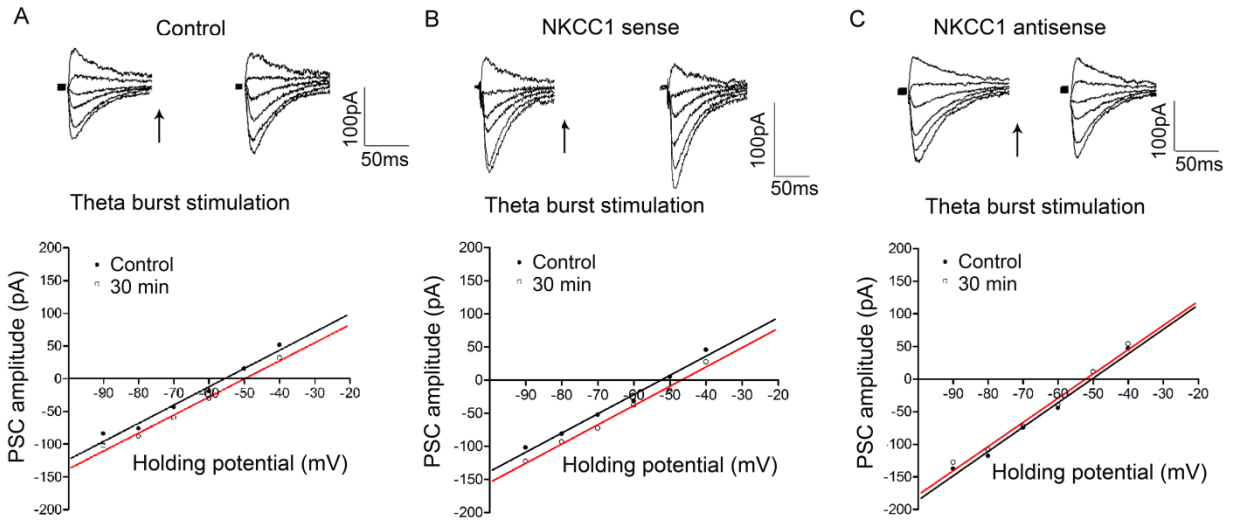

Figure 4-14 NKCC1 antisense ODNs block positive shifts in E_{GABA} induced by theta burst stimulation.

In A, B & C, the left records are controls and the right ones are taken 30 min following TBS. TBS-induced positive shift in E_{GABA} in one "control" neuron was shown in panel A. The effects of NKCC1 sense and antisense ODNs on TBS-induced shift in E_{GABA} were shown in B and C, respectively. Data in A, B and C were obtained from three different CA1 neurons and holding potential for each individual cell was -60 mV. Note that the NKCC1 antisense, but not sense ODNs, blocked the TBS-induced shifts in E_{GABA}. Slices were taken from 3-5 day old rats.

To investigate the involvement of postsynaptic Ca^{2+} in the TBS-induced shifts in E_{GABA} in neonatal hippocampal CA1 neurons, high BAPTA-containing intracellular pipette was used as well in one series of experiment. Thirty min following TBS of the input, E_{GABA} was not markedly shifted ($E_{GABA\text{-}control}$: -52.9±1.0, $E_{GABA\text{-}30min}$: -55.3±0.8 mV, n=6; p>0.05). However,

the PSC conductance was significantly decreased ($g_{psc\text{-}control}$: 5.6±0.2 nS, $g_{psc\text{-}30min}$: 3.8±0.2 nS, n=6; $p<0.05$), indicating other factors (changes on kinetics of Cl^- channel) may also be involved in this process.

Phosphorylation and dephosphorylation factors are able to modulate NKCC1 activity in cortical neurons (Schomberg et al. 2001). In one series of experiments, okadaic acid was applied into the incubating medium to pretreat the slice for 1 hour before recordings. Interestingly, 30 min following TBS of the input, E_{GABA} was not changed significantly ($E_{GABA\text{-}control}$: -51.8±1.5 mV, $E_{GABA\text{-}30min}$: -53.1±2.4 mV, n=6; $p>0.05$), indicating PP1 or PP2A seems to be involved in TBS-induced positive shift in E_{GABA} in immature neurons.

4.6 mGluRs are involved in TBS-induced shifts in E_{GABA} in both juvenile and neonatal rat hippocampal neurons

4.6.1 Electrophysiological data suggest that mGluRs seem to be involved in TBS-induced shifts in E_{GABA} in rat hippocampal neurons

Now that our previous (Yang et al. 2010) and present study suggest that an up-regulation of KCC2 and NKCC1 activity is involved in TBS-induced negative shift in juvenile neurons and positive shift in neonatal neurons, respectively, we investigated whether activation of mGluRs was involved by a 10 min bath application of group I and II mGluR antagonist MCPG (500 μM). MCPG, applied during TBS conditioning, blocked the TBS-induced shifts in either juvenile or neonatal neurons (see Fig. 4-15 and Table 4-4). In addition, E_{GABA} was unaffected by MCPG alone (no TBS) in both juvenile (from -58.6±2.5 mV to -59.8±2.3 mV, n=6, $P>0.05$) and neonatal (from -51.7±1.1 mV to -51.8±0.7 mV, n=6, $P>0.05$) neurons. Taken together, these results suggest that in rat hippocampal CA1 pyramidal neurons, activation of mGluRs is

involved in the up-regulation of KCC2 activity in juveniles or NKCC1 activity in neonates, leading to corresponding TBS-induced shifts in E_{GABA}.

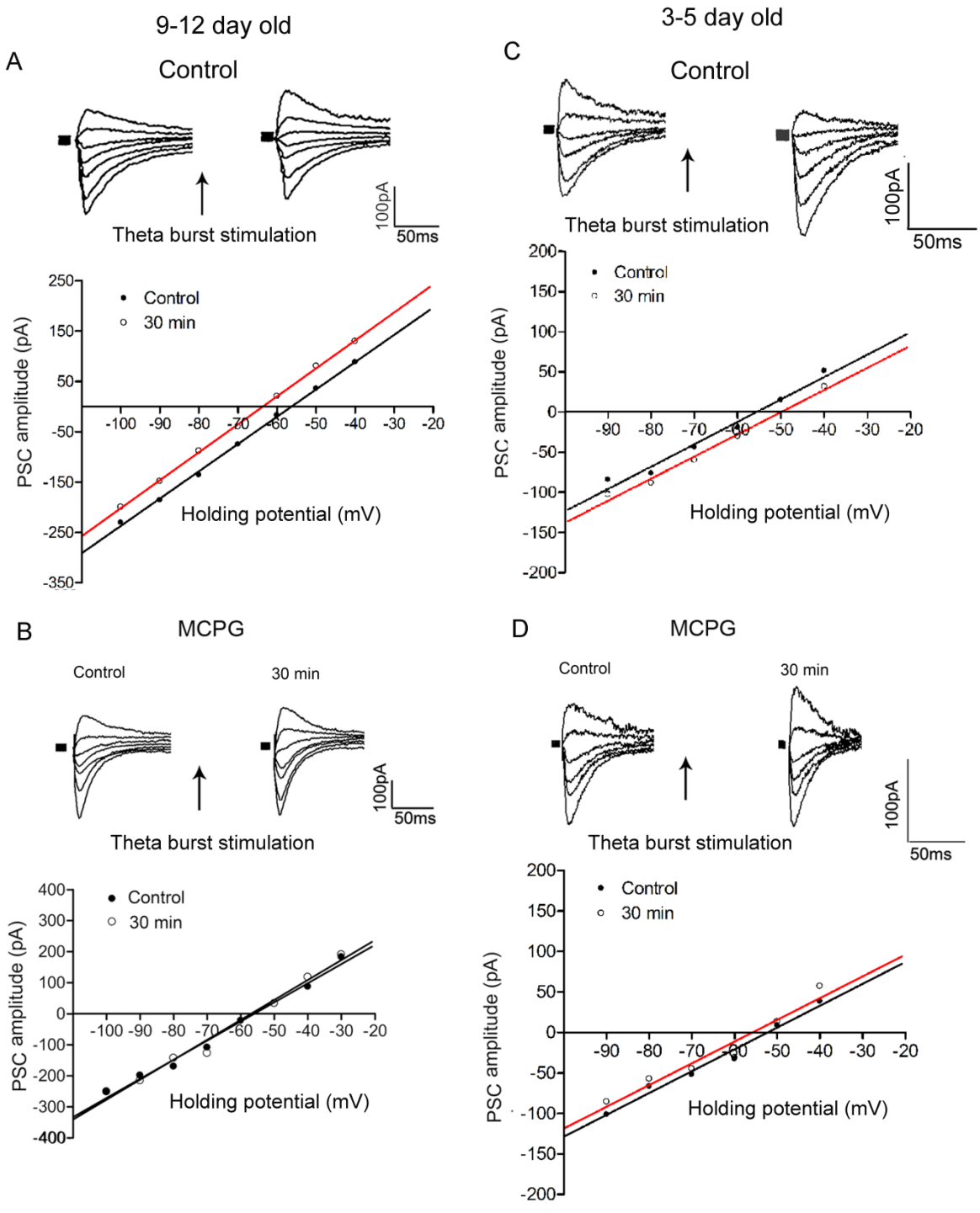

Figure 4-15 MCPG blocks TBS-induced shifts in E_{GABA} in both juvenile and neonatal rat hippocampal neurons

MCPG (500 μM) was applied into the superfusion medium 10 min prior to the TBS conditioning. The records show PSCs evoked at different holding potentials. I-V plots in each case represent PSC amplitudes at different holding potentials, before and after the conditioning stimulation. In all panels (A, B, C & D), the left records are controls and the right ones are taken 30 min following TBS conditioning. Panels A and B represent data obtained from 9-12 day old rat hippocampal neurons while panels C and D show data collected from 3-5 day old rats. In B & D, the effects of MCPG on the TBS-induced shifts in E_{GABA} are shown. Data in A, B, C and D were obtained from four different CA1 neurons and holding potential for each individual cell was -60 mV. Note that TBS induces a negative and a positive shift in $E_{GABA,}$ respectively, in juvenile and neonatal rat hippocampal neurons, which are both blocked by MCPG.

Table 4-4 Effects of several mGluR antagonists on TBS-induced shifts in E_{GABA} in juvenile and neonatal neurons

mGluR antagonist	Juvenile (9-12 day old)		Neonates (3-5 day old)	
	E_{GABA} before TBS (mV)	30 min following TBS (mV)	E_{GABA} before TBS (mV)	30 min following TBS (mV)
MPEP	-59.9±1.6 (6)	-68.5±1.5 (6)*	-54.7±1.1 (6)	-55.8±1.1 (6)
LY367385	-59.0±1.5 (7)	-66.8±1.1 (7)*	-54.1±1.0 (6)	-54.8±1.1 (6)
MCPG	-59.8±2.3 (6)	-59.0±2.5 (6)	-51.7±0.5 (6)	-53.3±0.7 (6)

Note: numbers inside brackets refer to *n* in individual study and * indicates $P< 0.05$.

4.6.2 Western blot and immunohistochemistry studies support the involvement of mGluRs in TBS-induced shifts in E_{GABA} in rat hippocampus

4.6.2.1 Expression of KCC2 in juvenile and neonatal rat hippocampus

In the hippocampus of the 3-5 day old rat, KCC2 like immunoreactivity was not detected in controls and with TBS conditioning, although slightly increased expression was seen in

MCPG or MCPG+TBS, which was not significantly different from controls. Nevertheless, in the hippocampus of the 9-12 day old rat, KCC2 was well expressed in controls and significantly increased upon TBS conditioning. KCC2 expression following MCPG or MCPG+TBS was comparable to control (see Fig. 4-16).

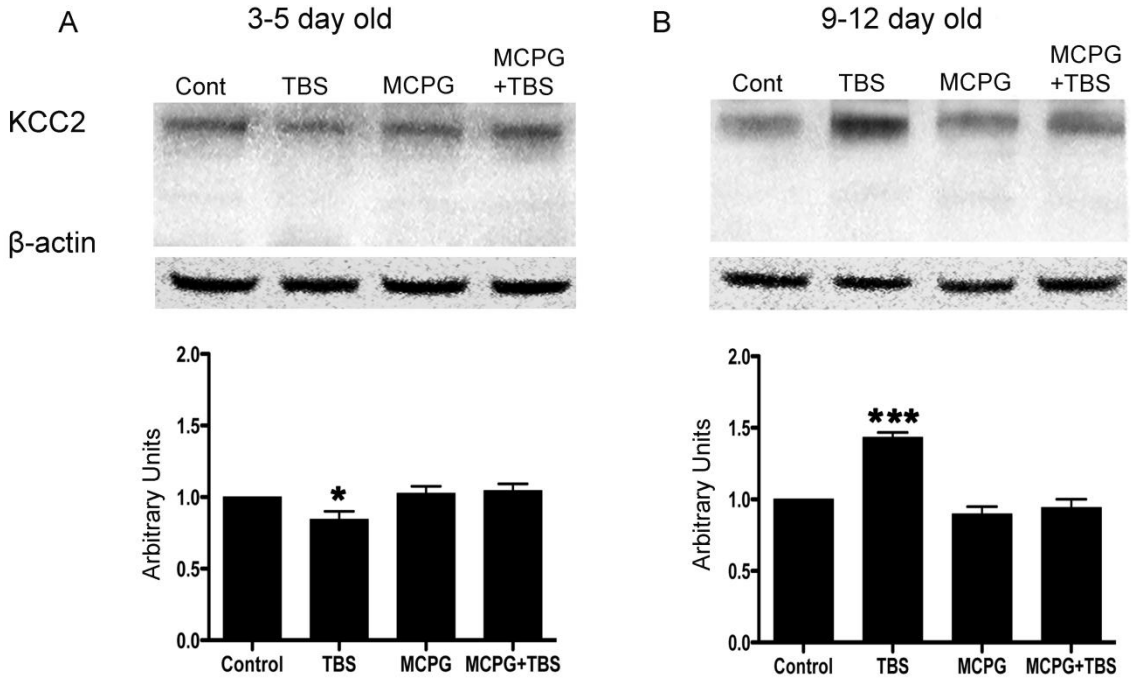

Figure 4-16 Western blot analysis of KCC2 in 3-5 day and 9-12 day old rat hippocampus

Total tissue lysate (20 μg) from CA1 region of hippocampus was subjected to immunoblot analysis using KCC2 specific antibodies. KCC2 expression was detected at the expected size of 140 kDa. Note the decreased expression of KCC2 following TBS in the CA1 area of 3-5 day old rat hippocampus (panel **A**) and increased expression of KCC2 in that of 9-12 day old rat hippocampus (panel **B)** while KCC2 expression was not significantly changed in MCPG and MCPG + TBS treated slices when compared to control in both 3-5 and 9-12 day old rat hippocampus. β-actin as loading control and densitometric analysis are shown in middle and bottom panel, respectively. Data are presented as mean ± SEM (n=5), * $P<0.05$,*** $P<0.001$.

4.6.2.2 Immunohistochemical localization of KCC2 in hippocampal brain slices

As illustrated in Fig. 4-17, in 3-5 day old rat hippocampal slices, KCC2 like immunoreactivity is selectively expressed in some neuronal populations in control and following TBS. Upon MCPG or MCPG+TBS treatments, KCC2 immunoreactivity was not significantly changed. In comparison to the 3-5 day old rat, KCC2 immunoreactivity was well expressed in control and further enhanced upon TBS in 9-12 day old rat hippocampus. In the case of MCPG treatment, immunoreactivity was diminished greatly, even to less than control levels, whereas in combined treatment with MCPG+TBS, KCC2 immunoreactivity was restored to control levels.

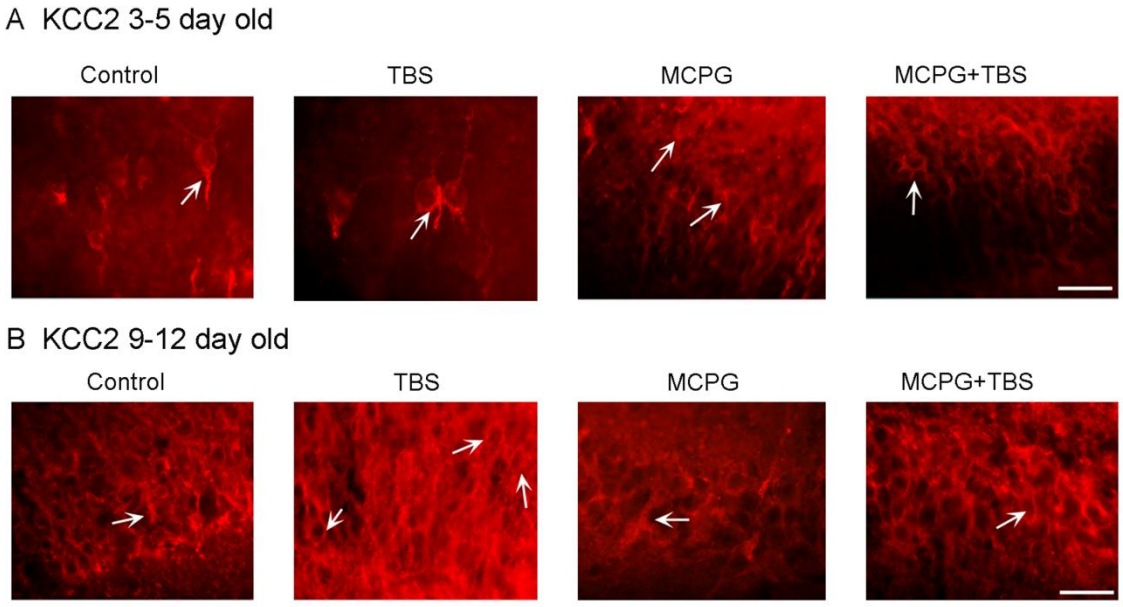

Figure 4-17 Representative photomicrographs illustrating the expression of KCC2 in the CA1 region of hippocampus from 3-5 and 9-12 day old rats

Note that the expression of KCC2 was not significantly changed in TBS and/or MCPG treated slices in 3-5 day old rat (panel A). KCC2 expression in 9-12 day old rat hippocampal neurons, however, significantly increased in TBS-treated slices. No changes were seen in MCPG or MCPG + TBS treated slices when compared to control slices (panel B). Scale bar: 10 μm.

4.6.2.3 Expression of NKCC1 in hippocampus of neonatal and juvenile rats

In order to quantify the changes of NKCC1 following TBS in both juvenile and neonatal hippocampal neurons, western blot analysis was further used as well in this study. As shown in Fig. 4-18, western blot show the expression of NKCC1 at the expected size of 170 kDa. Application of TBS resulted in an increase in NKCC1; whereas, NKCC1 immunoreactivity was not changed upon MCPG or MCPG+TBS treatment in 3-5 day old rat. In 9-12 day old rat, NKCC1 expression, in comparison to 3-5 old rat, was relatively higher in controls and increased in the presence of TBS, whereas, following MCPG or MCPG + TBS, the expression level of NKCC1 was comparable to that in controls.

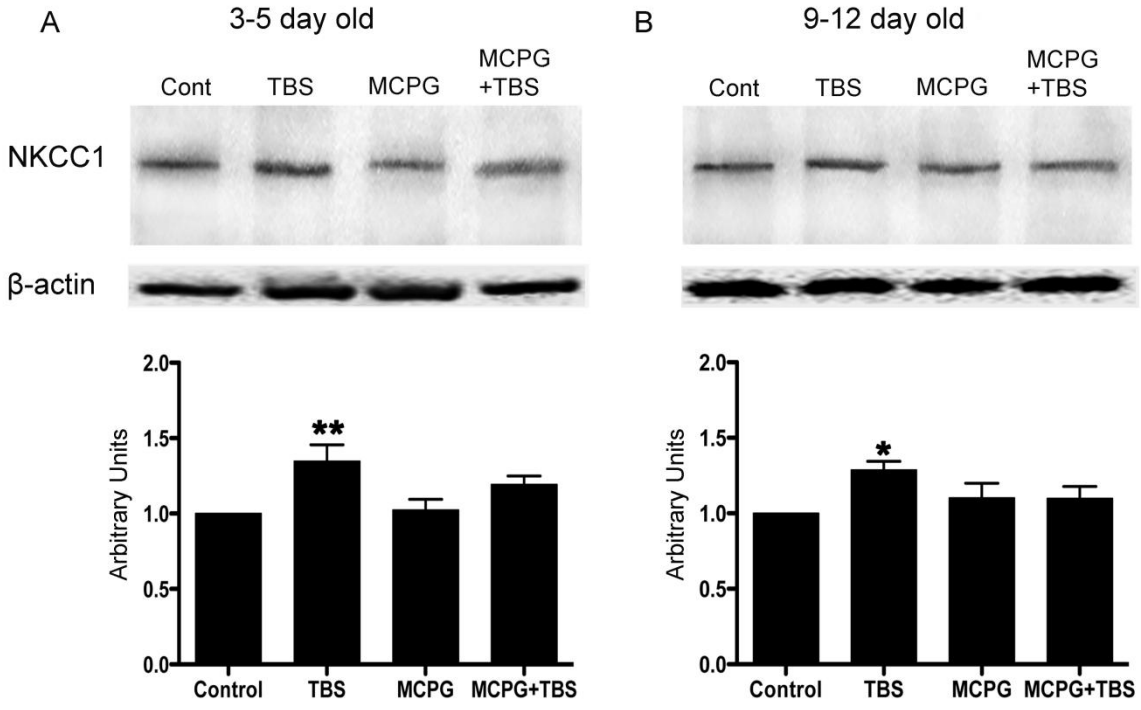

Figure 4-18 Western blot analysis of NKCC1 in 3-5 and 9-12 day old rat hippocampus

In A, western blot analysis showing the expression of NKCC1 in the CA1 region of hippocampus from control and treated 3-5 day old rat (upper panel). NKCC1 expression was seen at the expected size of 170

kDa. Densitometric analysis reveals the increase in NKCC1 expression in TBS treated slices, whereas, following MCPG or MCPG+TBS treatments, NKCC1expression was comparable to that in control. In B, western blot analysis depicting comparative changes in the CA1 region of hippocampus in 9-12 day old rats (upper panel). Note the increased expression of NKCC1 upon TBS treatment whereas no discernable changes were seen upon MCPG and MCPG + TBS treatments when compared to control. Data are presented as mean ± SEM (n=5), * $P<0.05$,** $P<0.01$.

4.6.2.4 Immunohistochemical localization of NKCC1 in hippocampal brain slices

As illustrated in Fig. 4-19, NKCC1 like immunoreactivity was well expressed in hippocampal slices from 3-5 day old rat. Upon treatment with TBS, NKCC1 immunoreactivity was increased, qualitatively speaking. Upon treatement with MCPG alone or in combination with TBS, NKCC1 seemed to be well expressed in hippoocampal slice without any variation although expression levels seemed relatively weaker than control and TBS treated slices. Similar distributional pattern of NKCC1 was seen in 9-12 day old rat hippocampal slices exhibiting somewhat enhanced immunoreactivity in presence of TBS and comparable expression in presence of MCPG alone or combination with TBS. These immunographs were used for qualitative, rather than quantitative, assessment; data from Western blots were, instead, used for quantification of values.

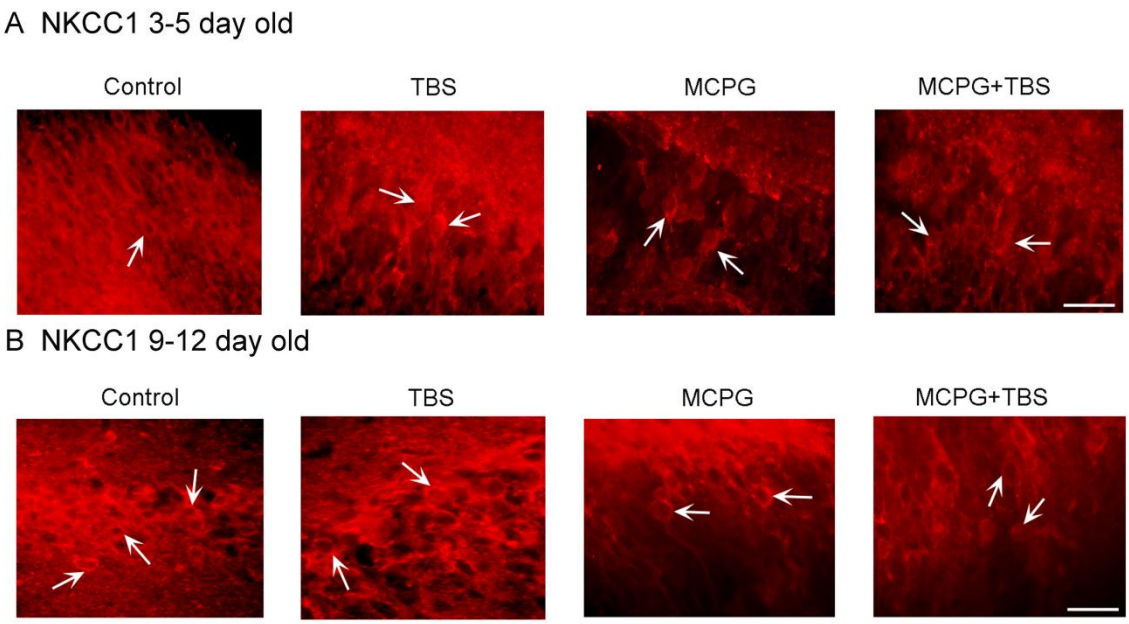

Figure 4-19 Representative immunofluorscence photomicrographs depicting the expression of NKCC1 in the CA1 region of hippocampus from 3-5 and 9-12 day old rats

In A, NKCC1 like immunoreactivity is well expressed, which was enhanced upon treatment with TBS, in the CA1 region of hippocampus from 3-5 day old rats. However, in the CA1 region of hippocampus from 9-12 day old rat, NKCC1 immunoreactivity did not seem to be changed following TBS. NKCC1 immunoreactivity seemed to be less than control following MCPG treatment or MCPG+TBS treatment in the CA1 region of hippocampus from both 3-5 and 9-12 day old rats. Arrows indicate NKCC1 like positive immunoreactivity in neuronal cells. Scale bar 10 μm.

Since the sections (400 μM thickness) used in immunohistochemistry experiments were much thicker than tissues routinely used in immunostaining, the immunofluorscence photomicrographs in the current study might appear not as clear as those in other studies. However, the quality of photomicrographs was optimized as best as we can. Even though some seemingly significant changes on the expression of either KCC2 or NKCC1 in the CA1 region of rat hippocampus were observed, we do not really know how meaningful those data would be. Therefore, quantification of those data has not been included in making our conclusions in this

thesis. Instead, quantitative values from Western blots were taken into consideration (see Fig.4-16 and Fig. 4-18).

4.6.3 Involvement of mGluR subtypes in TBS-induced shifts in E_{GABA} in rat hippocampus

4.6.3.1 Activation of mGluR1 or mGluR5 alone is sufficient to induce a negative shift in E_{GABA} following TBS in juvenile rat hippocampal neurons

To examine which mGluR subtype is involved in those processes, several mGluR antagonists (MPEP, LY367385, EGLU) were applied in series of experiments. Ten minutes application of EGLU (group II mGluR antagonist) did not block the TBS-induced shift in E_{GABA} ($E_{GABA\text{-}control}$: -57.7±0.6 mV, $E_{GABA\text{-}30min}$: -61.7±0.7 mV, n=6; $p<0.05$), suggesting group II mGluRs seem not to be involved in shifts in E_{GABA}. Following a 10 min application of either MPEP (a relatively selective mGluR5 antagonist, 10 μM) or LY367385 (a relatively selective mGluR1 antagonist, 100 μM), TBS-induced shifts in E_{GABA} were still observed in juvenile rat hippocampal neurons (see Fig. 4-20 and Table 4-4). E_{GABA} was not changed by either MPEP or LY367385 before TBS conditioning (data not shown). However, a 10 min co-application of MPEP and LY367385 blocked the TBS-induced shift in E_{GABA} in juvenile hippocampal CA1 neurons ($E_{GABA\text{-}control}$: -58.5±1.0 mV; $E_{GABA\text{-}30min}$: -61.0±0.9 mV, n=7, $P>0.05$, see Fig. 4-20), suggesting that the activation of mGluR1 or mGluR5 alone is sufficient to induce a negative shift in E_{GABA} following TBS in juvenile rat hippocampal neurons.

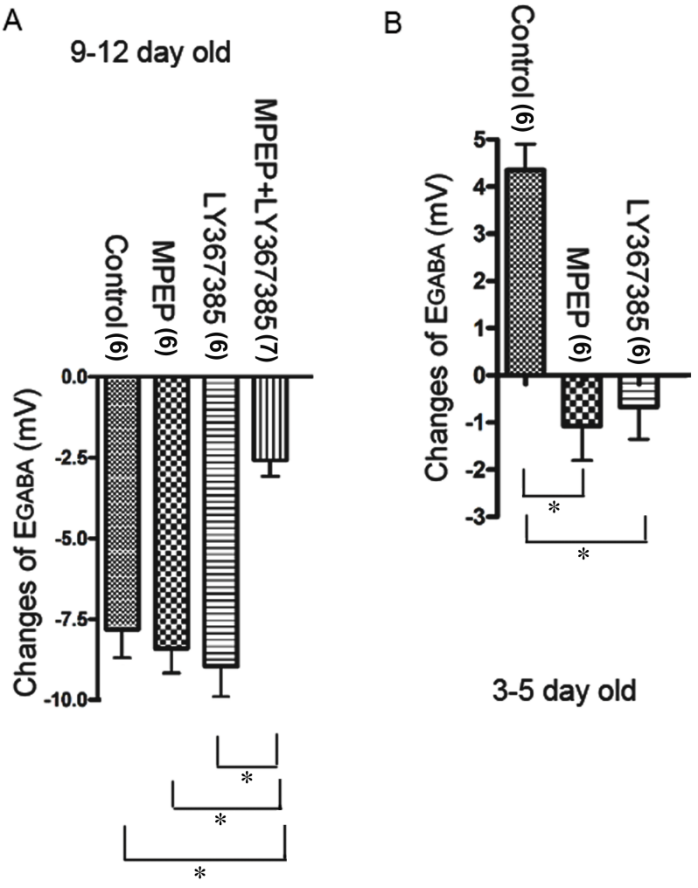

Figure 4-20 Different effects of group I mGluR antagonists on TBS-induced shifts in E_{GABA} in juvenile and neonatal rat hippocampal neurons

In panel A, on slices from 9-12 day old rats, two group I mGluR antagonists (MPEP and LY367385) did not block TBS-induced negative shifts in E_{GABA} when applied individually, while a co-application of these two antagonists significantly decreased the negative shift (numbers inside brackets refer to *n* in each study and * indicates $P< 0.05$, one-way ANOVA analysis). In panel B, either MPEP or LY367385 is able to inhibit TBS-induced positive shifts in $E_{GABA,}$ in 3-5 day old rat hippocampal neurons.

4.6.3.2 Activation of both mGluR1 and mGluR5 is needed for TBS-induced positive shift in E_{GABA} in neonatal rat hippocampal neurons

When neonatal slices were superfused with either MPEP or LY367385 for 10 min before TBS conditioning, E_{GABA} was not significantly changed (or even hyperpolarizing shifts were

observed) at 30 min following TBS (see Fig. 4-20 and Table 4-4), suggesting that, unlike in juvenile neurons, activation of both mGluR1 and mGluR5 is necessary for TBS-induced positive shift in E_{GABA} in immature neurons.

Chapter 5. Discussion

5.1 Significance of shifts in E_{GABA} in rat hippocampus

The balance between inhibition and excitation is crucially important for the integrative function of neural circuits in the CNS. In hippocampus, this balance is mainly maintained by GABA-ergic interneurons through feedforward and/or feedback inhibition on pyramidal cells to control the excitability of neuronal networks. In addition to these two types of GABA-ergic inhibition, changes in E_{GABA} can modulate the inhibitory and/or excitatory nature of GABA's action on hippocampal neurons. In contrast to the extensive studies on mechanisms involved in feedforward and feedback inhibition in hippocampus, the molecular mechanisms underlying changes in E_{GABA} have not attracted much attention until recent years (Fiumelli et al. 2005; Ouardouz and Sastry 2005; Ouardouz and Sastry 2000; Ouardouz et al. 2006; Xu and Sastry 2007; Yang et al. 2010).

Since the amplitudes and directions of the IPSC are subject to changes in E_{GABA}, it is necessary to monitor E_{GABA} regularly during studies on the long-term plasticity of the IPSC amplitude. Ignorance of changes in E_{GABA} can lead to erroneous interpretations. Patenaude et al. (Patenaude et al. 2003) reported that a theta-burst stimulation of inputs can induce LTP of the IPSC in hippocampal CA1 neurons. However, our previous studies suggest that this conditioning stimulation affects E_{GABA} instead (Ouardouz et al. 2006; Xu and Sastry 2007). Shifts in E_{GABA} can be caused by a disturbance of the transmembrance Cl^- gradient while recording in the whole-cell configuration. In current study, we examined if such recording conditions contribute to changes in E_{GABA}. Perforated-patch clamp recording was used in one series of experiments as a control. In these recordings, following TBS, E_{GABA} was also shifted in the negative direction in juvenile rat hippocampal neurons. Therefore, the TBS-induced shift in E_{GABA} using the whole-

cell clamp mode is not an artifact but a genuine phenomenon representing one type of activity-mediated plasticity of E_{GABA}. As a matter of fact, activity-mediated shifts in E_{GABA} can occur following various types of stimulations other than TBS. Results from this thesis work suggest that shifts in E_{GABA} seem to be associated with several types of activity-mediated plasticity of $GABA_A$-mediated IPSCs (such as FDD) in rat hippocampal neurons. Since the conditioning stimulations used in these studies effectively mimic physiological activity in mammalian CNS, shifts in E_{GABA} seem to naturally occur in many physiological conditions. Interestingly, results from studies on trauma, pain, epilepsy and other neurological disorders demonstrated that changes in E_{GABA} appear to be associated with the generation and process of those diseases as well (Coull et al. 2003; Khalilov et al. 2003; Laviolette et al. 2004; Perez Velazquez 2003; van den Pol et al. 1996). Therefore, shifts in E_{GABA} appear to be associated with both physiological and pathological processes in mammalian CNS.

In addition to activity-mediated plasticity, E_{GABA} undergoes age-dependent plasticity in rat hippocampus as well. However, the molecular mechanisms underlying changes in E_{GABA} are not yet well understood.

5.2 Shifts in E_{GABA} are involved in activity-mediated plasticity of IPSCs in rat hippocampus

Activity-mediated shifts in E_{GABA} were first reported in our laboratory in 2000 (Ouardouz and Sastry 2000). Since then, this special phenomenon has drawn the attention of several investigators in this field. Meanwhile, some concerns arose regarding to the measurement of E_{GABA}. There are two most frequently asked questions: 1) do changes in HCO_3^- gradient affect the calculation of E_{GABA} in hippocampal neurons? As mentioned above, a change in HCO_3^- gradient does not influence E_{GABA} owing to the small bicarbonate permeability (Kaila and Voipio

1987). However, the contribution of HCO_3^- to E_{GABA} was usually observed during sustained high frequency stimulation (HFS) in dendrites (Staley and Proctor 1999), suggesting that a sudden delivery of HFS may cause rapid breakdown of the Cl^-/HCO_3^- gradient across neurons and, hence, shifts in E_{GABA}. In the current study, however, application of acetazolamide, a membrane-permeant carbonic anhydrase inhibitor, did not block the shift in E_{GABA} caused by either TBS or repetitive stimulations (1 Hz). Application of acetazolamide alone did not significantly change the E_{GABA} during the control stimulation (0.05 Hz). Taken together, changes in HCO_3^- gradient seem not to contribute to shifts in E_{GABA} caused by either repetitive stimulations or TBS conditioning, in our studies. The apparent shifts in E_{GABA} observed in the conditioning stimulations were likely caused by changes in transmembrane Cl^- gradients. The second most frequently asked question is: can E_{GABA} be properly measured under whole-cell patch clamp recording? In the literature, there seems to be an exaggerated concern about the dialysis of the intracellular content in recording neurons and disruption of Cl^- homeostasis brought on by the whole cell recording approach. However, when K-gluconate electrodes and Cs-methylsulfonate electrodes were used in our previous studies (Ouardouz and Sastry 2005; Ouardouz et al. 2006; Xu and Sastry 2007), the E_{GABA} did not change significantly in controls over the recording time course (over 45 min) using whole-cell recordings. Sharp electrodes were also used in previous studies in this laboratory (Ouardouz and Sastry 2005) and no significant differences were observed between this method and the whole cell recording method. Therefore, dialysis does not seem to contribute in a major way to changes in E_{GABA} observed over the time course in our whole-cell recording experiments. Results from my recent study further supported the idea that stable control IPSCs can be obtained without significant shifts in E_{GABA} under 0.05 Hz stimulation (Yang et al. 2010). Moreover, perforated patch clamp recording technique was also used as an additional control. It turns out that E_{GABA} was not markedly changed during 0.05 Hz

stimulation but significantly shifted in a negative direction following TBS under perforated patch configuration, similar to what was found under whole cell patch configuration. Therefore, with low frequency stimulation (<0.05 Hz) and whole cell patch recording, changes in E_{GABA} can be reliably detected.

In recent years, there is a large body of evidence suggesting that shifts in E_{GABA} are involved in a variety of physiological or pathological disorders (Jedlicka and Backus 2006). It is tempting to investigate whether E_{GABA} undergoes activity-mediated plasticity following specific stimulations which mimic the firing activities of neurons associated with these disorders.

5.2.1 Frequency-dependent depression of IPSC

In the current study, significant depression of amplitudes of IPSCs was observed upon repetitive stimulation (0.1-1 Hz) of the input. However, the amplitudes of IPSCs are relatively stable during 0.05 Hz stimulation. Thus, with low frequency stimulation (<0.05 Hz), the IPSC drainage can be minimized to a tolerable range. Further studies suggest that FDD of the IPSC is associated with shifts in E_{IPSC}. Since the evoked IPSCs under various conditioning stimulations are completely abolished by the $GABA_A$ receptor antagonist bicuculline, we attribute these IPSCs to $GABA_A$-mediated currents. While we examined the post-conditioning effect on E_{GABA}, Ling and Benardo (Ling and Benardo 1995) reported that shifts in E_{GABA} can occur even during such conditioning stimulations. Interestingly, they found that E_{GABA} was shifted in a positive direction. However, E_{GABA} was observed to shift in a negative direction in our studies. There are several reasons which may account for this apparent discrepancy. First, E_{GABA} was checked during conditioning stimulations in their experiments (Ling and Benardo 1995). Since IPSC amplitude continuously changes under such circumstances and may not come to steady-state,

this method has room for error. In our experiments, after conditioning stimulations, the E_{GABA} was always checked at specific time point (30 min) using the control stimulation frequency (0.05 Hz). Second, we know that during a 0.05 Hz stimulation, the E_{GABA} does not shift and the IPSC amplitude is relatively stable when recorded for nearly 1 hour (Ouardouz and Sastry 2005; Xu and Sastry 2007). Third, the animals they used (about 4 weeks old) are two weeks (by average) older than the ones in our experiments (9-12 day old). It is known that E_{GABA} undergoes developmental modulation during the maturation of neurons (Ge et al. 2007). The control E_{GABA} in their study was also different from that in our study (Yang et al. 2010). Depending on the holding potential and the E_{GABA}, the direction of the PSC can be outward or inward and PSC amplitudes can increase or decrease in accordance to the direction and the shift in E_{GABA}. Since the holding potentials in both studies are around -60 mV, the evoked IPSC currents they observed are upward while downward in our experiments. In addition, the intracellular pipette solution and animal strains or ages vary between these two studies, which may also lead to the differences in steady-state E_{GABA}: while the extracellular and intracellular Cl- concentration was 133 mM and 8 mM, respectively, in their study, it was 131 mM and 10 mM in our study; 3-4 weeks old Sprague Dawley rats were used in their study but 9-12 day old Wistar rats were studied in ours. Nevertheless, there are some similarities between these two studies: Ling et al. (Ling and Benardo 1995) reported that the steady-state fast IPSCs amplitude was progressively reduced at low stimulation frequencies like 1 Hz caused by shifts in E_{GABA} with no change in the IPSC conductance and the IPSC depression was associated with changes in both E_{GABA} and IPSC conductance at higher stimulation frequencies (> 1Hz). In line with their finding, the fast IPSC amplitude in my study shows frequency-dependent depression upon low frequency (0.1- 1Hz) stimulations along with significant shifts in E_{GABA} but no apparent changes in IPSC conductance. Therefore, no matter in which direction E_{GABA} shifts during repetitive stimulations, the

frequency-dependent depression (use-dependent depression) of IPSC amplitude seems to occur in rat hippocampus.

FDD of IPSCs during repetitive stimulations may have fundamental implications on the genesis of epileptiform activity in hippocampal pyramidal cells. Epileptiform activity in hippocampal pyramidal neurons is reported to be associated with impairment of inhibition (McCarren and Alger 1985). These pyramidal neurons are able to fire burst potentials upon low frequency stimulation of inputs (Andersen and Lomo 1968; Krnjevic et al. 1982; Wong and Watkins 1982). Even though there are still controversies regarding how repetitive stimulation leads to epileptiform burst firing (Ben-Ari et al. 1979; Numann and Wong 1984), studies on the mechanisms underlying FDD of the IPSC may lead to a better understanding of epilepsy and other neurological disordered in which GABA-ergic inhibition is impaired. Among these factors, mechanisms involved in shifts in E_{GABA} associated with FDD of the IPSC have not attracted much attention until recently. Since these low frequency stimulations fall within the normal physiological range, further investigation on changes in E_{GABA} may provide insights into the mechanisms involved in variations in the modulatory role of the IPSC associated with changes in input activity.

In order to shed light on the mechanisms underlying shifts in E_{GABA} induced by repetitive stimulations, other experiments were carried out in this thesis. Since E_{GABA} is mainly determined by transmembrane Cl^- gradient in neurons, we decided to examine whether activity of KCC2 or NKCC1 are modulated during repetitive stimulation and thus lead to shifts in E_{GABA}. Both furosemide (KCC2 inhibitor) and bumetanide (NKCC1 blocker) (Blaesse et al. 2009) were applied in one of our studies to address this question. Our results suggest that modulation of

KCC2 but not NKCC1 appears to be associated with repetitive stimulation induced shifts in E_{GABA} in juvenile rat hippocampal CA1 neurons.

One intriguing question that arises from the above experiment is - why the effects of furosemide on E_{GABA} were not observed in control condition (0.05 Hz) where KCC2 is also present? The key issue is to examine the very factor which may trigger the involvement of KCC2 and hence shifts in E_{GABA}. In literature, it has been well established that KCC2 activity is tightly linked to changes in $[Cl^-]_i$. Repetitive stimulation of the input of neurons will lead to accumulation of Cl^- into the neuron (McCarren and Alger 1985), thus increasing $[Cl^-]_i$. When $[Cl^-]_i$ starts to increase, KCC2 will extrude Cl^- out of neurons, leading to a shift in E_{Cl-} in the negative direction. Therefore, $[Cl^-]_i$ may serve as a trigger for activity of KCC2. It's possible that the $[Cl^-]_i$ does not significantly change during 0.05 Hz stimulation while it dramatically changes during 1 Hz stimulation, justifying the lack of effects of furosemide at 0.05 Hz.

5.2.2 Short-term activity-mediated plasticity of IPSCs

Under physiological conditions, GABA-ergic transmission is labile: its efficiency is subject to the activities of either pre- or postsynaptic neurons (Storozhuk et al. 2005). Such modifications (use-dependent plasticity), even with a short time scale, can play an important role in both normal functioning of the neuronal network and pathological processes developing in the CNS. For example, use-dependent decrease in the strength of inhibition leads to initiation and spreading of epilepiform activity (Dichter and Ayala 1987).

Post-tetanic potentiation (PTP) is one type of short-term activity-mediated plasticity of IPSCs in rat hippocampus. In our study, following 100 Hz stimulation of the input, PTP of the IPSC could be induced in hippocampal CA1 neurons. Whether shifts in E_{GABA} were associated

with this phenomenon was also examined. Since the whole process only lasts for less than 2 min, it is not feasible to check E_{GABA} during this short-term plasticity while stimulating at 0.05 Hz. PTP of the IPSC in furosemide-treated cells is higher than that of the IPSC in no-drug control cells, indicating that PTP of the IPSC in controls is masked by concomitant changes in the E_{GABA}. The firing frequency of interneurons in the hippocampus is about 30-80 Hz and the frequencies used in many studies on PTP of the IPSC are close to this range. Therefore, changes in E_{GABA}, probably is a physiologically significant phenomenon.

In order to investigate the mechanisms underlying PPD of the IPSC, another type of short-term activity-mediated plasticity of IPSCs in rat hippocampus, $GABA_B$ antagonist (CGP55845) and $GABA_C$ antagonist (TPMPA) were applied in one series of experiment in the current study. Our data showed that TPMPA, but not CGP55845, blocked and even reversed PPD in hippocampal CA1 neurons, indicating that $GABA_C$ receptors seem to be involved in PPD of the IPSC in rat hippocampus. Higher concentrations of CGP55845 were not examined since TPMPA completely blocked PPD while not affecting $GABA_B$ receptors. Nevertheless, it is still possible that $GABA_B$ receptors may be involved in PPD but are not sensitive to CGP55845 at the concentration used (2 μM). Olpe et al. (Olpe et al. 1994) reported that CGP55845, at a concentration of 10 μM, only partially blocked PPD. It is unclear if CGP55845 selectively acts on $GABA_B$ receptors or has additional actions on $GABA_C$ receptors at these rather high concentrations. Interestingly, one of our previous studies suggests that the activation of $GAGA_B$ receptors does not contribute to the depression of the IPSC induced by paired stimulation of the input in deep cerebellar nuclei (DCN) (Morishita and Sastry 1995). $GABA_C$ receptors seem to be located on presynaptic elements in retina (Matthews et al., 1994; Hull et al., 2006) and hippocampus (Bormann 2000; Rozzo et al. 2002). If $GABA_C$ receptors are involved in modulating postsynaptic $GABA_A$ receptor activity, this may also account for PPD and for the

increase in the amplitude of the IPSC evoked by single stimulations. Further investigation is necessary to determine if $GABA_C$ receptors involved in PPD are pre- and/or post-synaptic.

In our study dealing with PPD of IPSCs, I/V plots for both first and second IPSC were linear and there was no effect of voltage on the degree to which PPD occurred. Analysis of the current-voltage (I/V) relationships of the IPSC revealed that there was no significant change in the reversal potential (E_{GABA}) from the first to second IPSC. Since desensitization of the $GABA_A$ receptor has been proposed to contribute to PPD (Thompson and Gahwiler 1989b) and depolarization is known to slow the rate of desensitization (Frosch et al. 1992; Hablitz 1992; Oh and Dichter 1992), the lack of a voltage-sensitive component of PPD suggests that desensitization of the postsynaptic $GABA_A$ receptor may not be involved in PPD of the IPSC observed in our study.

5.2.3 TBS induces shifts in E_{GABA} in both juvenile and neonatal hippocampal neurons

Theta-burst stimulation (TBS) has been widely used to induce synaptic plasticity owing to its physiological relevance (Larson et al. 1986; Staubli and Lynch 1987). Previous studies in our laboratory suggest that tetanic stimulation of inputs induce a negative shift in E_{GABA} of neonatal rat DCN neurons (Ouardouz and Sastry 2005; Ouardouz and Sastry 2000). Further investigation from our lab indicates that TBS is able to induce a negative shift in E_{GABA} in juvenile rat hippocampal CA1 neurons via an up-regulation of KCC2 activity (Xu and Sastry 2007). In this current study, TBS-induced negative shift in E_{GABA} in juvenile rat was further studied and the mechanisms involved investigated (Yang et al. 2010). Interestingly, TBS seems to induce a positive shift in E_{GABA} in neonatal rat hippocampal CA1 neurons via the up-regulation of NKCC1 activity when the NMDA receptors are blocked with APV (see Fig. 4-13).

KCC2 expression is relatively higher than that of NKCC1 in juvenile and adult rat hippocampus while NKCC1 expression is more dominant than that of KCC2 in neonatal rat hippocampus (Wang et al. 2002). Following TBS of the input, no matter which direction E_{GABA} was shifted, the GABA response seems to be strengthened in its existing position in both scenarios: a negative shift in E_{GABA} enhancing GABA inhibitory response in adults whereas a positive shift in E_{GABA} facilitating GABA excitatory response in neonates. Whether this intriguing phenomenon exists in *in vivo* studies is not known. Given that theta oscillation (θ-rhythm) exists in hippocampus generated either from CA1 pyramdial cells (Bland et al. 2002) or interneurons (Ylinen et al. 1995), the results in the present thesis are relevant in explaining contributions of theta oscillations to modulations of the neuronal network activity in the CNS.

5.3 Age-dependent plasticity of E_{GABA} in rat hippocampus

It has been widely accepted that GABA depolarizes rat hippocampal neurons and provides most of the excitatory drive to pyramidal cells during the first postnatal week (Cherubini, et al. 1991). This excitatory GABA response is due to the relatively higher $[Cl^-]_i$ and corresponding depolarized E_{GABA} in immature neurons (Ge et al. 2007). In agreement with this statement, results from both our previous and current studies suggest that E_{GABA} was set at a more depolarized level in neonatal hippocampal neurons compared to that in juvenile or adult hippocamapl neurons (Xu and Sastry 2007; Yang et al. 2010). Our data further supported the thinking that the developmental expression of two major Cl^- cotransporters (NKCC1 and KCC2) plays a critical role in modulating the plasticity of E_{GABA} during maturation of hippocampal neurons. Interestingly, it has been reported that, in new born granule cells of adult brain, E_{GABA} can still change during maturation (Ge et al. 2006). Therefore, E_{GABA} seems to undergo various types of plasticity throughout the development and maturation of the CNS and such flexibility in

GABA-ergic transmission endows the PSC with the ability to regulate excitatory transmission in distinct ways, depending on the direction or level of shifts in E_{GABA} (Yang et al. 2010).

The postnatal maturation of GABA-ergic inhibitory neurons is a delayed process compared to that of excitatory neuron (Micheva and Beaulieu 1997). In neonatal neurons, the excitatory action of GABA (Chen et al. 1996; Luhmann and Prince 1991; Misgeld et al. 1986) has been suggested to compensate for the lack of AMPA receptor-mediated EPSCs. Depolarization produced by $GABA_A$ receptors is sufficient to activate voltage-dependent Ca^{2+} channels (VGCC) and is strong enough to relieve the Mg^{2+} blockade of the NMDA receptor (Cherubini et al. 1991). In juvenile or adult neurons, GABA exerts its well-known inhibitory action and plays an important role in modulating excitatory synapses in the CNS.

The pattern of input neuronal activity has also been shown to play a critical role during maturation of the inhibitory circuitry (Benevento et al. 1995; Benevento et al. 1992; Blue and Parnavelas 1983). In the current study, our data suggest that E_{GABA} in neonatal neurons was set at a relatively depolarized level (< 5mV) compared to that in juvenile neurons. E_{GABA} remains stable during low frequency stimulation (< 0.05 Hz) in both juvenile and neonatal hippocampal neurons. However, following TBS, E_{GABA} shifts in two opposite directions in juvenile and neonatal neurons. Moreover, it seems that E_{GABA} changes during other low frequency stimulations (0.1, 0.5, 1 Hz) in juvenile hippocampal neurons. In neonatal neurons, however, E_{GABA} does not seem to significantly change 30 min following 1 Hz stimulation. Therefore, E_{GABA} might undergo different activity-mediated plasticity during the development of rat hippocampus. The shifts in E_{GABA} with as low frequencies as 0.1 to 1 Hz and as specific conditioning as theta-bursts in neonatal, juvenile and adult neurons (Xu and Sastry 2007; Yang et al. 2010) raise the possibility that GABA-ergic transmission is able to modulate neuronal excitability, excitatory transmission and network behavior in a variety of ways throughout life,

depending on input neuronal activity. Therefore, GABA-ergic transmission should no longer be viewed simply in static, excitatory (in neonates) and inhibitory (in adults) modes.

5.4 Regulation of E_{GABA} in rat hippocampal neurons

Our previous and current studies suggest that E_{GABA} undergoes both activity-mediated and age-dependent plasticities in rat hippocampus. Considering that changes in E_{GABA} have been reported to be associated with various neurological disorders, it has significant implications in understanding the underlying mechanisms involved in shifts in E_{GABA} under various conditions. Interestingly, a negative shift in E_{GABA} was reported in DCN following 10 Hz stimulation (Ouardouz and Sastry 2005). An enhanced expression or activation of KCC2 through an activation of protein kinase A, protein synthesis and protein phosphatases have been attributed to this plasticity in E_{GABA} (Ouardouz and Sastry 2005). It is unknown whether these factors contribute to various plasticities of E_{GABA} induced by several types of conditioning stimulations. Among activity-mediated plasticity of E_{GABA}, TBS-induced shifts in E_{GABA} were examined in more detail in neonatal and juvenile rat hippocampal neurons. Our data suggest that changes in the activity of KCC2 or NKCC1 are responsible for TBS-induced shifts in E_{GABA} in rat hippocampus. Moreover, mGluRs seem to be involved in the regulation of the activity of KCC2 or NKCC1, and thus, the modulation of activity-mediated and age-related plasticity of E_{GABA}. Therefore, discussion on mechanisms underlying the regulation of E_{GABA} mainly focused on TBS-induced plasticity in E_{GABA} in both juvenile and neonatal hippocampus.

5.4.1 Involvement of $GABA_B$ or $GABA_C$ receptors in the modulation of E_{GABA} in hippocampus

Since GABA exerts its function through $GABA_A$, $GABA_B$ and $GABA_C$ receptors, it is worth investigating whether activation of those three receptors affects the regulation of E_{GABA}. It has been proposed that GABA itself can lead to the shift in E_{GABA} and up-regulation of KCC2 requires the activation of $GABA_A$ receptors (Ganguly et al. 2001) even though seemingly contradicting findings were reported in two other studies (Ludwig et al. 2003; Titz et al. 2003). Diazepam, as an allosteric modulator of $GABA_A$ receptor, has been widely studied in literature in past decades (Delaney and Sah 1999; 2001; Giorgetti et al. 1998; Pawelzik et al. 1999; Thomson et al. 2000; Xu and Sastry 2007; Zhang et al. 1993). It is known that diazepam increases the amplitude of $GABA_A$-receptor mediated IPSCs and prolongs the decay time of IPSCs (Kang-Park et al. 2004; Pawelzik et al. 1999). This phenomenon is also further confirmed in this thesis work. However, the underlying mechanisms responsible for the modulation affects on IPSCs are not fully understood. In the current study, diazepam did not significantly shift the E_{GABA} during the low frequency stimulation, indicating that shifts in E_{GABA} do not contribute to the apparent changes in amplitudes of IPSCs induced by diazepam.

Several immunohistochemistry and northern blot studies show that both $GABA_B$ receptors and $GABA_C$ receptors are less expressed or functionally active in neonatal hippocampal neurons than in juvenile ones (Alakuijala et al. 2006; Correa et al. 2004; Rozzo et al. 2002). Therefore, the study on the involvement of $GABA_B$ and $GABA_C$ on the modulation of GABA-ergic transmission was conducted in juvenile rats in which both $GABA_B$ and $GABA_C$ receptors are expressed. It is well known that activation of $GABA_B$ receptors is able to suppress neurotransmitter release and dampen neuronal excitability (Davies and Collingridge 1993; Davies et al. 1991; Dutar and Nicoll 1988a; b; Mott and Lewis 1994). $GABA_B$ autoreceptors,

located on interneuron terminals, mediate an IPSP refractory period that reaches its maximium at ~ 200 ms (i.e. the theta period). This prolongation of IPSPs occurring at this interval facilitates the induction of LTP by the activation of the NMDA receptor current (Staubli and Lynch 1987). The exact mechanism underlying this feedforward IPSP suppression has been identified as a transient hyperpolarization of the terminal and a subsequent suppression of GABA release at the period of theta rhythm (Larson et al. 1986; Mott and Lewis 1994). During high frequency stimulation, the activated spines become strongly depolarized and the Mg^{2+} blockade of the NMDA receptor channel is removed, leading to Ca^{2+} influx into the neurons (Bliss et al. 2007). Therefore, results from the studies on LTP of EPSPs in hippocampus showed that $GABA_B$ receptors may be activated following TBS of the input to hippocampal CA1 pyramidal neurons. Interestingly, CGP55845, a potent $GABA_B$ antagonist, blocked TBS-induced negative shift in E_{GABA} in juvenile rat hippocampal CA1 neurons in current study. Since $GABA_B$ receptors are not fully functional in neonates (Correa et al. 2004), the involvement of $GABA_B$ receptors in TBS-induced positive shift in neonates was not examined in this thesis.

The study on effects of TPMPA on IPSCs in the current study suggests that activation of $GABA_C$ receptor does not change E_{GABA} under control stimulation (0.05 Hz). Considering that the chloride channels gated by $GABA_C$ receptors exhibit small single channel conductance (Bormann and Feigenspan 1995), it is not surprising that no significant changes in E_{GABA} was observed in hippocampal neurons upon application of TPMPA during low frequency stimulation. It seems that $GABA_C$ exerts its modulation affect via presynaptic mechanisms like inhibition of GABA release but not postsynaptic mechanism such as changes in E_{GABA} in hippocampal neurons.

Taken together, activation of $GABA_A$, $GABA_B$ and $GABA_C$ receptors did not significantly change E_{GABA} under 0.05 Hz stimulation. Moreover, under control (no drug control) conditions,

E_{GABA} does not seem to be changed either. Even furosemide does not markedly change E_{GABA} upon 0.05 Hz stimulation. Therefore, 0.05 Hz stimulation turns out to be an ideal frequency of stimulation in studies on plasticity of $GABA_A$-mediated IPSCs. Interestingly, if the stimulation frequency is increased to 0.5 or 1 Hz, E_{GABA} starts to shift in the negative direction in juvenile rat hippocampal neurons. Further studies suggest that up-regulation of KCC2 activity is responsible for this shift in E_{GABA}. Since the involvement of $GABA_B$ or $GABA_C$ receptors in 0.5 or 1 Hz stimulations was not examined, it is unknown whether activation of these two receptors is responsible for shifts in E_{GABA} induced by the two types of conditioning stimulations.

5.4.2 Regulation of KCC2 activity in TBS-induced shift in juvenile hippocampal neurons

Both our previous (Xu and Sastry 2007; Yang et al. 2010) and current studies suggest that TBS is able to induce a negative shift via an up-regulation of KCC2 activity in juvenile rat hippocampal neurons. Since the expression of KCC2 is much higher in juvenile rat hippocampal neurons than neonatal hippocampal neurons, discussion on the regulation of KCC2 activity is mainly focused on TBS-induced shift in E_{GABA} in juvenile hippocampal neurons. Further investigation in the current study suggests that activations of $GABA_B$ receptor, G-protein, mGluRs and postsynaptic Ca^{2+} are involved in this process as well. It is possible that those factors exert their modulatory effects on E_{GABA} via regulating KCC2 activity.

5.4.2.1 Modulation of KCC2 activity at functional levels

In the current study, it was found that $GABA_B$ receptor antagonist (CGP55845) and group I and II mGluRs antagonist (MCPG) blocked TBS-induced negative shift in E_{GABA} in juvenile

hippocampal neurons. Since this TBS-induced negative shift in E_{GABA} is blocked by either KCC2 inhibitor or KCC2 antisense ODN, we propose that both $GABA_B$ receptors and mGluRs are involved in the regulation of KCC2 activity following TBS of the input in juvenile hippocampal neurons. Moreover, in GDP-β-S (G protein inhibitor) loaded juvenile hippocampal neurons, E_{GABA} was not significantly shifted 30 min following TBS. Given that both $GABA_B$ receptors and mGluRs belong to group C family (metabotropic neurotransmitter receptor) of G-protein coupled receptors (GPCR) (Bonsi et al. 2005), it is not surprising that the TBS-induced shift in E_{GABA} was abolished in GDP-β-S loaded neurons.

Activation of presynaptic $GABA_B$ receptors or mGluRs (group II and III) mainly leads to a decrease in the release of GABA (Mott and Lewis 1994; Thompson et al. 1993) or glutamate into synaptic cleft (Endoh 2004). This leads to the question of where is the cross-link for the two different signaling pathways involving activation of either $GABA_B$ receptor or mGluRs. It has been reported that activation of postsynaptic $GABA_B$ receptors facilitates the formation of inositol trisphosphate (IP3) and causes Ca^{2+} release from the internal stores in postsynaptic cells (Komatsu 1996). Activation of group I/II mGluRs may cause Ca^{2+} influx through VGCC and increase the Ca^{2+} release from internal stores as well (Woodhall et al. 1999). Fiumelli et al (Fiumelli et al. 2005) reported that both Ca^{2+} influx through VGCC and Ca^{2+} release from internal stores contribute to the down-regulation of KCC2 activity and the resultant shift in E_{Cl^-} via Ca^{2+}-dependent PKC pathway. Therefore, the increase in $[Ca^{2+}]_i$ may play a critical role in the modulation of KCC2 activity in both cases. As a matter of fact, when high BAPTA (Ca^{2+} chelator) was loaded into cells, the shifts in E_{GABA} following TBS were not observed in juvenile hippocampal neurons in current study, suggesting that postsynaptic Ca^{2+} is involved in the modulation of KCC2 in neurons. However, since the conductance of I/V plots were markedly changed, other factors (such as decreases in channel conductance or changes in characteristics of

Cl^- channel) may also be involved.

Another arising question is - how Ca^{2+} modulates the activity of KCC2 in juvenile hippocampal neurons. A rapid regulation of KCC2 function can be achieved by altering the status of phosphorylation at consensus sites for PKC or tyrosine kinase (Payne et al. 1996). In agreement with these findings, serine/threonine phosporyaltion was suggested to be responsible for the regulation of KCC2 activity in oocytes (Strange et al. 2000) while tyrosine kinases may either up-regulate or down-regulate KCC2 function under different conditions in hippocampal neurons (Kelsch et al. 2001; Rivera et al. 2004). Moreover, phosphorylation of KCC2 was shown to increase its surface expression by decreasing endocytosis and possibly increasing its insertion rate, leading to an overall increase in this cotransport function (Lee et al. 2010). Elevation of postsynaptic Ca^{2+} following stimulation has been shown to alter the membrane trafficking or posttranslation modification of KCC2. Interestingly, Russell et al. (Russell 2000) proposed that CCCs are generally modulated by phosphorylation/dephosphorylation events. Compared with the extensive studies on the modulation effects of kinases phosphorylation on KCC2 function, the dephosphoryaltion of KCC2 has not attracted much attention until recently. The possible involvement of deposphorylation of KCC2 in TBS-induced shifts in E_{GABA} was tested in one series of experiment in the current study. Okadaic acid, one protein serine/threonine phosphatase 1 and 2A inhibitor, has been widely used to investigate the involvement of phosphatases in various biological processes in cells. In hippocampal slices preincubated with okadaic acid for one hour before recording, shifts in E_{GABA} were still observed following TBS of the input in juvenile hippocampal neurons. Moreover, the extent of this negative shift in E_{GABA} (-6.8 mV by average) is comparable to that observed (-7.8 mV) under control condition. Since dephosphorylation of KCC2 is able to promote endocytosis and reduces the activity of KCC2 in HEK-293 cells (Lee et al. 2010), the same mechanism may also exist in hippocampal neurons. If

KCC2 activity was enhanced by the application of okadaic acid due to the inhibition on the dephosphorylation process of KCC2 protein, a more negative shift in E_{GABA} would be expected in our experiments. In addition, okadaic acid did not significantly change E_{GABA} before TBS conditioning, indicating that dephosphorylation of KCC2 did not contribute to the regulation of KCC2 under control condition. Taken together, dephosphorylation of KCC2 does not seem to contribute to the up-regulation of KCC2 involved in TBS-induced shift in E_{GABA} in juvenile neurons.

5.4.2.2 Regulation of KCC2 activity at translational levels

KCC2 mRNA level undergoes developmental regulation during the maturation of central neurons (Rivera et al. 1999). This finding has been further confirmed in the current study (see Fig. 4-16 & 17). In addition to the modulation of KCC2 function, any factor influencing the regulation of KCC2 expression may as well cause changes in the activity of KCC2 and subsequent shifts in E_{GABA} during development of the CNS.

Antisense RNA and DNA techniques have been developed as an effective approach to the modulation of a specific gene expression *in vitro* and *in vivo*. To obtain evidence for a strictly causal relationship between KCC2 expression and shifts in E_{GABA} in juvenile hippocampal neurons, antisense RNA techniques were applied in our experiments. Our data suggest that KCC2 antisense ODN, but not KCC2 sense ODN, blocked TBS-induced shifts in E_{GABA} in hippocampal neurons. There may be concerns regarding the method of application of antisense or sense ODNs in this study. First, is the time window enough for KCC2 antisense or sense ODNs to interact with target mRNA and exert their functions? In my experiment, antisense or sense mRNA was allowed to take effect for almost 1 hour after being loaded into recording cells.

Within the initial 20 min after the rupture of cell membrane, ODNs are most likely able to diffuse from intracellular pipette, equilibrate with cell plasma and combine with target mRNA within the cell. The following 10 min control recording was performed to monitor the stability of the recording and prolong the acting time of those ODNs. Thirty min after the delivery of TBS, E_{GABA} was checked by the approach mentioned in methods section. Therefore, the total time window for monitoring the effects of those ODNs on KCC2 expression was almost 1 hour. Considering that KCC2 mRNA is subject to a continuous kinetic modulation and the turnover rate for the protein is extremely rapid (its turnover rate is around 10 min) (Khazipov et al. 2004; Lee et al. 2007; Rivera et al. 2004; Wardle and Poo 2003), the time window set in this protocol is likely long enough to detect the changes, if any, on KCC2 expression in the cells. Second, are those ODNs stable under our laboratory conditions throughout the whole-cell recording? The length of the ODNs used in this study was 20 bases, which is longer than the minimum length (12-15 bases) for using RNA antisense technique (Neckers and Whitesell 1993). Even though the actual mechanism and location of translation inhibition in cells by ODNs remain controversial, it has been well established that ODNs directed at the 5'cap region or translation initiation site are most effective at inhibiting gene expression in cell culture (Daaka and Wickstrom 1990). Realistically speaking, the major problems with the application of ODNs are extracellular degradation and limited penetration into cells (Neckers and Whitesell 1993). However, in our study, ODNs were directly loaded into recording electrode so that they can rapidly diffuse into the cell. Moreover, those ODNs were phosphorothionated-protected, which greatly increases the stability of ODNs within the cell. The volume of patch pipette and cell plasma is much less than extracellular medium, the degradation of ODNs is negligible under our method. Therefore, by intracellularly loading antisense or sense ODNs into the cells, the stability and efficiency of ODNs were protected throughout the whole cell recording. Third, is the dose of ODNs used in

current study toxic to cells? The ideal concentration for ODNs should be able to maximize the efficiency while minimizing the toxicity when loaded into cells. Antisense ODNs against KCC2 mRNA was applied at a concentration of 5 μM for 8-15 h in cultured medium (Rivera, 1999). In our studies, a lower concentration (100 nM) of test ODNs was chosen since those ODNs were to be loaded directly into the recording neurons (Ouardouz and Sastry 2005; Yang et al. 2010). With adequate control recording taken and enough attention taken to common confounding variables, this antisense technique yielded reliable results in our studies.

It is worth noting that activity of KCC2 may also be regulated by posttranslational mechanisms (Stein et al. 2004). This will raise the possibility that changes in KCC2 activity and subsequent shift in E_{GABA} do not necessarily correlate with expression of the KCC2 protein (Stein et al. 2004). Therefore, further investigations are needed to elucidate on the correlation between expression of KCC2 and activity of KCC2 under certain conditions. Since KCC2 expression can be enhanced within a certain time window in response to various conditions such as neuronal hyperexcitability and/or trauma (Rivera et al. 2005), studies targeting the mechanisms involved in the regulation of KCC2 expression under those conditions will lead to a better understanding of physiological or pathological conditions associated with some neurological disorders.

5.4.3 Regulation of NKCC1 activity in TBS-induced shift in juvenile hippocampal neurons

As most of studies on the involvement of NKCC1 in activity-mediated plasticity of E_{GABA} were carried out under TBS protocols in current study, the discussion on the mechanisms underlying the regulation of NKCC1 activity was mainly focused on TBS-induced shifts in E_{GABA} in rat hippocampus. Since NKCC1 undergoes developmental expression during the

maturation of the CNS, this study was carried out in both juvenile and neonatal rats. Our data suggest that TBS is capable of inducing a positive shift in E_{GABA} in neonatal hippocampal neurons via the up-regulation of NKCC1 activity. Activations of mGluRs and postsynaptic Ca^{2+} seem to be involved in this process.

5.4.3.1 Modulation of NKCC1 activity at functional levels

Despite an important role of NKCC1 in the accumulation of Cl^- into the cells, little is known of how this cotransporter is regulated in neurons. However, it has been proposed that function of NKCC1 can be modulated through three major routes: phophorylation, protein-protein interactions and a direct stimulation effect of $[Cl^-]_i$ (Flatman 2002). Moreover, there is a large body of evidence suggesting that changes in protein phosphorylation are, at least partly, involved in the detection, signal transduction and response to those stimuli (Altamirano et al. 1988; Flatman 1991; Muzyamba et al. 1999; Pewitt et al. 1990). It has been accepted that phosphorylation of the N- and/or C-terminal domain of NKCC1 protein is capable of modulating its activity (Flemmer et al. 2002; Haas and Forbush 2000; Matthews et al. 1998). Therefore, phosphorylation of NKCC1 is suggested to be the final pathway through which this cotransporter is regulated (Lytle 1997; Lytle and Forbush 1992; O'Donnell et al. 1995; Tanimura et al. 1995; Torchia et al. 1992).

In the current study, our data suggest that postsynaptic Ca^{2+}, activation of mGluRs and phosphatases PP1 and PP2 are involved in TBS-induced positive shift in E_{GABA} in neonatal rat hippocampal neurons. Further investigation by using antisense technique revealed that up-regulation of NKCC1 activity was responsible for TBS-induced positive shift in E_{GABA}. However, two critical questions remain. First, how those factors including postsynaptic Ca^{2+}, mGluRs and protein phosphatases participate in the regulation of NKCC1 activity and resultant shifts in

E_{GABA} following TBS in neonatal hippocampal neurons? As mentioned above, there is an increasingly comprehensive body of evidence suggesting that phosphorylation of NKCC1 is the final pathway for the regulation of this cotransporter in cells. It has been generally accepted in literature that activation of group I mGluRs leads to a rise in intracellular Ca^{2+} in neurons (Schoepp et al. 1990; Woodhall et al. 1999). This statement was further supported by two major findings from a study carried out by Schomberg et al.: 1) BAPTA-AM completely eliminated the 1-Aminocyclopentane-*trans*-1,3-dicarboxylic acid (trans-ACPD) and DHPG-mediated stimulation of NKCC1; 2) group I mGluRs antagonist CPCCOEt abolished the increase in $[Ca^{2+}]_i$ induced by DHPG in cortical neurons (Schomberg et al. 2001). Even in hippocampal stratum oriens/alveus interneurons, mGluRs agonist ACPD is able to induce oscillatory membrane depolarizations and rises in intracellular Ca^{2+} (Woodhall et al. 1999). Therefore, postsynaptic Ca^{2+} may play a critical role in the regulation of NKCC1 activity in neonatal hippocampus. As a matter of fact, inhibition of phosphatase activity by okadaic acid was found to significantly stimulate NKCC1 activity in either squid giant axon (Altamirano et al. 1995) or cortical neurons (Schomberg et al. 2001). Moreover, Schomberg et al. concluded that Ca^{2+}/CaM-dependent pathway directly or indirectly modulates the phosphorylation of NKCC1 in cortical neurons (Schomberg et al. 2001). Therefore, it seems that the postsynaptic Ca^{2+} plays an important role in modulating the activity of NKCC1 via phosphoryaltion in cells. In current study, however, okaidac acid blocked the positive shift in E_{GABA} following TBS in neonatal hippocampal neurons, suggesting that phosphatase 1 or 2A appears to be involved in the regulation of NKCC1 activity.

Generally speaking, protein phosphorylation and dephosphorylation are two opposite processes which modulate the activity of one specific protein. Phosphorylation is carried out by various kinases while dephosphorylation is achieved by several types of phosphatases (Flatman

2002). In the case of NKCC1, a multiple kinase model has been accepted to elucidate the mechanisms involved in the regulation of NKCC1 in various systems (Flatman 2002). However, it is yet unclear whether phosphorylation by one kinase (PKA or PKC) is reversed by one particular phosphatase or whether it is antagonized by the action of several phosphatases, and *vice versa*. Therefore, the exact mechanisms underlying the phosphorylation and dephosphorylation of NKCC1 still remain unknown. Since the up-regulation of NKCC1 activity was suggested to be caused by the stimulation of Ca^{2+}/CaM kinase but not PKA or PKC kinases in cortical neurons (Schomberg et al. 2001), whether the same mechanism exists in rat hippocampus neurons awaits further investigation. However, our data do not rule out the possibility that dephosphorylation of NKCC1 is also involved in the regulation of NKCC1 in hippocampal neurons. If NKCC1 activity was stimulated by the application of okadaic acid due to the inhibition on the phosphorylation of NKCC1, a more positive shift in E_{GABA} is expected to be observed following TBS in neonatal hippocampal neurons. However, according to the multiple kinase/phosphatase model, the dephosphorylation of NKCC1 may be carried out by several phosphatases. Apparently, okadaic acid is not able to inhibit all the phosphatases in hippocampal neurons. In other words, dephosphorylation of NKCC1 may still be enhanced through other phosphatases. Moreover, it is also possible that the preincubation with okadaic acid results in changes in the activity of other Cl^- cotransporters or Cl^- channels, which leads to a disruption of Cl^- gradient across the membrane.

The second question is - where does postsynaptic Ca^{2+} come from in central neurons? Generally speaking, postsynaptic Ca^{2+} comes from three sources: voltage-gated channels, intracellular Ca^{2+} stores and NMDA receptors (Sun and Murali 1998; Woodhall et al. 1999). Results from several studies conducted by Schomberg et al. suggested that activation of NMDA receptors, AMPA receptors and group I mGluRs contribute to the stimulation of NKCC1 activity

in cortical neurons. In our study, since APV and DNQX were present in the superfusion medium throughout the recording, whether activation of ionotropic glutamate receptors (NMDA and AMPA receptors) is involved in the regulation of NKCC1 activity was not examined. Nevertheless, E_{GABA} did not change significantly in the presence of NMDA and AMPA antagonists under control condition, suggesting that activation of ionotropic glutamate receptors may not be necessary in the regulation of NKCC1 in neonatal hippocampal neurons. Stomberg et al. showed that BAPTA substantially reduce the basal level of NKCC1 activity even though this effect is not statistically significant (Schomberg et al. 2001). In one series of our experiments, intracellularly loaded BAPTA did not significantly change passive characteristics of recorded neuron and E_{GABA} was not markedly shifted during the control condition either. However, 30 min following TBS, the positive shift in E_{GABA} was completely blocked and even reversed in several neurons, indicating that postsynaptic Ca^{2+} was involved in the regulation of NKCC1 activity and subsequent shifts in E_{GABA} following TBS, in neonatal neurons. In another series of experiment, group I and II mGluRs antagonist MCPG significantly eliminated TBS-induced shifts in immature hippocampal neurons, pointing out that activation of mGluRs is also involved in this process. Since the activation of group I mGluRs was found to induce an increase in postsynaptic Ca^{2+} via activation of VGCC or synthesis of IP3 (Chavis et al. 1996; Schoepp et al. 1990), the increase in postsynaptic Ca^{2+} following TBS in neonatal hippocampal neurons come from, at least, two sources.

Ca^{2+}, as a second messenger, plays multiple roles in various biological processes in cells such as enzyme activation, metabolism, gene expression, neurite outgrowth, cell differentiation (Tsien et al. 1988; Vanoverberghe et al. 2004). The conductance of I/V plots was significantly changed following TBS in BAPTA-loaded neurons, suggesting that many other factors may also be involved in the regulation of NKCC1 and subsequent shifts in E_{GABA} following TBS in

hippocampal neurons. Since $[Ca^{2+}]_i$ participates in various signaling pathways in the cells, it is a challenging mission to figure out which signal transduction pathway(s) is (are) responsible for the regulation of NKCC1 following TBS in immature hippocampal neurons. Given that Ca^{2+} is actively participating in the phosphorylation and dephosphorylation of protein in other systems (Lyons and Shaw 1980), it is possible that $[Ca^{2+}]_i$ contributes to both phosphorylation and dephosphorylation of NKCC1 in rat hippocampal neurons as well. Some specific techniques such as microfluorimetric measurement (fura-2) of $[Ca^{2+}]_i$ may be helpful in shedding some light on the relationship between $[Ca^{2+}]_i$ and regulation of NKCC1 in rat hippocampal neurons.

5.4.3.2 Regulation of NKCC1 activity at transcription levels

In recent years, the expression of NKCC1 in the CNS has attracted lots of attention in this research area (Lu et al. 1999; Payne et al. 2003; Wang et al. 2002). Results from these studies point out that NKCC1 gene or mRNA is developmentally regulated in the CNS (Clayton et al. 1998). For instance, Wang et al. reported NKCC1 was abundantly expressed in premature hippocampal CA1-3 neurons and hardly detected after 2 weeks (P15) in the same region of hippocampus (Wang et al. 2002). In good agreement with these studies, the same developmental expression pattern of NKCC1 was demonstrated in the current study. Any factor which influences the expression of NKCC1 during the maturation of neurons may also affect the regulation of NKCC1 activity. There is evidence that NKCC1 gene expression is transcriptionally regulated (Suske 1999; Wierstra 2008). It has been reported that disruption of the gene encoding NKCC1 leads to a negative shift in E_{GABA} in dorsal root ganglion cells (Sung et al. 2000), which is due to a loss of active Cl^- accumulation (Alvarez-Leefmans et al. 1988; Alvarez-Leefmans et al. 2001).

In one series of experiments in the current study, knock-out of NKCC1 led to a complete

blockade of the positive shift in E_{GABA} following TBS. In sense NKCC1 or scrambled NKCC1 ODNs loaded neurons, the positive shift in E_{GABA} was still observed following TBS. Taken together, those data suggest that TBS-induced positive shift in E_{GABA} is caused by the up-regulation of NKCC1 activity in immature neurons. Since several technical issues related to antisense technique have already been discussed above in the section on the regulation of KCC2 activity, only two issues will be addressed here. First, is the observation period (1 hour) long enough to assess the activity of NKCC1 in our experiments? The turnover rate of NKCC1 in corneal endothelial cell is reported to be around 100 s^{-1} (Kuang et al. 2001), however, even higher turnover rate (~ 4000 s^{-1}) of NKCC1, which is much higher than that of KCC2 turnover rate (about 1000 s^{-1}), has been reported in duck red blood cells (Haas and Forbush 1986). Considering the homogeneity of NKCC1 among various species, even though there is no exact value available on the turnover rate of this cotransporter in rat hippocampal neurons, we presumed that the turnover rate of NKCC1 was comparable to that of KCC2 in rat hippocampal neurons. Since the recordings last about one hour, the time window is long enough for any changes in the activity of NKCC1 to be observed, if any. Second, why was scrambled NKCC1 mRNA used in this study? In the antisense experiment targeting KCC2, two types of ODNs were used: KCC2 sense and antisense ODNs. KCC2 antisense but not KCC2 sense ODNs blocked TBS-induced negative shift in E_{GABA}, suggesting that up-regulation of KCC2 is responsible for this shift in E_{GABA}. Therefore, this antisense approach seems to work well under our experimental conditions. Even though there is no evidence that scrambled ODNs provide superior controls, the single-or double-based mismatched complementary ODNs are shown to be useful and confirmatory (Stein 2001). Thus, in another set of experiment targeting NKCC1, this approach was further improved by adding scrambled NKCC1 ODNs into the test ODNs. If the observed effects of antisense NKCC1 ODNs on change in E_{GABA} are significantly distinct from

those seen using control ODNs (both sense and scrambled NKCC1 ODNs), an antisense mechanism of down-regulation in NKCC1 activity would be confirmed.

Interestingly, PKC is also able to modulate the level of NKCC1 mRNA expression in cultured cells (Farokhzad et al. 1999; Liedtke et al. 2001), suggesting that regulation of NKCC1 activity through phosphorylation occurs at both functional levels and transcriptional levels. However, whether this mechanism exists in acute hippocampal slices is as yet unknown.

5.4.4 Function of mGluRs on the modulation of KCC2 and NKCC1 activity

Apart from the mGluRs expressed in pyramidal neurons in hippocampus, mGluRs are also located both on the cell soma and the axonal terminals of inhibitory interneurons. Activation of mGluRs (mostly group I mGluRs) located on the soma leads to an enhanced excitation of the interneurons, and thereby, an increase in inhibitory input activity. Conversely, activation of mGluR located on the axon terminals results in an inhibition of inhibitory transmitter release (Anwyl 1999). Therefore, the effects of mGluRs on inhibitory synapses may vary under different conditions. In present study, TBS was found to be able to induce two-opposite-direction shifts in E_{GABA} in both juvenile and neonatal rat hippocampal neurons. Interestingly, it appears that mGluRs play a dual role in regulating E_{GABA} in both cases via either KCC2 (juvenile) or NKCC1 (neonates). Our results indicate that changes in the activity of KCC2 or NKCC1 are responsible for TBS-induced shifts in E_{GABA} in rat hippocampus. Given that changes in KCC2 or NKCC1 activity and resultant shift in E_{GABA} have been reported in many physiological or pathological disorders (Hasbargen et al. 2010; Munakata et al. 2007; Munoz et al. 2007; Wake et al. 2007; Wang et al. 2006), our findings may have implications for them.

5.4.4.1 mGluRs are involved in TBS-induced shift in E_{GABA} in juvenile rat hippocampus via regulation of KCC2 activity

Although various mechanisms have been proposed to account for the modulation of KCC2 in central neurons (Fiumelli et al. 2005; Ganguly et al. 2001; Kelsch et al. 2001; Lee et al. 2007; Rivera et al. 2004; Woodin et al. 2003), the exact mechanism underlying the regulation of KCC2 is still unclear. Recently, activation of group I mGluRs has been suggested to be responsible for the regulation of KCC2 activity in hippocampal CA3 neurons (Banke and Gegelashvili 2008). In line with this thinking, data from the current study indicates that mGluRs are involved in TBS-induced shifts in E_{GABA} via an up-regulation of KCC2 activity in juvenile hippocampal CA1 neurons. In addition, a significant increase in the expression of KCC2 (compared to either control or MCPG treated slices) was detected in TBS-treated juvenile rat hippocampus in western blot and a marked increase in the population of immunostained neurons for KCC2 (compared to either control or MCPG treated slices) was seen. However, one question remains to be answered - "how does mGluR modulate KCC2 activity following TBS in hippocampal CA1 neurons?" There are several lines of electrophysiological and morphological evidence supporting mGluRs as a candidate for regulating KCC2 activity in hippocampal neurons: a) immunohistochemical studies demonstrate that both group I mGluRs (Lujan et al. 1996) and KCC2 (Gulyas et al. 2001) are expressed in somatic and dendritic membranes of hippocampus pyramidal neurons; b) Fiumelli et al. reported that KCC2 function is regulated by intracellular Ca^{2+} via PKC-dependent phosphorylation of KCC2 or its regulatory proteins in hippocampal cultured cells (Fiumelli et al. 2005). This statement was further supported by our data obtained by high BAPTA loaded recording pipettes; c) activation of group I mGluRs leads to an increase in $[Ca^{2+}]_i$ via VGCC (Schomberg et al. 2001) or from internal stores (Woodhall et al. 1999). Moreover, there is evidence that Ca^{2+} is involved in the gene expression of KCC2 in the CNS

(Galanopoulou et al. 2003; Toyoda et al. 2003). Therefore, postsynaptic Ca^{2+} participate in the modulation of KCC2 activity at both function and expression levels through the activation of mGluRs. Ca^{2+}-dependent PKC phosphorylation has been suggested to be the major pathway involved in the regulation of KCC2 function (Fiumelli et al. 2005; Payne et al. 1996).

The possible involvement of dephosphorylation process in the modulation of KCC2 activity was examined by pretreating slices with okadiac acid. Our data suggest that the dephosphorylation of KCC2 is not responsible for the up-regulation of KCC2 following TBS in hippocampal neurons. Therefore, the modulation effect of mGluR on KCC2 activity following TBS can be proposed as a three step process as below (see Fig. 5-1): 1) high frequency stimulation (such as TBS) of the input leads to an increase in the release of glutamate into synaptic cleft; 2) excessive glutamate caused by TBS may activate both presynaptically located or extrasynaptically located (Boudaba et al. 2003) group I mGluRs and results in a sudden increase in postsynaptic Ca^{2+} either from VGCC or internal Ca^{2+} store; 3) Ca^{2+}-dependent PKC phosphorylation contributes to the up-regulation of KCC2 at functional level and Ca^{2+}-dependent regulation results in the enhancement of KCC2 expression at translation level, leading to the up-regulation of KCC2 in central neurons.

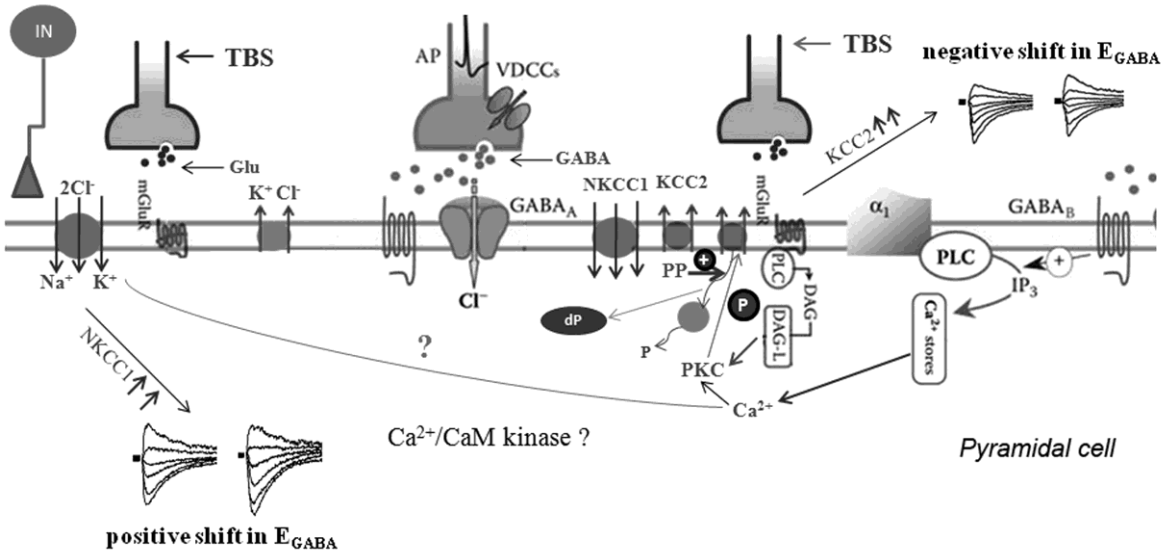

Figure 5-1 Schematic of signaling pathway involved in TBS-induced shifts in E_{GABA} in rat hippocampal neurons

Following TBS, the increased release in glutamate leads to activation of mGluRs (group I in this case). Activation of mGluRs can up-regulate the activity of NKCC1 or KCC2 presumably via phosphorylation or dephosphorylation of two Cl^- cotransporters (a speculation at this time). An up-regulation of KCC2 activity contributes to a negative shift in E_{GABA} in juvenile neurons, while the up-regulation of NKCC1 leads to a positive shift in E_{GABA} in neonatal neurons.

5.4.4.2 mGluRs contributes to TBS-induced shift in E_{GABA} in neonatal rat hippocampus via modulation of NKCC1 activity

As mentioned above, NKCC1 plays an important role in the maintenance of E_{GABA} and determination of postsynaptic responses to GABA in neonates. Synergistic excitatory action of $GABA_A$ receptor and glutamergic receptors has already been suggested in the neonatal hippocampus (Ben-Ari et al. 1997). Group I mGluRs are expressed in hippocampal neurons in the early developmental stage (Lujan et al. 1996; Romano et al. 1996) and the expression of

NKCC1 is set at a higher level in this stage than later stage (beyond postnatal 2 weeks). Both ionotropic glutamate receptors (NMDA and AMPA) and group I mGluRs are suggested to be involved in the regulation of NKCC1 activity in immature cortical neurons (Schomberg et al. 2001; Sun and Murali 1999; 1998). Therefore, it is of interest to check whether mGluRs contribute to TBS-induced shifts in E_{GABA} via the up-regulation of NKCC1 in neonatal hippocampal neurons. Moreover, our okaidac acid and intracellular BAPTA experiments suggest the involvement of postsynaptic Ca^{2+} and phosphotases (PP1 or PP2A) in the up-regulation of NKCC1 activity following TBS in neonatal hippocampal neurons. Activation of group I mGluRs leads to the increase in $[Ca^{2+}]_i$ which in turn stimulates the PKC-dependent pathway in the phosphorylation of NKCC1. In addition, it has been reported that Ca^{2+} dynamics plays a critical role in PP1- and PP2A- modulated series of events in cells (Kong et al. 2006). Therefore, the signaling cascades for the modulation of mGluRs on NKCC1 activity in neonatal neurons could be proposed. The whole signaling transduction pathway is similar as what was proposed in last section (modulation of mGluR on KCC2 activity).

5.4.4.3 Subtypes of mGluRs involved in TBS-induced shifts in E_{GABA}

There is a large body of evidence in literature suggesting that group I mGluRs play an important role in synaptic plasticity in the CNS. Group I mGluRs are suggested to be involved in the induction of LTD in hippocampal CA1 region (Watabe et al. 2002), dentate gyrus (Camodeca et al. 1999) and corticostriatal (Calabresi et al. 1992; Calabresi et al. 1993; Gubellini et al. 2001; Sung et al. 2000). Our present data indicate that group I and/or II mGluRs are involved in TBS-induced shifts in E_{GABA} in rat hippocampus. However, our recent study on EGLU (a selective group II antagonist) suggests that group II mGluRs seem not to be involved

in TBS-induced shifts in E_{GABA} in juvenile hippocampus (data not shown). Surprisingly, either mGluR1 or mGluR5 alone seems to be sufficient for TBS-induced negative shift in E_{GABA} in juvenile hippocampus. Nevertheless, in immature neuron, co-activation of both mGluR1 and mGluR5 seems to be necessary for TBS-induced positive shift in E_{GABA}. Both mGluR 1a and mGluR 5 are expressed in the cultured immature hippocampal neurons (Lujan et al. 1996; Romano et al. 1996; Schomberg et al. 2001). The extrasynaptic localization of mGluR1 and mGluR5 in both pyramidal cells and interneurons (Lujan et al. 1996), perhaps, can facilitate the coordinated and synergistic activation of these two receptors. In addition, the cooperativity may occur somewhere between downstream elements (such as G-proteins and second messengers) of their respective signal transduction systems. At present, it is not known which group I mGluR may be localized on Schaffer collateral terminals. Previous studies showed that mGluR1a does not appear to be localized on excitatory or inhibitory nerve terminals in the hippocampus (Baude et al. 1993; Martin et al. 1992). However, the subcellular distribution patterns for the other mGluR1 splice variants (mGluR1b/mGluR1c) are not known (Gereau and Conn 1995).

5.4.4.4 Significance of the dual modulation role of mGluRs on KCC2 activity and NKCC1 activity in neonates

mGluRs seem to be involved in differentially modulating the TBS-induced shifts in juvenile and immature hippocampal neurons via regulation of KCC2 or NKCC1 activity, respectively. The dual modulatory effects of mGluRs on KCC2 activity in juvenile or NKCC1 activity in immature neurons are of particular significance: a) stimulation of NKCC1 activity caused by the activation of mGluRs in immature hippocampal neurons could increase $[Cl^-]_i$ which reinforces the depolarizing GABA response, contribute to K^+ re-accumulation in neurons after an action potential and promote Na^+ influx for Na^+-K^+-ATPase function (Walz 1992); b)

elevation of KCC2 activity by mGluRs facilitates the Cl^- extrusion from juvenile or adult neurons, which renders GABA to exert the classical inhibitory effects in the CNS; c) since abnormal activity of KCC2 or NKCC1 is associated with neurological disorders involving cell damage and neuronal death, changes in mGluR activity would have significant implications for their modulation of neuronal network excitability in the CNS and for development of rational therapeutic strategies.

5.5 Future direction

In this thesis, various types of activity-mediated plasticity of E_{GABA} were examined and underlying mechanisms investigated. Among the activity-mediated plasticity of E_{GABA}, theta-burst stimulation induced shifts in E_{GABA} in both juvenile and neonatal hippocampal neurons via an up-regulation of either KCC2 or NKCC1 activity were examined in more detail. Activation of mGluRs seems to play a dual role in regulating KCC2 in juvenile rat neurons and NKCC1 in neonates following TBS. However, several issues are yet to be resolved. First, since postsynaptic Ca^{2+} has been suggested to be involved in TBS-induced shifts in E_{GABA} in both juvenile and neonatal hippocampal neurons, it will be interesting to measure the changes of $[Ca^{2+}]_i$ by microfluorimetric technique along with electrophysiological recording. It will provide more direct evidence on the involvement of $[Ca^{2+}]_i$ in the modulation of either KCC2 or NKCC1 activity following TBS. Second, even though my thesis work suggests that phosphorylation of KCC2 or NKCC1 seems to account for the regulation of their activity following TBS in hippocampal neurons, the exact kinase (or kinases) which may participate in the phosphorylation of KCC2 or NKCC1 was not examined. Probably various kinases including PKA inhibitor, PKC inhibitor and Ca^{2+}/CaM blockers can be examined. Third, it remains unclear how dephosphorylation of NKCC1 contributes to the regulation of NKCC1 in hippocampal neurons.

As mentioned above, multiple kinase/phosphatases are involved in the phosphorylation or dephosphorylation of NKCC1 activity in the CNS. More selective inhibitors of kinase/phosphatases will be extremely useful in this type of investigation. Finally, the current study shows that group I but not group II mGluRs are responsible for the regulation of either KCC2 or NKCC1 following TBS in hippocampal neurons. Moreover, coordinated or synergistic activation of mGluR1 and mGluR5 on the TBS-induced shift in E_{GABA} seems to exist in juvenile but not neonatal hippocampal neurons. It is tempting to examine which factor accounts for the crosslink of their respective signal transduction systems.

5.6 Plasticity of E_{GABA} and implications for the CNS

Considering that GABA is a major transmitter in most regions of the mammalian CNS involved in the modulation of cell excitability and neuronal network activity, plasticity in GABA-ergic transmission can have significant consequences for CNS function in general. Since E_{GABA} changes with age leading to the amino acid being excitatory in the neonatal and inhibitory in the juvenile and adult brain, age should be taken into consideration in developing therapeutics involving GABA as a transmitter. As mentioned elsewhere in Discussion, the dual excitatory and inhibitory behavior of GABA will have implications for plasticity of excitatory transmission, as an excitatory transmitter helping glutamatergic synaptic plasticity via removal of Mg^{2+} block of NMDA channels and as an inhibitory transmitter, helping with the Mg^{2+} block of the NMDA channel and hence, impeding glutamatergic synaptic plasticity.

In addition to the age-related plasticity of E_{GABA}, as reported in this thesis, there is presynaptic activity-mediated plasticity of E_{GABA} which complicates GABA-ergic transmission. As the activity changes, so does the E_{GABA}, and hence, depending on the membrane potential of the target neuron, GABA can be either inhibitory or excitatory. Whether it is a transient or a

long-lasting shift in E_{GABA}, such changes will have significant implications for neuronal excitability and network activity and, thus, on CNS behavior in general. In other words, even in juvenile and adult neurons, GABA cannot be considered as an inhibitory transmitter but as a dynamic system setting CNS activity at different levels depending on activity in the input neurons. This poses difficult problems in designing GABA-ergic therapeutic agents for treating CNS disorders. The traditional view of GABA being an inhibitory transmitter must be replaced with a more realistic, multifaceted and dynamically changing CNS modulator.

5.7 Conclusion and summary

Equilibrium potential of GABA (E_{GABA}) undergoes both activity-mediated plasticity and age-dependent plasticity in rat hippocampus. Various types of activity-mediated plasticity of $GABA_A$-mediated IPSCs have been induced under different conditioning stimulations in this thesis work and shifts in E_{GABA} appear to be involved in (or associated with) some types of plasticity such as FDD and PTP of $GABA_A$-mediated IPSCs in hippocampal neurons. Repetitive low frequency stimulations (ranging from 0.05 to 1Hz) are able to induce a negative shift in E_{GABA} via an up-regulation of KCC2 activity in juvenile rat hippocampal neurons. Our previous and current studies suggest that theta-burst stimulation is capable of inducing shifts in E_{GABA} in both juvenile and neonatal rat hippocampal neurons. Further investigation indicates that the TBS-induced shifts in E_{GABA} were due to the up-regulation of either KCC2 or NKCC1 activity in juvenile or neonatal hippocampal neurons, respectively. Moreover, our studies demonstrate that activation of mGluRs and postsynaptic Ca^{2+} seem to be involved in TBS-induced shifts in E_{GABA} in both juvenile and neonatal rat hippocampal neurons. Activation of $GABA_B$ receptor and G-proteins appears to be involved in TBS-induced negative shift in E_{GABA} in juvenile hippocampal neurons as well. Even though the exact signaling pathway of the phosphorylation or

dephosphorylation of either KCC2 or NKCC1 remains unknown, Ca^{2+}-dependent PKC pathway and/or Ca^{2+}/CaM kinases are believed to be responsible for the phosphorylation of KCC2 in juvenile hippocampal neurons and phosphoryaltion of NKCC1 in neonatal hippocampal neurons. The most intriguing finding in the current study is that mGluRs (group I mGluRs) play a dual role in modulating TBS-induced shifts in E_{GABA} via an up-regulation of either KCC2 activity in juvenile hippocampus leading to the preservation of GABA's inhibitory role or NKCC1 activity in neonatal hippocampus enhancing GABA's excitatory role. In addition, a coordinated effect of mGluR1 and mGluR5 on the regulation of TBS-induced shift in E_{GABA} is observed in neonatal but not in juvenile hippocampus.

Age-dependent plasticity of E_{GABA} was also observed in rat hippocampus. The relatively depolarized level of E_{GABA} in neonates is believed to contribute to the developmental switch of GABA response from excitatory to inhibitory during the maturation of the CNS. Interestingly, both KCC2 and NKCC1 undergo development expressions in rat hippocampal neurons and are subject to control by mGluRs and TBS conditioning. Considering that changes in E_{GABA} have been reported in various neurological disorders in recent years, results from this thesis work may have critical implications on a better understanding of normal brain functions and treatment of neurological diseases.

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