**CONTRIBUTION OF METABOTROPIC GLUTAMATE
RECEPTORS TO SYNAPTIC TRANSMISSIONS OF INFERIOR
COLLICULUS NEURONS**

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in Partial Fulfillment of the requirements for the degree of Doctor of Philosophy
(Specialization in Neuroscience)

by

Rasoul Farazifard

Institute of Neuroscience
Department of Psychology
Carleton University
Ottawa, Canada

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Abstract

The central nucleus of the inferior colliculus (ICC) integrates excitatory and inhibitory inputs from lower as well as higher auditory structures. In this study, the effects of metabotropic glutamate receptor (mGluR) activation on intrinsic membrane properties and fast glutamatergic and GABAergic transmission, AMPA and GABA_A receptor-mediated responses respectively, of ICC neurons were investigated. Whole-cell patch clamp recordings were made from brain slices of young rats. AMPA and GABA_A receptor-mediated EPSCs and IPSCs respectively were elicited by stimulation of the lateral lemniscus.

The agonist for mGluRs I and II, ACPD, and that for mGluRs III, SOP, did not affect intrinsic membrane properties of the ICC neurons. ACPD and LY379268 (the agonist for mGluRs II) significantly reduced the EPSCs and IPSCs, and the effects were dose-dependent. The effects of LY379268 on the EPSCs and IPSCs were reversed by mGluR II antagonist, LY341495. The specific agonists for mGluRs I and III, DHPG and SOP, respectively, did not affect the EPSCs and IPSCs. The reduction of the synaptic responses by LY379268 was accompanied by a substantial increase in a ratio of the 2nd to 1st EPSCs and IPSCs to paired-pulse stimulation. These results suggest that only mGluR II regulates glutamatergic and GABAergic synaptic transmissions in the ICC, probably through a presynaptic mechanism due to a reduction of transmitter release.

The EPSCs and IPSCs induced by 10 stimulus pulse train at 5, 30 and 100 Hz showed different forms of synaptic modulation, which was frequency-dependent. Application of LY379268 not only suppressed the EPSCs and IPSCs over the train

but also removed or changed the frequency-dependence of the synaptic modulation. These results suggest that mGluRs II activation can modulate short-term plasticity of excitatory and inhibitory synaptic transmission in the ICC.

TBOA, the glutamate transporter blocker, had almost the same effect as LY379268 on the EPSC and IPSC modulation over the stimulus trains. That effect could be reversed by LY341495. TBOA also increased temporal summation, charge transfer across the membrane and decay time of the EPSCs. These results suggest that glutamate transporters are involved not only in glutamate clearance but also in activating mGluRs II in the ICC.

Dedicated to my lovely wife and son.

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List of Abbreviations

ACh	acetylcholine
ACPD	(±)-1-Aminocyclopentane-trans-1,3-dicarboxylic acid
ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP	action potential
APV	2-amino-5-phosphonovaleric acid
AUC	area under the curve
cAMP	cyclic adenosine monophosphate
CN	cochlear nucleus
CNS	central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CoIC	commissure of the inferior colliculus
CPP	3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid
DCN	dorsal cochlear nucleus
DHPG	(RS)-3,5-Dihydroxyphenylglycine
DNLL	dorsal nucleus of the lateral lemniscus
EAAT	excitatory amino acid transporter
EGLU	(2S)-α-Ethylglutamic acid
EPSC	excitatory post synaptic potential
EPSP	excitatory postsynaptic potential
GABA	γ-aminobutyric acid

GluR	AMPA receptor subunit
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid
IC	inferior colliculus
ICC	central nucleus of the inferior colliculus
ICD	dorsal cortex of the inferior colliculus
ICX	external cortex of the inferior Colliculus
I_h	hyperpolarization-activated inward current
IP ₃	inositol 1,4,5-trisphosphate
IPSC	inhibitory post synaptic current
IPSP	inhibitory postsynaptic potential
LL	lateral lemniscus
LSO	lateral superior olive
LTD	long term Depression
LTP	long term potentiation
LY341495	(2 <i>S</i>)-2-Amino-2-[(1 <i>S</i> ,2 <i>S</i>)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid
LY379268	(1 <i>R</i> ,4 <i>R</i> ,5 <i>S</i> ,6 <i>R</i>)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid
MCPG	(α)-methyl-4-carboxyphenylglycine
MGB	medial geniculate body
mGluR	metabotropic glutamate receptors
NBQX	(1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide)

NMDA	<i>N</i> -methyl-D-aspartate
NTB	nucleus of the trapezoidal body
PBS	phosphate buffered saline
PKC	protein kinase C
PPD	paired pulse depression
PPF	paired pulse facilitation
PSP	post synaptic potential
R_{in}	input resistance
RMP	resting membrane potential
RM-ANOVA	repeated measure ANOVA
RT-PCR	reverse transcription polymerase chain reaction
SOC	superior olivary complex
SOP	L-2-Amino-3-hydroxypropanoic acid 3-phosphate
τ	time constant
TBOA	DL-threo-b-Benzoyloxyaspartic acid
vGluT	glutamate vesicular transporters
VNLL	ventral nucleus of the lateral lemniscus

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1. Introduction

Glutamate and γ -aminobutyric acid (GABA) are respectively the two most abundant neurotransmitters mediating excitation and inhibition in the brain. An individual neuron usually receives excitatory as well as inhibitory inputs. Balanced activation of excitatory and inhibitory pathways to the same neuron is critical not only for normal neuronal function but also for maintenance and survival of the neuron (reviewed in Moulder et al., 2006). One of the key mechanisms to achieve balanced excitation and inhibition is through modulation of synaptic transmission by endogenous neurotransmitters via autoreceptors or heteroreceptors. Besides ionotropic glutamate receptors, glutamate also activates metabotropic glutamate receptors (mGluRs). The mGluRs activate a number of signaling pathways. In the auditory system, their physiological functions involve the modulation of neuronal excitability, feedback regulation of neurotransmitter release, synaptic integration, and plasticity in form of long-term potentiation (LTP) and long-term depression (LTD) (Alexander and Godwin, 2005 and 2006; Bandrowski et al., 2001 and 2002; Lu, 2007; Schwarz et al., 2000). In addition, they may play a role in pathological conditions of hyperexcitability (audiogenic seizure) or hypoexcitability (hearing loss) through control of glutamate and GABA releases.

Along the auditory pathway, inferior colliculus (IC) has a great role in sensory processing. It has been shown that the highest level of glucose transport protein can be found in the IC (Zeller et al., 1997), and activation of mGluRs can increase the glucose use of the IC by more than 20% (Lam et al., 1999). The especially high metabolic rate in the IC must support its essential functions in detection of sound and

representation of sound in space. Divergent and convergent ascending as well as descending auditory pathways are terminated in the IC (Herbert et al., 1991; Huffman and Henson, 1990; Saldana et al., 1996; Winer et al., 1998) where both the excitatory and inhibitory neurotransmitters work together to integrate the signals, and provide the functions. It is well known that ionotropic glutamate and GABA receptors contribute to the neurotransmission of the IC neurons, however, there is a lack of information about the role of mGluRs in the regulation of this neurotransmission.

An understanding of the interplay between ionotropic and metabotropic actions in the IC is important for understanding mechanisms that may underlie normal auditory processing and pathological conditions in the IC. There is only one brief *in vivo* report about the role of mGluRs in bat IC. The report showed that antagonists for groups I and II but not III, mGluRs had effects on the rate-level function of the IC neurons in response to temporally complex sounds (Voytenko and Galazyuk, 2008). More studies are required to address the functional role of mGluRs in auditory processing. The present study examined if functional mGluRs are present on presynaptic or postsynaptic site in central part of IC (ICC) *in vitro*, and investigated roles of mGluRs in synaptic transmission. The contribution of glutamate transporter to activation of mGluRs and synaptic transmission was also investigated.

1.1. Auditory system

The auditory system consists of ascending and descending pathways. Auditory signals received by inner and outer hair cells in the cochlea are sent to the cochlear nucleus (CN) and superior olivary complex (SOC) in the brainstem through

the auditory nerve. The CN is divided into three main domains: anterior ventral cochlear nucleus, posterior ventral cochlear nucleus and dorsal cochlear nucleus (DCN). The SOC is located in the caudal portion of the pons, and is divided into three main nuclei: medial superior olivary nucleus, lateral superior olivary nucleus (LSO) and nucleus of the trapezoidal body (NTB). The neuronal signals carrying auditory information then go to the IC in the midbrain by fibers of the lateral lemniscus (LL). There are three major nuclei embedded in the LL: ventral nucleus (VNLL), dorsal nucleus (DNLL), and intermediate nucleus of the LL (Helfert et al., 1991; Tanaka et al., 1985; Whitley and Henkel, 1984). The IC receives afferents bilaterally from the SOC and contralaterally from the CN. The IC mainly projects to the thalamus. Finally, the signals reach the auditory cortex from projection of the medial geniculate body (MGB) in the thalamus (Demanez and Demanez, 2003).

1.1.1. Inferior Colliculus (IC)

The IC is a critical structure for auditory processing and integration of auditory information with motor and other sensory information. Functionally, the IC is involved in integration and routing of multimodal sensory perception, including startle and ocular reflexes. The IC plays an important role in a number of pathophysiological conditions that engage hearing. For instance, it has been reported that the IC is involved in and responsible for occurrence of audiogenic seizures (Faingold, 2002; Yasuda et al., 2000). Some studies have also shown that the IC is involved in tinnitus and age-related hearing loss (Syka, 2002, for review).

Auditory information from the brainstem nuclei is analyzed and transformed by cells in the IC, and relayed to the thalamus and further to the cortex. The IC is located below the superior colliculus, and can be found on the dorsal surface of the midbrain. The IC can be divided into three anatomical subdivisions: ICC, dorsal cortex (ICD) and external cortex (ICX). The ICC is encapsulated by the ICD dorsally and by the ICX ventrally, laterally and rostrally. The subdivisions of the IC are different in their responses to acoustic stimuli in addition to the cell type and size, dendritic field shape and organization (Huffman and Hensor, 1990). Therefore, this anatomical classification can be related to sensory neurophysiology. The ICC is composed of a series of cochleotopically ordered layers (Oliver and Shneiderman, 1989). Neurons in the ICX and ICD are organized in three layers, showing a cortical-like neuronal architecture. In rats, the ICC can be distinguished from the ICD and ICX by the large number of myelinated fibers passing through the ICC (Koch and Groth, 2003).

1.1.2. Ascending and descending projections of the IC

The IC receives both ascending and descending auditory projections from lower brainstem nuclei and higher auditory structures as well as contralateral IC and non-auditory structures. The ascending auditory system is composed of a large number of nuclei connected through a series of parallel pathways (Fig. 1.1). The IC receives its major ascending input from the CN, SOC, and LL as well as the contralateral IC (Batra and Fitzpatrick, 2002; Cant and Benson, 2006; Moore, 1988; Nordeen et al., 1983).

The IC provides the principal source of innervations to the MGB and so indirectly to the auditory cortex. Projections from the IC to the MGB are both excitatory and inhibitory (Bartlett et al., 2000; Peruzzi et al., 1997; Winer et al., 1998). The ICC sends some fibers to the ipsilateral ICD and ICX, called “intrinsic connection” (Saldana and Merchan, 1992). It has been suggested that the most of these intrinsic connections are excitatory (Fremouw et al., 1999; Yang et al., 2000). Moreover, the ICC also sends some projections to the contralateral IC. Indeed, the right and the left ICs in the auditory midbrain are connected to one another by a bundle of fibers, the commissure of the IC (CoIC). Studies have shown that this commissural projection connects corresponding frequency regions in the two sides, and mainly originates from excitatory neurons (Malmierca et al., 1995; Saldana and Merchan, 1992). It is suggested that a smaller number of inhibitory neurons may also project via the CoIC (Moore et al., 1998).

The responses of IC neurons are modulated by descending projections from higher auditory and non-auditory centers. Traditionally, corticofugal fibers were believed to project mainly to the ICD. However, there are some anatomical evidences suggesting that a substantial number of fibers from the primary auditory cortex project to the ICC, and appear to be tonotopically organized (Bajo et al., 2007; Lim and Anderson, 2007; Saldana et al., 1996). It is believed that the cortical projections to the IC are excitatory as the corticocollicular pathway contains glutamatergic and/or aspartatergic fibers (Feliciano and Potashner, 1995). There are some descending projections from the IC to the lower auditory structures. It has been shown that the IC has descending projection to the DNLL and VNLL. High frequency regions in the

ICC send descending projections to the medial parts of the NTB (MNTB) and DCN while low-frequency regions in the ICC project to the lateral parts of the NTB and the DCN (Malmierca et al., 1995).

1.1.3. Characteristics of neurons in the ICC

The ICC is described by the presence of fibrodendritic lamina distinguishable in Golgi staining (Oliver, 2005). This laminar organization has been observed in bat (Zook et al., 1985), cat (Morest and Oliver, 1984), rat (Faye-Lund and Osen, 1985), and guinea pig (Malmierca et al., 1995). Morphological study has shown that there are two major cell types in the ICC of cat, disc-shaped neurons and stellate cells. The stellate cells contribute about 25% of all ICC neurons. They have radiating dendritic arbors which usually extend beyond the single fibrodendritic lamina into adjacent lamina (Oliver, 2005). The most general cell type in the ICC is disc-shaped neurons. They have parallel dendritic fields, and contribute to a discrete lamina appearance. In the rat, two types of neurons including flat neurons and less flat neurons, instead of disc-shaped and stellate neurons, have been categorized (Malmierca et al., 1993, Oliver, 2005).

The processing of information is different in the ICC, ICD and ICX which are distinguished morphologically as well as by different inputs and outputs. The ICC neurons have sharper frequency tuning, lower threshold, shorter spike latency and higher rate of spontaneous activity than ICD and ICX neurons. The ICC and ICD neurons respond more frequently in a sustained manner than the ICX neurons. The ICX neurons have higher threshold and shorter response latency than the ICD

neurons (Syka et al., 2000). The ICC neurons code interaural time difference, interaural intensity difference, and interaural phase difference (Irvine, 1992; Wenstrup et al., 1988). It is believed that localization of high frequency sounds are mainly through interaural intensity difference mechanism while localization of low frequency sounds are by use of the interaural time difference mechanism (Pollak et al., 2003).

The ICC neurons show a variety of firing patterns during constant-current injections using intracellular or patch-clamp recordings. An important distinction is made based on the amount of accommodation. Many cells show sustained firing during current injection whereas other cells accommodate rapidly or show onset firing. In addition, cells can be classified based on firing regularity or on the presence of rebound, burst, or build-up firing in response to constant-current injections (Bal et al., 2002; Koch and Grothe, 2003; Peruzzi et al., 2000; Sivaramakrishnan and Oliver, 2001; Tan et al., 2007). Sivaramakrishnan and Oliver (2001) recorded the responses of the ICC neurons in brain slice preparation. They categorized the ICC neurons into six groups according to their firing patterns including sustained regular, onset, build-up/pauser, rebound regular, rebound adapting and rebound transient. *In vivo* patch clamp recording has also shown the same firing patterns in the IC neurons, however, burst onset was found instead of single-spike onset (Tan et al., 2007). The neurons with different firing pattern differ in their temporal coding abilities. For example, the onset neurons are able to follow trains of short current pulses at high rates up to 200 spikes/sec whereas the maximal rate generated by neurons with sustained discharge patterns is only 40 spikes/sec (Condon et al., 1996; Peruzzi et al., 2000). The build-up

neurons show a typical delay in firing, which became more pronounced when the depolarizing current is preceded by a hyperpolarization current (Sivaramakrishnan and Oliver, 2001). Both *in vivo* (Tan et al., 2007) and *in vitro* (Koch and Grothe, 2003) studies have shown that almost half of the IC neurons have an obvious depolarizing sag, suggesting that they have the hyperpolarization-activated current, I_h . The I_h current also contributes to the different firing patterns. The sag has been detected more frequently in the burst and adapting neurons than in the build-up or sustained neurons (Tan et al., 2007), and the I_h current in the onset and adapting neurons is larger than that in the sustained neurons (Koch and Grothe, 2003). Resting membrane potential (RMP) of neurons with I_h is more depolarized. The neurons with I_h more likely generate rebound spikes, and usually show long-lasting afterhyperpolarization following long depolarization (Tan et al., 2007).

1.2. Synaptic receptors

In the auditory system like other parts of the brain, excitatory neurotransmission is mainly mediated by the action of glutamate on AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and NMDA (N-methyl-D-aspartate) receptors whereas inhibition mostly depends on two neurotransmitters, GABA and glycine. However, other neuroactive substances such as acetylcholine, serotonin and norepinephrine also have some role in shaping the responses of the IC neurons (Kelly and Caspary, 2005).

1.2.1. GABA receptors

The activity of inhibitory neurons is critical to set the spatiotemporal conditions required for information processing (Mohler, 2006). GABA as the major inhibitory neurotransmitter in the vertebrate central nervous system, mediates synaptic inhibition through its receptors. These receptors can be divided into two major types: ionotropic receptors that are ligand-gated ion channels, and metabotropic receptors (GABA_B receptors) that are G-protein coupled receptors and act via second messengers (Johnston, 2005). The family of ionotropic GABA receptors is divided into two subfamilies: GABA_A and GABA_C receptors; on the basis of their structures and differences in their physiological and pharmacological properties (Bormann, 2000; Johnston, 2005).

The GABA_A receptors are usually found on postsynaptic sites of neurons. Most of the inhibitory effects of GABA are mediated by the GABA_A receptors. Different GABA_A receptor subunits are arranged to form a ligand-gated ion channel with distinct biophysical and pharmacological properties (Brickley et al., 1999; Fisher and Macdonald, 1997). Binding of GABA to the GABA_A receptors triggers opening of the receptor (channel) that gates Cl⁻ and, to a lesser extent, HCO³⁻ (Krushaar and Backus, 2002). Most neurons in the adult brain maintain a low intracellular Cl⁻ concentration due to the activity of a K⁺/Cl⁻ cotransporter. Therefore, GABA_A receptor activation in most cases produces a net entry of Cl⁻ into the neurons. The Cl⁻ entry elicits inhibitory postsynaptic potentials (IPSP) due to the hyperpolarizing effect of Cl⁻ influx, shunting of excitatory synaptic inputs, and consequently decreases probability of generation of action potentials (APs). It has been shown that

the GABA_A activation can also evoke depolarization in early developing neurons of various brain areas including auditory brain stem neurons (Kandler and Friauf, 1995).

It is believed that the GABA_B receptors are present at both presynaptic and postsynaptic sites. The GABA_B receptors have 7 transmembrane domains. The receptor is a heterodimer of subunit GABA_{B1} and GABA_{B2}. The GABA_{B1} subunit is involved in agonist activation of the receptor while GABA_{B2} subunit is involved in G-protein activation. It has been shown that GABA_B autoreceptors and heteroreceptors usually inhibit neurotransmitter release by suppressing Ca²⁺ conductance and increasing K⁺ conductance (Bowery et al., 2002).

1.2.2. Function of GABA receptors in the IC

The major inhibitory transmitters in the IC are GABA and glycine. The glycinergic inhibitory inputs to the IC originate extrinsically while GABAergic inputs originate extrinsically as well as intrinsically (Fubara et al., 1996; Gonzalez-Hernandez et al., 1996; Oliver and Shneiderman, 1989; Roberts and Ribak, 1987; Zhang et al., 1998). Oliver et al. (1994) have shown that up to 20% of the neurons in the IC of cat are GABAergic, and GABA immunoreactive neurons have different soma size, orientation and axosomatic endings from GABA immunonegative neurons. In rat, the GABAergic neurons account up to 30% of the ICC neurons, and are larger than non-GABAergic neurons (Merchan et al., 2005). GABAergic neurons of the IC can be divided into two main groups according to their distinct firing types. Tonic type (including regular sustained, pauser and adapting) neurons repetitively fire in response to a depolarizing current pulse while transient and onset type neurons

generate APs just at the onset of current pulses. Both groups can show I_h current in response to hyperpolarizing current injection (Ono et al., 2005). In the IC, GABA is released in calcium-dependent manner (Moore and Moore, 1987), and taken up by a high-affinity uptake system (Suneja et al., 1995). It is believed that inhibition mediated by GABA plays an important role in the shaping of neuronal responses to simple and complex acoustic stimuli in the IC (Nataraj and Wenstrup, 2005). Alteration of normal GABA neurotransmission in central auditory pathways causes auditory dysfunction (Casparly et al., 1990). In addition, a deficit in GABA-mediated inhibition in the IC neurons has been shown to be a critical key in genetic and induced forms of audiogenic seizures (Faingold, 2002).

The morphological presence of the GABA_A receptors in the IC has been shown by some studies (Csapary et al., 1990; Fubara et al., 1996; Glendenning and Baker, 1988). In bat, neurons with GABA_A receptors are distributed mostly in the dorsomedial, and lightly in the ventrolateral regions of the IC (Fubara et al., 1996). It has been shown that activating or blocking of the GABA_A receptors can modify the responses of IC neurons in bat (Park and Pollak, 1993), cat (Watanabe and Simada, 1973), rat (Faingold et al., 1989; Zhang and Kelly, 2001 and 2003), owl (Fujita and Konishi, 1991) and guinea pig (Le Beau et al., 1996). *In vivo* recordings have revealed that GABA_A receptor antagonists increase the firing rate of the IC neurons (Zhang and Kelly, 2001 and 2003) while iontophoretic application of GABA into the IC results in reduction of neuronal discharge rate that can be reversed by co-application of bicuculline, a GABA_A receptor antagonist (Faingold et al., 1989). Although GABA_A agonists reduce the firing rate of the IC neurons, they can have

opposing effects on rate-level functions, i.e. they can increase the firing rate at high stimulus intensities (Rees, 1992; Sivaramakrishnan et al., 2004). It has been shown that the tuning curve of the IC neurons become wider during bicuculline application and are narrowed during GABA application (Jen and Wu, 2005).

Ma et al. (2002a) have shown that muscimol (a GABA_A receptor agonist) significantly increases membrane conductance of the ICC neurons, and decreases the neuronal firing rate produced by depolarization current *in vitro*. Activation of GABA_A receptors basically increases Cl⁻ influx in the IC neurons, and mostly evokes inward or biphasic currents in the neurons (Frech et al., 1999). These currents depend on HCO₃⁻, and are characterized by a continuous shift of GABA_A reversal potential to positive direction. Electrical stimulation of the LL, the major source of afferent projection to the ICC, typically induces postsynaptic potentials (PSPs) in the ICC neurons (Wagner, 1996). The PSPs usually include a short latency excitatory postsynaptic potential (EPSP) followed by a longer latency IPSP. The IPSP can be separated from the EPSP by perfusion of the glutamate receptor antagonist to the ICC. The IPSP is mostly mediated by GABA_A receptors, however, it may include a glycinergic component (Moore et al., 1998). It has been shown that GABA_A receptors-mediated inhibitory postsynaptic current (IPSC) in the ICC neurons can follow high frequency (10-100 Hz) stimulation of the LL. However, the response shows depression at low rates and facilitation at higher rates (Wu et al., 2004).

Presence of GABA_B receptor in the IC has been confirmed by a few studies. Using quantitative receptor autoradiography technique, Milbrandt et al. (1994) have shown that GABA_B receptor binding is significantly higher in the ICD than in the

ICC and ICX. It has been shown that activation of the GABA_B receptors depresses the amplitude of inhibitory and excitatory synaptic responses in the ICC while it does not have any significant effect on cell membrane potential and conductance or firing rate of neurons (Ma et al., 2002a; Sun et al., 2006).

1.2.3. Glutamate receptors

L-glutamate is the predominant excitatory neurotransmitter in the vertebrate central nervous system. Glutamate exerts its physiological effects by binding to a number of different types of glutamate receptors. The glutamate receptors can be divided into two functionally distinct categories: those that mediate their effects via coupling to G-protein and second messenger systems, i.e. metabotropic glutamate receptors, and those that mediate their effects via ionotropic ligand-gated ion channels (Simeone et al., 2004). The metabotropic glutamate receptors are coupled to either inositol 1,4,5-trisphosphate (IP₃) or cAMP-signaling cascades (Jonas, 2000). The ionotropic glutamate receptors are heterotetrameric cation channels, and have been categorized based on their pharmacology and functions. They contain three distinct subtypes: AMPA, NMDA and kainate receptors. The ionotropic glutamate receptors are mostly concentrated at postsynaptic sites (Palmer et al., 2005).

1.2.4. Function of AMPA receptors in the IC

AMPA glutamate receptors mediate the majority of fast excitatory transmission in the brain. Most of the AMPA receptors are tetramer, and are composed of four subunits, GluR1-GluR4 (or GluRA-GluRD). The subunit

compositions vary depending on the brain region. The AMPA receptor channels have rapid onset, offset, and desensitization kinetics (Sprengel, 2006). They gate mostly Na^+ in response to ligand binding. This influx of ions causes a fast excitatory postsynaptic response, and exhibits moment to moment synaptic signaling (Jonas, 2000). The AMPA channels are mostly Ca^{2+} impermeable. Only AMPA receptors that lack the GluR2 subunit are permeable to Ca^{2+} (Cull-Candy et al., 2006). Several types of auditory neuron have AMPA receptors with unusually high permeability to divalent cations (Ca^{2+}) (Parks, 2000). Those receptors have very rapid desensitization and deactivation rates, and it is believed that the latter property is specific to the auditory pathway (Gardner et al., 1999; Parks, 2000).

In the IC, glutamate is the main excitatory neurotransmitter. Petralia and Wenthold (1992) reported moderate to high levels of immunoreactivity for GluRs 1, 2/3 and 4 while Sato et al. (1993) found low levels of GluR1 and GluR4 transcripts and high levels of GluRs 2 and 3 in the rat IC. In barn owl, it is claimed that the IC subdivisions can be distinguished based on glutamate receptors immunoreactivity. There are high level of GluR4 and moderate level of GluR1 in the ICX, and low level of GluR1 and 2/3 and moderate level of GluR4 in the ICC (Levin et al., 1997). Caicedo and Eybalin (1999) described immunoreactivity in neuronal cell bodies in the ICC of rats as light for GluR1, moderate for GluR2 and GluR2/3, and light for GluR4. It is suggested that most of the AMPA receptors in the ICC are impermeable to Ca^{2+} since they express high levels of GluR2 subunit (Schmid et al., 2001). Caicedo et al. (1998) have shown that permeability of the AMPA receptors to

calcium is very low in the ICC neurons, moderate in the ICD neurons, and high in the ICX neurons.

Researchers have used whole-cell patch-clamp recordings with drug application to study biophysical properties and pharmacology of the AMPA receptors in IC neurons. Brain slice studies of neurons in the rat ICC indicate that postsynaptic excitatory responses evoked by electrical stimulation of the LL consist of two components, an early, rapid response mediated by AMPA receptors and a later, a slower one mediated by NMDA receptors (Ma et al., 2002b; Wu et al., 2004). This phenomenon has also been observed in mouse (Wagner, 1996) and gerbil (Moore et al., 1998). For more accurate assessment of the kinetics of these excitatory responses, the inhibitory responses should be isolated and blocked by the GABA and glycine receptor antagonists. It has been shown that the AMPA component of excitatory postsynaptic responses can be blocked by AMPA receptor antagonists such as CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f] quinoxaline-7- sulfonamide), which confirms the role of AMPA receptors in early excitatory response (Ma et al., 2002b). The current-voltage relationship for AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) is linear, which shows that AMPA receptors are not voltage dependent (Ma et al., 2002b). AMPA receptor-mediated EPSCs in the ICC neurons induced by electrical stimulation of LL can follow repetitive stimulation at 10-100 Hz. Although the EPSCs undergo response depression during the repetitive stimulation, GABA_A receptor-mediated inhibition can decrease the degree of the EPSCs depression (Wu et al., 2004). It has been reported that activation of the GABA_B receptors by baclofen

can reduce the EPSC amplitude in the ICC neurons, and it is suggested a modulatory role for GABA_B receptors over AMPA receptors (Sun et al., 2006). Electrical stimulation of the CoIC can also produce post synaptic responses in the IC neurons. These responses are mostly excitatory but can be inhibitory or both excitatory and inhibitory (Li et al., 1999). Malmierca et al. (2005) recorded sound-evoked responses of neurons in one IC while blocking the excitation in the contralateral IC by injection of kynurenic acid (non-selective antagonist of glutamate receptors). They found that the responses of the IC neurons to auditory stimulation were declined. This result indicates that the IC receives glutamatergic projection from contralateral IC probably through CoIC.

In vivo studies have shown that application of AMPA and NMDA receptor antagonists lead to a decrease in firing rate of the ICC neurons. The antagonists have a selective influence on early and late components of tone-evoked responses (Zhang and Kelly, 2001 and 2003). It is reported that the AMPA antagonists have more effects on suppressing the early than late responses of the neurons that display sustain activity to a 100 ms tone (Kelly and Zhang, 2002). *In vivo* patch clamp recordings have indicated that almost all IC neurons show spontaneous EPSPs (Tan et al., 2007), which are probably mediated by AMPA receptors. These results suggest the importance of AMPA receptors in shaping of neuronal responses in the IC.

1.2.5. Function of NMDA receptors in the IC

NMDA receptors are located in the postsynaptic membrane (Rao and Finkbeiner, 2007). The NMDA receptors are composed of different subunits, NR1,

NR2, and NR3 (Monyer et al., 1992). The activation and deactivation kinetics of NMDA receptors are much slower than those of AMPA receptors. By contrast to the AMPA receptors, permeability to Ca^{2+} is a feature of the NMDA receptors. The NMDA receptors are responsible for most Ca^{2+} influx in response to synaptic activity (Monyer et al., 1992). Ca^{2+} entering through the NMDA receptors initiates Ca^{2+} dependent intracellular signaling pathways. Ca^{2+} is also crucial for inducing synaptic plasticity. Evidences show that the NMDA receptors not only are involved in short-term plasticity but also regulate long-term structural plasticity at the level of gene expression. The NMDA receptor signaling appears to have a major role in dendritic growth and formation of new synapses (Rao and Finkbeiner, 2007).

Some studies have indicated that in addition to the AMPA receptors, the NMDA receptors are important for shaping excitatory responses in the IC, and both receptor types probably play roles in auditory information processing. Zhang and Kelly (2001 and 2003) have shown that NMDA receptor antagonists decrease the firing rate of the ICC neurons in anaesthetized rat. They reported that the NMDA antagonists could significantly suppress the late part of the response of sustained neurons, which generate spikes during a long auditory stimulus. In brain slice preparation, Ma et al. (2002b) electrically stimulated the LL and recorded post synaptic responses of the ICC neurons in rat. They found that the NMDA receptor antagonist, APV (2-amino-5-phosphonopentanoic acid) or CPP (3-(2-carboxy piperazin-4-yl) propyl-1-phosphonic acid) can block the late component of the EPSPs. It was concluded that the late part of excitatory responses is mediated by the NMDA receptors (Wu et al., 2004). It has been shown that the amplitude and duration of the

NMDA responses are partially regulated by activation of the GABA_A receptors (Sivaramakrishnan and Oliver, 2006), and NMDA receptor-mediated responses are suppressed by activation of GABA_B receptors (Sun et al., 2006). These results suggest that the NMDA responses can be regulated by both GABA_A and GABA_B receptors.

1.2.6. Metabotropic glutamate receptors

The mGluRs couple to G-proteins to modulate neuronal response through intracellular second messengers (Hollmann and Heinemann 1994; Jonas, 2000). They are important for modulating neuronal excitability, synaptic plasticity and feedback regulation of neurotransmitter release (Ferraguti and Shigemoto, 2006). Molecular cloning has shown eight members of this family, called mGluR1 to mGluR8 (or mGlu1–8). The distribution of mGluR1, mGluR3, mGluR5 and mGluR7 is extensive throughout the brain whereas that of mGluR2, mGluR4 and mGluR8 is more restricted to specific brain regions. Expression of mGlu6 has been reported mostly in the retina (Laurie et al., 1997). The mGluR1 and mGluR5 are highly expressed at postsynaptic sites in rodents and primates (Hanson and Smith 1999; Poisik et al., 2003; Testa et al., 1998). The mGluR2 and 3 are localized at both pre- and post-synaptic sites (Pin and Acher, 2002). The mGluR4, 6, 7, and 8 are predominantly expressed at presynaptic sites, where they regulate neurotransmitter release (Schoepp, 2001). Metabotropic glutamate receptors are also present in glial cells, where they play important roles in neuroprotection, glial-neuronal communication, and glutamate

release (Winder and Conn, 1996; Yao et al., 2005; Ye and Sontheimer, 1999). Figure 1.2 shows the localization of different mGluRs in a hypothetical synapse.

The mGluRs are classified into three groups (mGluR I-III) based on sequence similarity, second messenger system involvement and relative pharmacology (Conn and Pin, 1997; Tanabe et al., 1992). They have different transduction mechanisms depending on the receptor subtype, the cell types in which they are expressed, and the associated effector molecules.

Group I	Group II	Group III
mGluR1, GluR5	mGluR2, mGluR3	mGluR4, mGluR6, mGluR7, mGluR8

The mGluRs I (mGluR1 and mGluR5) activate phospholipase C to generate diacylglycerol and IP₃. Their activation increases intracellular Ca²⁺ level by releasing Ca²⁺ ions from intracellular stores, stimulates protein kinase C (PKC) activity and inhibits K⁺ conductance (Conn and Pin, 1997). Staub et al. (1992) have shown that application of mGluR I agonist promotes an inward current in Purkinje cells of cerebellum, which is associated with an increase in input conductance of the cell membrane, and a rise in cytosolic Ca²⁺ concentration. The mGluRs I can mediate both excitatory and inhibitory postsynaptic responses. At the cerebellar parallel fiber-Purkinje cell synapse, high-frequency presynaptic activity can induce a slow EPSC that is mediated by the mGluR I (Reichelt and Knöpfel, 2002). The slow mGluR mediated EPSC can be induced at climbing fiber synapses in cerebellum too (Otis et al., 2004). Activation of mGluR I can generate not only excitatory but also inhibitory responses in hippocampal neurons. It is suggested that the mGluR I-mediated EPSC

is evoked by activation of Ca^{2+} activated non-selective cationic channel in CA1 (Charpak et al., 1990) while mGluR I-mediated EPSC is generated by blocking of a K^+ channel in CA3 (Congar et al., 1997). The mGluR I activation can also provoke a long lasting IPSP in forebrain neurons by activating G-protein-coupled, inwardly-rectifying potassium channels (Dutar et al., 2000). The activation of mGluR I mediates a slow IPSP in ventral midbrain dopaminergic neurons by releasing calcium from intracellular stores, and increasing potassium conductance (Fiorillo and Williams, 1998). The mGluRs I activation can both up- and down- regulate NMDA receptor activity, depending on the particular cell type. Grishin et al. (2004) have shown that the activation of mGluR I potentiates NMDA currents in CA1 but depresses the currents in CA3. Such bidirectional modulation may explain the involvement of the mGluRs I in both LTP and LTD (Camodeca et al., 1999; Gubellini et al., 2003). It has been shown that the mGluR I mostly depresses glutamate release by either activating K^+ channels (White et al., 2003) or inhibiting Ca^{2+} channel activity (Mutoh et al., 2005). Activation of mGluR I may also facilitate the neurotransmitter release in certain area of the brain. For example, Park et al. (2004) have shown that activation of presynaptic mGluR I increases glutamate release in spinal cord. It has been shown that activation of mGluR I can increase the amplitude of GABA_A receptor-mediated IPSP in hippocampus (Poncer et al., 1995). The mGluRs I are also able to enhance the release of ACh on nerve terminals of striatal cholinergic interneurons (Marti et al., 2001).

The mGluRs II (mGluR2 and 3) are negatively coupled to adenylate cyclase, and inhibit cAMP formation (Conn and Pin 1997; Pin and Acher, 2002). The mGluRs

III (mGluR4, mGluR6, mGluR7 and mGluR8) are also negatively coupled to the adenylate cyclase. Activation of mGluRs II and III mostly exerts an inhibitory action on L, N and P/Q type Ca^{2+} channels, and an excitatory action on hyperpolarizing K^{+} conductance (Anwyl, 1999). The stimulation of the mGluRs II and III usually produces a presynaptic inhibitory action on the release of several neurotransmitters, including glutamate and GABA (Gubellini et al., 2004). The mGluRs III activation reduces the release of glutamate through a G-protein-mediated inhibition of presynaptic Ca^{2+} channels. In contrast, the inhibition mediated by the mGluRs II in the hippocampal stratum results from activation of K^{+} channels (Capogna, 2004). It has been shown that presynaptic the mGluRs II/III function as heteroreceptors, and activation of those receptors attenuates GABA release from inhibitory terminals (Poncer et al., 1995). It has been also reported that mGluR II agonist inhibits the release of ACh from striatum. This effect can be prevented by an mGluR II selective antagonist, EGLU ((2S)- α -Ethylglutamic acid) (Marti et al., 2001). There are some studies that show the mGluRs II/III are involved in synaptic plasticity. The mGluR II can enhance the NMDA receptor current in pyramidal neurons of prefrontal cortex via a mechanism dependent on the PKC activation (Tyszkiewicz et al., 2004). Potent mGluR II agonists were found to inhibit LTP induction and maintenance while mGluR II antagonist was found to block LTD induction in the dentate gyrus *in vitro* (Huang et al., 1997). The mGluR II antagonists are also able to inhibit a late phase LTD in CA1 *in vivo* (Manahan-Vaughan, 1997).

1.2.7. Function of mGluR in the auditory system

Electrophysiological studies on the mGluRs have shown that mGluRs activation results in a large number of diverse cellular actions in the mammalian CNS (for review, Anwyl, 1999). There are a few studies which examined the effect of the mGluR activation on responses of auditory neurons. In the CN, activations of mGluR I, II and III lead to depression of GABA_A receptor-mediated IPSC amplitude but they do not change RMP or AP properties of the neurons. Activations of groups I and II mGluRs but mGluR III reduce the frequency of miniature IPSC recorded from CN neurons (Lu, 2007). It has been shown that the mGluRs I, II and III agonists inhibit high-voltage-activated Ca²⁺ channel current but they do not change Na⁺ or K⁺ currents in the CN neurons (Lu and Rubel, 2005). Sanes et al. (1998) have shown that the mGluRs I/II and III agonists initially increase spontaneous and sound-evoked discharge rates, and chronically cause depression or enhancement of the sound-evoked response of the CN neurons *in vivo*.

It has been found that application of a common agonist for the mGluRs I and II, ACPD, leads to a long lasting depolarization effect on LSO neurons (Kotak and Sanes, 1995). Wu and Fu (1998) have shown that applications of ACPD and L-AP4 as respective agonists for the mGluRs I/II and III decrease amplitude of EPSP in the LSO neurons whereas they do not change the input resistance (R_{in}) of the neurons. It has been shown that activation of the mGluR I can increase the release of dopamine from the lateral olivocochlear efferent fibers (Doleviczenyi et al., 2005).

In the MNTB, the mGluRs I/II and III agonists are able to decrease EPSC amplitude (Barnes-Davies and Forsythe, 1995), and the mGluR II agonist decreases

amplitude of GABA_A receptor-mediated IPSP (Nishimaki et al., 2007). Takahashi et al. (1996) recorded calcium current from presynaptic terminal in the MNTB, and showed that activation of the mGluR III inhibits the current passing through P/Q voltage-gated Ca²⁺ channels. It has been shown that application of ACPD as the agonist of the mGluRs I/II depolarizes MGB neurons, and inhibits inwardly rectifying K⁺ current on these neurons (Schwarz et al., 2000).

In the auditory cortex, it has been shown that activation of the mGluRs I/II reduces amplitudes of AMPA and NMDA receptors-mediated EPSPs and GABA_A and GABA_B receptors-mediated IPSPs (Bandrowski et al., 2001 and 2002). Kudoh et al. (2002) have shown that blocking of the mGluRs I/II by their antagonist, MCPG, causes suppression in induction of LTD in supragranular layers of the auditory cortex.

All of the above data obtained from auditory neurons suggest that the mGluRs may provide a key role in regulating not only glutamate but also GABA transmission. So far, there is no study to show the role of the mGluRs in synaptic regulation of IC neurons. However, Voytenko and Galazyuk (2008) showed that blocking of the mGluRs can change the responses of the IC neurons to complex sound stimuli *in vivo*. It has been shown that bilateral microinjections of the mGluRs II and III agonists, and mGluR I antagonist into the IC can suppress sound-induced seizures in genetically epilepsy-prone rats (Chapman et al., 1999 and 2001; Tang et al., 1997). The presence and distribution of metabotropic glutamate receptors in the IC may explain these effects. It has been found that distribution of the mRNA for mGluR1 is scattered and low in rat IC (Shigemoto et al., 1992). Non-specific mRNA immunolabeling of mGluR2/3 revealed light to moderate labeling for these receptors in the more rostral

areas as well as in the central region of rat IC (Petrulia et al., 1996). However, no significant expression of the mGluR2 mRNA or immunoreactivity for the mGluR2 has been observed in rat (Ohishi et al., 1993a and 1998) and mouse ICs (Tamaru et al., 2001). Moderate expression of mGluR3 mRNA and weak immunolabeling for the mGluR3 have been reported in both rat and mouse ICs (Ohishi et al., 1993b; Tamaru et al., 2001). Staining intensity of mGluR5 immunoreactivity has been found being very high in the ICD (Romano et al., 1995; Shigemoto et al., 1993) and moderate in the ICC and ICX (Romano et al., 1995). Kinzie et al. (1995) have reported low distribution of metabotropic receptor mGluR7 mRNA in the IC, however, no significant immunoreactivity was found for two alternative splicing variants of metabotropic glutamate receptor mGluR7, mGluR7a and mGluR7b, in the IC (Kinoshita et al., 1998). Using RT-PCR technique, it has been shown that all four genes encoding the mGluRs III (mGluR4, mGluR6, mGluR7 and mGluR8) are present in the IC, however, they are expressed at very low quantity (Yip et al., 2001). In summary, all groups I, II and III mGluRs have been found in the IC. The mGluRs I (mGluR1 and 5) are expressed at low to moderate levels throughout the IC. For group II, it seems that only subtype mGluR3 is present at moderate level, and there is no subtype mGluR2 in the IC. The mGluRs III are present at very low level in the IC.

1.2.8. Glutamate transporters

In addition to diffusion and breakdown of glutamate after its release, glutamate molecules may be reuptaken by glutamate transporters on glial and neuronal membranes to terminate the glutamate actions. Glutamate is reuptaken by

excitatory amino acid transporter (EAAT). Reuptaking of glutamate by the transporters has some physiological function. By clearance of glutamate, it terminates the activation of glutamate receptors. It improves signal to noise ratio of synaptic transmission by setting a lower limit for the glutamate concentration in extracellular space. It can reload the synaptic vesicles in synaptic terminals for future release by recycling glutamate. Finally, it can prevent glutamate neurotoxicity due to excessive exposure of neurons to glutamate (Shimamoto, 2008). The glutamate transporter uses electrochemical gradient forces across the cell membrane to transport glutamate molecules. It is proposed that three Na^+ ions and one H^+ ion accompany one glutamate molecule into the neurons and one K^+ ion flows out of the neurons. Therefore, there is a more positive inward charge in the neurons (Levy et al., 1998; Zerangue and Kavanaugh, 1996). Five subtypes of the EAAT have been identified (EAAT1-5) till now (Arriza et al., 1997; Fairman et al., 1995). The EAAT1 can be found in astrocytes throughout the whole brain. It was believed that the EAAT2 can be expressed only in astrocytes, however, it has been shown that it can be also found on presynaptic terminals of neurons (Chen et al., 2004). The maintenance of glutamate homeostasis in the brain mostly depends on the action of the EAAT1 and EAAT2. The EAAT3 and EAAT4 are expressed in postsynaptic membrane of neurons (Fig. 1.2). The EAAT3 is distributed throughout the brain while the EAAT4 can be found mainly in cerebellum (Danbolt, 2001). The EAAT5 can only be found in the retina at photoreceptor and bipolar cell terminals (Arriza et al., 1997).

There are few studies showing the role of glutamate transporters in regulating of synaptic transmission in auditory structures. For example, Turecek and Trussell

(2000) have shown that repetitive stimulation of auditory nerve fibers leads to more depression of EPSC amplitudes when blocking glutamate transporter than in normal condition in avian cochlear nucleus magnocellularis. It has been suggested that the glutamate transporters are necessary not only for removal and metabolism of glutamate but also for maturation and onset of auditory system (Jin et al., 2003). It has been shown that blockade of glutamate transporter in young rats causes a tonic activation of NMDA receptors and depression of AMPA response amplitude in calyx of Held synapse. It is believed that the latter effect is due to activation of mGluR II by spill-over of glutamate from synaptic cleft (Renden et al., 2005). There is no study to show the normal physiological function of glutamate transporters in the IC. However, Tadros et al. (2007) have shown that the EAATs are upregulated with age and hearing loss in the CBA mouse inferior colliculus (CBA mouse is an animal model for human presbycusis).

Figure 1.1.

Figure 1.1. The major ascending auditory pathways. In the auditory system, inferior colliculus receives projections from almost all brainstem nuclei and sends auditory inputs to cortex via thalamus. These projections are both excitatory and inhibitory. The excitatory synapses are mediated by glutamate (solid lines), and inhibitory synapses are mediated by GABA (solid and bold lines) and glycine (broken lines). The nuclei which receive inputs from only one ear are involved in monaural auditory processing while those obtaining inputs from both ears are binaural (adopted from Pollak et al., 2003).

Figure 1.2.

Figure 1.2. Hypothetical synapse illustrating the general glutamatergic and GABAergic receptors in pre- and post-synaptic sites. The ionotropic glutamate receptors; AMPA, NMDA and kainate receptors, largely function to mediate fast synaptic transmission. The glutamate vesicular transporters (vGluT1 and vGluT2) load glutamate into vesicles presynaptically. The glial, astrocyte and postsynaptic glutamate transporters (excitatory amino-acid transporters, EAAT) are thought to mediate the uptake of glutamate and consequently, termination of synaptic transmission. The metabotropic glutamate receptors (mGluR) have a diverse synaptic localization. The mGluRs modulate neurotransmitter release and postsynaptic excitability through pre- and post- synaptically function, respectively (adopted from Swanson et al., 2005).

2. Purpose of the study

In auditory physiology, the processing of acoustic signals has traditionally been explained by excitatory and inhibitory interactions of divergent and convergent projections within the auditory system. Here, I reviewed the results from a number of studies indicating that the IC is a major center of integration in the ascending as well as descending auditory pathways, where both excitatory and inhibitory amino acid neurotransmitters play a key role. An important question is how the balance between excitation and inhibition in the IC is achieved, and what factors are involved. A few *in vitro* studies have previously shown that the mGluRs adjust both inhibitory and excitatory synapses' strengths along the auditory pathway. However, there is a lack of knowledge in the role of the mGluRs in the IC. Therefore, I investigated the importance of the mGluRs in regulating the synaptic transmission in the ICC. My first aim was to find whether there is any group of the mGluRs on postsynaptic site in ICC neurons. This aim was achieved by evaluating the changes in intrinsic properties of the neurons following activation of three groups of the mGluRs. My second aim was to examine the presence and the role of the mGluRs on excitatory and inhibitory terminals that synapse to the ICC neurons. This aim was gained by recording the evoked inhibitory and excitatory synaptic responses of the ICC neurons following activation of the mGluRs. Paired-pulse stimulations were used to suggest the presynaptic action of the mGluR. Multiple pulses at different stimulation frequencies were applied to the LL to investigate the modulatory role of the mGluRs in neurotransmitter release, and to examine how the mGluRs play a role in temporal integration of excitatory and inhibitory inputs into the ICC neurons. My final aim was

to investigate the role of glutamate transporters in activation of the mGluRs and regulation of neuronal transmission in the ICC. This aim was achieved by blocking glutamate transporter and recording excitatory and inhibitory responses of the ICC neurons following single or multiple stimulations of the LL.

2.1. Effect of mGluRs activation on intrinsic membrane properties of ICC neurons

The first purpose of this study was to investigate the role of the mGluRs in shaping the active and passive membrane properties of the ICC neurons. The mGluRs can be expressed at both presynaptic and postsynaptic sites. Activation of the mGluRs at postsynaptic sites has a wide range of electrophysiological effects including membrane depolarization (Schwarz et al., 2000), changes in I_h current (Brager and Johnston, 2007), inhibition of potassium currents, and modulation of neuronal firing pattern (Derjean et al., 2003). It is well known that the ICC neurons show different membrane properties and firing patterns which can be directly attributed to activation of the I_h current and potassium conductance. Therefore, if there is any postsynaptic mGluR in the ICC, its activation should change both passive as well as active membrane properties of the neurons. For this experiment, different protocols of intracellular current injection were utilized to examine the membrane properties of the ICC neurons. Then mGluRs I, II and III agonists were applied, and the membrane properties of the neurons were re-examined. In order to have comparable results to other studies, and establish my own data base about membrane properties and firing patterns of the ICC neurons, responses from a large sample of the ICC neurons were recorded in normal condition.

2.2. Effect of mGluRs activation on synaptic responses of ICC neurons

It has been shown that electrical stimulation of the LL can produce both excitatory and inhibitory postsynaptic responses in the ICC neurons (Wu et al., 2004; Ma et al., 2002a). If there is any functional mGluRs in the ICC, activation of the mGluRs should change the synaptic response of the neurons. I recorded postsynaptic currents generated by stimulation of the LL. The postsynaptic currents mediated by GABA_A and AMPA receptors were pharmacologically isolated by using appropriate receptor antagonists. The effect of mGluRs I, II and III agonists were tested on postsynaptic responses. Paired-pulse stimulation as well as normal and low calcium mediums were applied to get evidence regarding the presence of presynaptic mGluRs in the ICC neurons.

2.3. Effect of mGluR II activation on temporal integration of inputs into ICC neurons

Cochleas commonly receive sounds with different frequencies, and transform them to neuronal signals. Neurons in each auditory structure receive and integrate these signals through their synaptic connections with other neurons, and send them to the next auditory nuclei. Auditory neurons are able to respond to high frequency stimuli with a high fidelity. Fast synaptic responses are primary requirements to achieve this task. Joshi et al (2007) suggest that the mGluRs are essential for high-fidelity neurotransmission at calyx of Held synapse. It has been shown that ICC neurons in normal rat can follow different frequency signals (10-100 Hz) coming from the LL when AMPA and GABA_A receptors are activated. However, the responses undergo some modifications at this frequency range (Wu et al., 2004).

Short-term plasticity and modulation has also been reported at many synapses in the CNS (reviewed by Zucker and Regehr, 2002). This plasticity is mostly believed to be due to modulation of neurotransmitter release from presynaptic terminals. Many studies have shown that the mGluRs are involved in modulation of neurotransmitter release. Because only mGluR II was found to be active in this study, the contribution of the mGluR II to temporal integration and short-term plasticity of signals in the ICC neurons was examined. To do this, the LL was stimulated by multi-pulses at different frequencies, and the integration and modulation of AMPA and GABA_A receptor-mediated responses were compared before and after application of mGluRs II agonist.

2.4. Role of glutamate transporters in activation of mGluR II

In this study, it was found that only the mGluRs II were presynaptically active. Because the mGluRs II are mostly localized outside of synaptic cleft on presynaptic sites, they can probably be activated by spill-over of glutamate from synaptic cleft into the synaptic periphery (Anwyl, 1999). Spill-over of glutamate can be induced by high frequency stimulation of presynaptic neurons and/or blocking of glutamate transporters on glial cells and neurons. To test the role of the glutamate transporters in activation of the mGluRs II in the ICC, the LL was stimulated with multi-pulses at different frequencies to elicit AMPA and GABA_A responses. The responses were recorded before and after blocking the glutamate transporters. Then antagonist of the mGluRs II was added to the solution to examine whether spill-over of glutamate could have activated the mGluRs II.

3. Methods and materials

3.1. *Animals*

Young Long Evans rats aging 8-17 days old were used. They were purchased from Charles River Co (Quebec, Canada), and kept in standard room temperature ($21 \pm 1^\circ\text{C}$) and 12 hrs day/night cycles. All animal procedures followed the guidelines of the Canadian Council on Animal Care, and were approved by the animal care committee of Carleton University.

3.2. *Brain Slice preparation*

Animals were anaesthetized using inhaled isoflurane until areflexic, and were then euthanized by decapitation. The brain was carefully removed and put in oxygenated artificial cerebrospinal fluid (ACSF) at room temperature ($20\text{-}22^\circ\text{C}$). ACSF was consisted of CaCl_2 (2.4 mM), Glucose (10 mM), HEPES (3 mM), KCl (3 mM), KH_2PO_4 (1.2 mM), MgSO_4 (1.3 mM), NaCl (129 mM) and NaHCO_3 (20 mM) in deionized water. In low Ca^{2+} solution (1.0 mM CaCl_2), 1.4 mM MgCl_2 was added to the ACSF. The pH of the ACSF was adjusted to 7.4, and saturated with 95% O_2 and 5% CO_2 . A thick block of the auditory midbrain was made by cutting the brain tilted $15\text{-}20^\circ$ from coronal plane toward the caudal side. Using vibrotom tissue slicer (Campden, UK), midbrain section was further cut at 200 μm thick through the IC. The slice containing ICC and LL was transferred to the recording chamber. ACSF temperature and flow rate were maintained at $29\text{-}30^\circ\text{C}$ and 10-12 ml/min, respectively.

3.3. Electrophysiology and visual patch clamp recording

Using a Narishige PP-830 vertical puller, patch electrodes were made from glass micropipettes with 1.1 mm outer diameter and 0.8 mm inner diameter. (Kimax-51, Kimble). The resistance of the electrodes was about 4-6 M Ω when filled with internal patch pipette solution. This solution was composed of potassium gluconate (130 mM), MgCl₂ (2 mM), KCl (5 mM), GTP (0.3 mM), ATP (2 mM), EGTA (1.6 mM) and HEPES (10 mM) in deionized water. For recording synaptic responses, QX-314 (0.5 mM) was added to the electrode solution to block Na⁺ channels and AP generation. The internal solution was filtered with 0.2 μ m filter, and its pH was adjusted to 7.2.

Neurons were visualized with an upright light microscope (Zeiss, Axioskop 2, FS plus) by a 40x water immersion objective with differential interference contrast (DIC) optics. Signals were obtained by using Axopatch 200A or B amplifier. The signals were initially filtered at 5 kHz and digitized (Digidata 1310A) with sampling rate of 10 kHz, and acquired by pClamp 8.02 software. The whole cell capacitance and series resistance were compensated nearly 75-90% after achieving a whole-cell configuration.

3.4. Drugs

Bicuculline (10 μ M) were used as GABA_A receptor antagonist. CNQX (10 μ M) (6-cyano-7-nitroquinoxaline-2,3-dione) and APV (100 μ M) (amino-5-phosphonovaleric acid) were respectively used as AMPA and NMDA receptor antagonists. Strychnine (0.5 μ M) and kynurenic acid (5 mM) were respectively used as antagonist of glycine

receptors and general glutamate receptor antagonist. ACPD ((±)-1-Aminocyclopentane-trans-1,3-dicarboxylic acid) (10-200 μM) was used to activate both mGluRs I and II. DHPG ((RS)-3,5-Dihydroxyphenylglycine) (100 μM), LY379268 ((1R,4R,5S,6R)-4-Amino-2-oxabicyclo[3.1.0] hexane-4,6-dicarboxylic acid) (5-100 nM) and SOP (L-2-Amino-3-hydroxypropanoic acid 3-phosphate) (100 μM) were respectively used as mGluRs I, II and III agonists. LY341495 ((2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid) (10 nM) were used as mGluRs II antagonist. TBOA (DL-threo-b-Benzyloxyaspartic acid) was used as a non-specific glutamate transporter blocker at 50 μM. Strychnine, bicuculline, L-AP5, CNQX, and kynurenic acid were purchased from Ascent scientific, United Kingdom. ACPD, SOP, LY379268, LY341495 and TBOA were obtained from Tocris, U.S.A.. DHPG were purchased from Wako Chemicals, U.S.A.. The mGluR reagents are summarized in the table below.

	Agonist	Antagonist
Group I	ACPD*, DHPG	
Group II	ACPD*, LY379268	LY341495
Group III	SOP	

* ACPD is a non-selective agonist of the mGluRs I and II.

3.5. Examination of membrane properties of neurons

To test the membrane properties, the amplifier was set on current clamp mode. The RMP of a neuron was measured during the first 2-5 minutes following obtaining

of the whole cell patch configuration. Neurons with the RMP negative to -55 mV were accepted for further recording.

To test input resistance (R_{in}) and firing patterns of neurons, current pulses of -300 to 300 pA were injected into the neurons in 10 pA increments steps with a duration of 200 ms. To study the possible diversity of firing patterns, a 200 ms prehyperpolarizing current (which could induce at least 20 mV change in membrane potential) was injected into the neurons prior to a depolarizing current injection of 0-200 pA (duration of 300 ms in 10 pA increments).

RMP of neurons was measured when there was no current injection into the neurons. A membrane potential at which a neuron could generate only one or a couple of APs was defined as AP threshold. The amplitude of an AP was obtained from the membrane potential at the AP threshold to the peak of the AP. Change in membrane potential elicited by current injection was obtained by measuring between the RMP and the peak hyperpolarization or subthreshold depolarization, respectively. Values were then used to plot current-voltage relationship. From this curve, the slope was calculated. This slope represented the R_{in} of the cell membrane, measured in M Ω . The time constant (τ) of the cell membrane was estimated by measuring the time required for the membrane potential to reach 63% of the way toward its final value in response to a 100 pA hyperpolarizing current pulse.

3.6. Examination of synaptic transmission properties

For recording of synaptic responses, a bipolar stimulating electrode, which was built from paired tungsten wires with a tip separation of 50 μ m, was placed along

the LL just below the ICC. After establishment of a reliable whole cell configuration, the amplifier was set on voltage clamp mode. Postsynaptic responses in ICC neurons were evoked by electrical stimulation of the LL. For this purpose, a square pulse with 0.1 ms duration was generated by a stimulator (Grass S-8800, U.S.A.) and delivered through a stimulus isolator unit to the LL. The intensity of the stimulation was ranged from 5-50 V.

To isolate AMPA receptor-mediated responses, strychnine, bicuculline and APV were added to the normal ACSF. GABA_A receptor-mediated responses was isolated by adding strychnine and kynurenic acid (or a combination of APV and CNQX instead) to the bath solution to block glycine and glutamate receptors, respectively. The membrane potential of neurons was clamped at -60mV and -40mV for AMPA and GABA_A responses, respectively. After isolation of the desired receptor-mediated response, the stimulus intensity that could produce approximately two third of the maximum response amplitude was chosen to elicit synaptic response for the rest of recordings. For paired-pulse stimulation, two pulses of equal characteristics (i.e. duration and intensity) were used. To examine temporal synaptic integration, the LL was stimulated with a train of 10 pulses at 5, 30 and 100 Hz. Three responses of a neuron with each protocol were recorded, and the average of the three responses was used for further analysis.

The amplitude of a synaptic response was measured from the baseline to the peak of the response. Rising and decay times of synaptic responses were determined by measuring the time that the current increased or decreased from 10% to 90% of the peak amplitude, respectively. In paired- and multi-pulses stimulation protocols,

amplitude of responses was measured from the baseline of the first response to the peak of each response. The time course at the 50% amplitude of a response was defined as the half-width duration. To calculate the area under the curve (AUC) for a multiple synaptic response evoked by multiple pulses, the baseline of the first response was set at 0 pA. Then the area under the curve was calculated from the beginning of the 1st response till the 10th response reached to the baseline again.

3.7. *Histology*

Some neurons were labeled with fluorescent dye, Lucifer Yellow (2 mM). The slices containing stained neurons were fixed in 4% paraformaldehyde over night, and subsequently washed in 0.1 M PBS. Using 20x and 40x objectives, the neurons were visualized with a Perkin Elmer Ultraview Vox spinning disk confocal system (PerkinElmer Inc., Waltham, MA). A Z-stack of confocal images at 0.3-0.5 μ m intervals was collected and a two-dimensional reconstruction of the neuron was generated with Volocity software 5.2.0 (Improvision Ltd., Waltham, MA). For a few neurons, images were taken at one plane of the sections, and Z-stack was not used. The obtained images were photographed and analyzed.

3.8. *Data analysis*

Data were considered for analyzing if the R_{in} of a neuron remained relatively constant during the course of recording. If changes of the series resistance were more than 15% of the initial value, the response of the neuron were discarded from the analysis.

Microsoft Excel (2000) (Microsoft Ltd., US) and SPSS 17.0 for Windows were used to analyze the data. The data are presented as mean $\pm$ S.E.M. For statistical analysis, student's *t*-test was applied when there were two independent groups of data. Paired *t*-test was used when there were two groups of data from same subjects (before and after a drug). One-way ANOVA with Tukey's *post-hoc* was applied when there were more than two groups, and they were independent. Repeated measure (RM) ANOVA with a *post-hoc* paired *t*-test analysis was used when there were more than two groups of data from same subjects (dependent data). Mauchly's test was applied to test the sphericity of the data in the RM-ANOVAs. Greenhouse–Geisser correction was applied to the degrees of freedom when Mauchly's test was significant. The difference with $p < 0.05$ was considered to be significant.

4. Results

Whole-cell patch clamp recordings were obtained from 313 ICC neurons. The RMPs of these neurons were stable throughout the recording. The mean RMP was -61.3 ± 0.3 mV (range: -57.0 to -65.5 mV, $n=16$). Spontaneous firing and synaptic activities were very rare in these neurons. Intrinsic membrane properties and synaptic responses of the ICC neurons were respectively examined under current-clamp and voltage-clamp modes.

4.1. *Properties of ICC neurons*

4.1.1. *Intrinsic membrane properties of ICC neurons*

Voltage responses of 113 neurons were recorded in responses to depolarization and hyperpolarization current injections. The neurons were categorized as regular, adapting, onset/transient, build-up/pauser and bursting, based on the appearance of their firing patterns. Most of the neurons showed a regular firing pattern ($n=47$, 42%). These neurons could generate a train of spikes during suprathreshold depolarization current injection meanwhile their interspike intervals were uniform or equal throughout the spike train. Responses of a regular neuron are illustrated in figure 4.1.A₁ and A₂. In response to negative current injection, the regular neurons were hyperpolarized. Some of the regular neurons (35/47) showed a voltage sag during hyperpolarization current injections. The sag was a gradual reduction of the membrane hyperpolarization while responding to a fixed level of hyperpolarizing current. The generation of the sag is believed to be due to the activation of I_h current (Koch and Grothe 2003). These neurons showed a

depolarizing rebound when the hyperpolarization current injection was stopped (Fig. 4.1.A₁). An AP (or several APs) could be produced on the rebound if it was large enough to reach the threshold (Fig. 4.1.A₂). In the regular neurons, the voltage changes in response to hyperpolarizing currents were measured at the peak of hyperpolarization and at the steady state. Figure 4.1.C shows the current-voltage relationships of 10 regular neurons.

The adapting neurons (n=25, 22%) could also produce a train of spikes but their late interspike intervals were gradually longer than the preceding interspike intervals throughout the depolarizing current injection (Fig. 4.1.B₁ and B₂). Neurons were considered as an adapting type if the last interspike interval was at least 50 % longer than the first interspike interval in 200 ms suprathreshold depolarizing current, which could induce the generation of 4-8 spikes. Some of the adapting neurons (19/25), like the regular neurons, showed the sag and depolarizing rebound in response to hyperpolarization current injections. Current-voltage relationships at the peak and steady state for 10 adapting neurons, derived from their responses to various levels of negative current injection, are illustrated in figure 4.1.D.

The onset neurons (n=14) only generated one AP at the beginning of depolarizing current injection (Fig. 4.2.A₁ and A₂) while the transient neurons (n=5) generated one full size AP at the beginning of the depolarizing current injection, which could be followed by a few tiny spikes (Fig. 4.2.B₁ and B₂). The sag and depolarizing rebound in response to hyperpolarization current injection were observed in all of the onset and transient neurons. Because of some similarities between the onset and transient neurons (such as generation of one full size AP

regardless of the suprathreshold depolarizing current level, occurrence of the sag and rebound in response to hyperpolarization current injection, insignificant differences in R_{in} and RMP), they were categorized in one group ($n=19$, 17%). Figure 4.2.C shows the current-voltage relationships at the peak and steady state for the onset/transient neurons, derived from their responses to various levels of the negative current injection ($n=10$).

The build-up/pauser neurons ($n=14$, 12%), like the regular and adapting neurons, could produce a train of spikes in response to depolarizing current injections. However, there was not any spike at the beginning of the depolarizing current for a while, and APs were generated after a delay in the build-up neurons ($n=6$, Fig. 4.3.A₁₋₃). In the pauser neurons, there was a pause between the first and the second APs so the first interspike interval was longer than the subsequent interspike intervals ($n=8$, Fig. 4.3.B₁₋₃). By increasing the depolarizing current level, the delay in the build up neurons and the duration of the pause in the pauser neuron progressively became shorter. The spike generation patterns of some of the build-up/pauser neurons were very similar to the pattern of regular or adapting neurons in response to depolarization current injections. However, their firing pattern could be distinguished from the regular and adapting firing patterns if a depolarization current was preceded by a hyperpolarizing current injection (Fig. 4.3.A₃ and B₃). It is believed that the build-up and pauser neurons belong to the same type of neurons because of having A-type K^+ current (Sivaramakrishnan and Oliver, 2001). Most of the build-up/pauser neurons (12/14) did not produce any evident sag or rebound in response to hyperpolarization current injection. Current-voltage relationships at the peak and steady state for 10

build-up/pauser neurons, derived from their responses to various levels of negative current injection, are presented in figure 4.3.C.

The bursting neurons ($n=8$, 7%) showed a big depolarizing hump at the beginning of depolarization current injection. There were always a couple or more APs (depending on the depolarizing level) with very short interspike intervals on the hump. The first spike was a full size AP, and the following spikes were usually smaller than the first one. Following the bursting firing, the neurons could generate multiple full size APs throughout the current injection (Fig. 4.4.A₁ and A₂). In response to hyperpolarizing current, the bursting neurons could generate sag and rebound. If the rebound was big enough to generate AP, there were always more than one AP on the rebound (bursting firing). If the depolarizing current injection was preceded by a hyperpolarizing current, the bursting neurons still could produce a bursting activity which was followed by a few normal size APs (Fig. 4.4.B₁ and B₂). Current-voltage relationships at the peak and steady state for the bursting neurons ($n=8$), derived from their responses to various levels of negative current injection, are presented in figure 4.4.C.

The intrinsic membrane properties (including RMP, R_{in} , τ , AP amplitude, AP half-width and AP threshold) of five groups of neurons with different firing patterns are presented in table 4.1. Each of these properties was compared among the groups using one-way ANOVA test. It was revealed that there was no significant difference ($p>0.05$) among the groups for RMP ($F_{(4, 47)}=0.6$), AP amplitude ($F_{(4, 47)}=0.8$), AP half-width ($F_{(4, 47)}=2.0$), and AP threshold ($F_{(4, 47)}=2.2$). One-way ANOVA followed by Tukey's *post-hoc* test showed significant differences in R_{in} ($F_{(4, 47)}=16.4$, $p<0.001$)

and τ ($F_{(4, 47)}=6.7$, $p<0.001$) among the groups. The regular neurons had the highest R_{in} and the longest τ while the onset/transient neurons had the lowest R_{in} and the shortest τ among the groups. Significant differences between groups are presented in table 4.1.

4.1.2. Histology of ICC neurons

Seven ICC neurons whose intrinsic membrane properties were identified, were labeled with Lucifer Yellow. These neurons had regular or adapting firing patterns with or without robust depolarizing rebound. Four out of the seven neurons showed similar morphology and were classified as bipolar cells (Fig. 4.5A₁ and A₂). The bipolar neurons had a cell body with fusiform shape. Their primary dendrites emerged from opposite ends of the cell body. Their dendritic branches were extended in a direction roughly parallel to the long axis of the cell body as well as the fibrodendritic laminae in the ICC. Among the four bipolar cells, one regular non-rebound, two regular rebound and one adapting rebound cells were identified. The other three cells were classified as multipolar cells (Fig. 4.5B₁ and B₂). These cells had a cell body with multipolar shape, and their primary dendrites emerged radially from the cell body. The most dendritic branches were oriented across the fibrodendritic laminae. Among the three multipolar cells, one was adapting non-rebound, and the other two were regular rebound and adapting rebound cells.

4.2. Effects of mGluRs activation on ICC neurons

4.2.1. Effects of mGluRs activation on intrinsic membrane properties

To test the effect of the mGluR activation on the intrinsic properties of the ICC neurons, the control responses were obtained by injecting depolarizing and hyperpolarizing currents. After that, ACSF containing either ACPD (a general agonist for mGluRs I and II) or SOP (agonist of mGluR III) was applied, and the responses of the neurons were re-examined. Then, ACSF containing either ACPD or SOP was stopped, and the normal ACSF was run again to wash out the drugs, and voltage responses of the neurons to current injections was recorded once more. All of the neurons tested in this part produced multiple APs when membrane depolarization was higher than the threshold level for generation of APs, so they were regular or adapting neurons. In response to hyperpolarizing current injection, some neurons (11/16, 69%) showed a sag of the membrane potential at high levels of current injection, and generated a rebound depolarization immediately after hyperpolarization.

Figure 4.6.A illustrates the responses of one representative neuron to the positive and negative current injections. In control ACSF, this neuron had an adapting firing pattern in response to positive current injection, and produced sag and rebound in response to the hyperpolarizing current injection. Application of ACPD at 100 μ M did not affect the firing pattern of this neuron (Fig. 4.6.A, middle panel). ACPD also did not have any effect on the sag and rebound characteristics. Current-voltage relationships of the sample neuron were not altered by ACPD either (Fig. 4.6.B). Generally, ACPD did not have any significant effect either on the firing pattern and current-voltage relationships ($n=8$, Fig. 4.6.C) or on the sag and rebound properties of

the ICC neurons ($n=6$). Other intrinsic membrane characteristics, i.e., RMP, R_{in} , APs amplitude, half-width and threshold, were also not significantly affected by ACPD (Table 4.2, paired t -test, $p>0.05$).

Responses of a sample neuron to the positive and negative current injection protocols in control ACSF were depicted in figure 4.7.A, left panel. This neuron showed a sustained firing pattern in response to the depolarizing current injection but did not produce any sag and depolarizing rebound in response to the hyperpolarizing current injection. Application of SOP (agonist for mGluR III) at 100 μ M did not have any significant effect on the firing pattern and frequency of the neuron (Fig. 4.7.A, middle panel). Current-voltage relationship of this neuron was not affected by adding SOP into the ACSF (Fig. 4.7.B). Similar to ACPD, SOP had no significant effect on the firing patterns and current-voltage relationships of the ICC neurons ($n=8$, Fig. 4.7.C). SOP did not affect the sag and rebound characteristics of the neurons ($n=5$). RMP, R_{in} , AP threshold and AP amplitude of the neurons were not significantly influenced by administration of SOP (Table 4.2, paired t -test, $p>0.05$).

4.2.2. *Effects of mGluRs activation on AMPA receptors-mediated EPSC*

Electrical stimulation of the LL usually induces mixed excitatory (AMPA and NMDA receptor-mediated), and inhibitory (GABAergic and glycinergic) responses in the ICC neurons (Ma et al., 2002; Sun et al., 2006). Therefore, for this set of experiments, AMPA receptor-mediated EPSCs were pharmacologically isolated by suppressing GABA_A, glycine and NMDA receptors-mediated responses with their specific receptor antagonists (see material and methods). Figure 4.8.A shows isolated

AMPA responses of a sample neuron hold at different membrane potentials. The averages of the EPSC amplitudes hold at different potentials were depicted in figure 4.8.B. The relationship between the EPSC amplitudes and the membrane potentials was linear. Polarity of the EPSC in the ICC neurons was reversed at a membrane potential of approximately 2.3 ± 5.8 mV ($n=6$).

After isolation of the AMPA receptor-mediated responses, a few control responses were recorded from the ICC neurons. To test the effect of mGluRs activation on the AMPA receptor-mediated responses, ACPD was added to the control solution. Figure 4.9.A shows responses of a neuron in control, during application of ACPD (50 μ M) and after washing out the drug. ACPD reversibly reduced the EPSC amplitude in that neuron. Using paired *t*-test, it was found that ACPD significantly reduced the average of the EPSC amplitudes ($n=12$, $p<0.01$, Fig. 4.9.B). The effect of ACPD on the AMPA receptor-mediated responses was dose dependent. ACPD at 10, 20, 50 , 100 and 200 μ M decreased the EPSC amplitudes to 79.0%, 66.4%, 59.6%, 50.0% and 44.8% of the control responses, respectively ($n=5$, Fig 4.9.C).

To figure out which group(s) of the mGluRs were activated by ACPD, and were involved in reduction of the EPSC amplitudes, DHPG was used as the agonist for mGluR I. The AMPA receptor-mediated responses of a neuron before and after application of DHPG (100 μ M), and after washing out the agonist are presented in figure 4.10.A. DHPG did not have any remarkable effect on the AMPA responses of this neuron. The averages of the EPSC amplitudes ($n=8$) in the control, DHPG and wash solutions were not significantly different (paired *t*-test, $p>0.05$, Fig.4.10.B).

This result suggests that ACPD suppressed the AMPA receptor-mediated responses likely through the activation of the mGluR II. To confirm further whether AMPA responses were modulated by the mGluRs II, LY379268 was used as the mGluR II agonist. Adding this drug at 20 nM to the control solution substantially reduced the amplitude of the AMPA response in ICC neurons (Fig. 4.11.A). After application of LY379268 (20 nM) for 10 min the average of the EPSC amplitude was reduced to $54.2 \pm 4.1\%$, and returned back to $80.6 \pm 9.1\%$ of the control level following drug washout for 20 min ($n=7$, Fig. 4.11.B). Similar to ACPD, LY379268 had a dose-dependent effect on the average of the EPSC amplitudes. LY379268 at 5, 10, 20, 50 and 100 nM decreased the EPSCs to 76.6%, 61.7%, 49.5%, 41.0% and 38.4% of the control responses, respectively ($n=10$, Fig. 4.11.C). The concentration range that produced 50% suppression of the EPSC amplitudes was 10-20 nM. LY379268 at 20 nM did not significantly change rising time, decay time and half width of the AMPA responses ($n=7$, Fig. 4.11.D). A few neurons whose AMPA responses were reduced by LY379268, were labeled with Lucifer Yellow. It was found that the AMPA responses of both the bipolar and multipolar neurons were affected by activation of the mGluR II. Figure 4.11.E shows a stained multipolar cell whose AMPA response decreased by LY379268.

As it can be seen in figure 4.11.B, the effect of LY379268 on the AMPA response was not completely reversed, that could be due to the chronic effect of the drug and/or the presence of high affinity connection between the drug and the receptors, which retarded washing out the drug thoroughly. Therefore, some neurons were treated with both LY379268 and LY341495, the mGluR II antagonist (10 nM).

Figure 4.12.A shows responses of a sample neuron in control solution and after adding LY379268 into it, which led to decrease of the EPSC amplitude. The inhibitory effect produced by LY379268 was reversed by adding LY341495 to the solution. The changes of the EPSC amplitude of the neuron were plotted as a function of time in figure 4.12.B. After recording of control responses for 5 min, LY379268 was added to the bath solution, which decreased the amplitude of the AMPA response. Washing out the drug for 30 min did not bring the amplitude of the response back to the control level. Adding the mGluR antagonist, LY341495, dramatically increased the EPSC amplitude close to the control level. Further adding LY379268 to the LY341495 solution did not have any notable effect on the AMPA response. The averages of the EPSC amplitudes in the control and LY379268 solutions, and in a combination of LY379268 and LY341495 solution are presented in figure 4.12.C. LY379268 significantly decreased the AMPA response amplitude (paired *t*-test, $p < 0.01$). Adding LY341495 to the agonist solution brought the response back to the control level ($n=7$). LY341495 (100 nM) alone did not have any significant effect on the intrinsic membrane properties or the EPSCs of the ICC neurons ($n=7$, paired *t*-test, $p > 0.05$).

To find if activation of the mGluR III had any effect on the AMPA responses of the ICC neurons, SOP at 100 μ M was applied as an agonist for mGluR III. Figure 4.13.A shows AMPA responses of a representative neuron before and after SOP application, and after washing out the drug. SOP did not notably affect the AMPA response of this neuron. The average amplitude of AMPA responses ($n=7$) was not significantly changed by SOP (paired *t*-test, $p > 0.05$, Fig. 4.13.B).

4.2.3. Presynaptic mechanism of mGluR II and AMPA receptors-mediated EPSC

The above results suggested that the AMPA receptor-mediated responses could be modulated by activation of only mGluR II but not groups I and III. To test whether the reduction of the EPSCs was due to the activation of the mGluR II through a presynaptic mechanism, paired-pulse stimulation with a 200 ms inter-pulse interval was applied to the LL. In control solution, the paired-pulse stimulation induced two EPSCs, which in most cases the 2nd EPSC had smaller amplitude than the 1st one. Adding LY379268 (20 nM) to the control solution decreased both the 1st and 2nd EPSCs, with more reduction of the first one (Fig. 4.14.A). The 1st response in LY379268 solution was normalized (gray traces) to the 1st response in the control solution (black traces), and two traces were then superimposed, further illustrating the smaller reduction of the 2nd EPSC during LY379268 administration (Fig. 4.14.A, bottom traces). In 9/10 cells tested, the ratio of the 2nd to the 1st EPSCs was increased in the LY379268 solution compared to that in the control solution (Fig. 4.14.B). The average ratios of the 2nd EPSC amplitudes to the first ones (n=10) was significantly larger during application of LY379268 (0.950 ± 0.065) than that in the control solution (0.796 ± 0.037) (paired *t*-test, $p < 0.01$, Fig. 4.14.C). The reduction of the 1st EPSC was highly correlated with the increase of the paired-pulse ratio during LY379268 application (n=10, $r = 0.83$, $p < 0.01$, Fig. 4.14.D).

To get more evidences of presynaptic effect of LY379268 on AMPA responses, the effect was also tested in a solution with a low extracellular Ca^{2+} concentration (1.0 mM). The EPSCs were always smaller in 1.0 mM Ca^{2+} solution than that in the normal Ca^{2+} solution (2.4 mM), however, when using paired-pulse

protocol, the second response was less affected by lowering Ca^{2+} concentration (Fig. 4.15.A, top trace). The paired-pulse ratio in the low Ca^{2+} solution was significantly larger than that in the normal Ca^{2+} solution (student's *t*-test, $p < 0.05$, Fig. 4.15.B). The effect of LY379268 on the EPSCs in the low extracellular Ca^{2+} solution was similar to that in the normal extracellular Ca^{2+} solution. LY379268 (20 nM) reduced the EPSC amplitudes in response to paired-pulse stimulation but suppressed the first one more than the second one (Fig. 4.15.A). To have a better visualization of the smaller reduction of the 2nd EPSC during LY379268, the 1st response in the LY379268 solution was normalized (gray traces) to the 1st response in the control solution (black traces), and then two traces were superimposed (Fig. 4.15.A, bottom traces). The ratio of the 2nd to 1st EPSCs was increased in 8/10 cells tested in the low Ca^{2+} solution (Fig. 4.15.C). The average ratio of the 2nd to 1st EPSCs ($n=10$) was significantly increased from 0.924 ± 0.023 to 1.019 ± 0.044 by LY379268 application (paired *t*-test, $p < 0.05$) in the low Ca^{2+} medium (Fig. 4.15.D).

4.2.4. Effects of *mGluRs* activation on *GABA_A* receptors-mediated IPSC

The IPSCs mediated by *GABA_A* receptors in the ICC neuron were pharmacologically isolated by suppressing glycine and ionotropic glutamate (AMPA and NMDA) receptors. Figure 4.16.A illustrates isolated *GABA_A* receptor-mediated responses of a sample neuron hold at different membrane potentials. The averages of the IPSC amplitudes hold at different membrane potentials were depicted in figure 4.16.B. The relationship between the IPSC amplitudes and the membrane potentials

was relatively linear. Polarity of the IPSC in the ICC neurons was reversed at a membrane potential of -72.7 ± 2.8 mV ($n=6$).

To test the effect of the mGluRs activation on the GABA_A receptor-mediated responses, a few control responses were recorded from the ICC neurons. Then ACPD (50 μ M) was added to the control solution, and the responses of the neurons were recorded. Finally, the drug was washed out, and the responses were recorded one more time. Figure 4.17.A illustrates responses of a neuron in the control solution, during application of ACPD and after washing out the drug. The IPSC of the neuron was suppressed by applying ACPD, and backed to the control level after washing out the ACPD. Administration of ACPD (50 μ M) substantially decreased the average of the IPSC amplitudes ($n=10$, paired *t*-test, $p<0.001$, Fig. 4.17.B). The effect of ACPD on GABA_A receptor-mediated responses was dose dependent (Fig 4.17.C). The EPSC amplitudes decreased to 63.9%, 46.5%, 34.1%, 33.5% and 34.0% of the control responses by application of ACPD at 10, 20, 50, 100 and 200 μ M, respectively ($n=5$). To examine if the effect of ACPD was due to the activation of the mGluR I or II, DHPG and LY379268 were applied, respectively. GABA_A receptor-mediated responses of a neuron in the control, DHPG (100 μ M) and wash solutions are presented in figure 4.18.A. DHPG did not have any significant effect on the GABA_A responses of this neuron. Using paired *t*-test, it was found that the averages of the IPSC amplitudes ($n=9$) in the control, DHPG and wash solutions were not significantly different ($p>0.05$, Fig.4.18.B).

In contrast to DHPG, application of LY379268 greatly reduced the IPSC amplitudes. GABA_A responses of a neuron in the control solution, and during and

after application of LY379268 (20 nM) are presented in figure 4.19.A. The agonist dramatically reduced the IPSC amplitude. By washing out LY379268, the response amplitude was relatively returned back to the control level. Application of LY379268 (20 nM) for 10 min reduced the IPSC amplitude to 51.8% of the control level (Fig. 4.19.B). The IPSC was returned back to 77.0% of the control level after 20 min drug washout (n=6). The effect of LY379268, like ACPD, on the IPSC amplitude was dose-dependent. The IPSCs were reduced to 86.7%, 54.9%, 37.6%, 30.1% and 30.4% of the control levels at 5, 10, 20, 50 and 100 nM LY379268, respectively (n=8, Fig. 4.19.C). The concentration of LY379268 that produced a 50% reduction in the IPSC amplitude was estimated to be 10-20 nM. LY379268 (20 nM) did not significantly affect rising time, decay time and half width of the GABA_A responses (n=10, Fig. 4.19.D). A few neurons whose GABA_A responses were reduced by LY379268, were labeled with Lucifer Yellow. It was found that GABA_A responses of both the bipolar and multipolar neurons were changed by LY379268. Figure 4.19.E illustrates a stained multipolar cell whose GABA_A response was decreased by activation of the mGluR II.

The suppressive effect of LY379268 on the GABA_A receptor-mediated responses was completely reversed by the mGluR II antagonist, LY341495 (10 nM). Figure 4.20.A shows responses of a neuron in the control and after adding LY379268 into the control solution, which led to a decrease in the GABA_A response amplitude. The inhibitory effect produced by LY379268 was reversed by adding LY341495 to the solution. The changes of the IPSC amplitude were plotted as a function of time for that neuron in figure 4.20.B. After recording the control responses for 5 min,

LY379268 was added to the bath solution, which reduced the amplitude of the GABA_A response. Washing out the drug for 30 min did not take the amplitude of the response back to the control level. Adding the mGluR II antagonist, LY341495, considerably improved the GABA_A response amplitude close to the control level. Further adding LY379268 to the solution did not change the GABA_A response. The averages of the IPSC amplitudes (n=7) in the control and LY379268 solutions, and in the combination of LY379268 and LY341495 solution are presented in figure 4.20.C. LY379268 significantly decreased the average of the IPSC amplitudes (paired *t*-test, $p<0.01$). Adding LY341495 to the agonist solution brought the response back to the control level. LY341495 (100 nM) alone did not have any significant effect on the GABA_A response properties of the ICC neurons (n=6, paired *t*-test, $p>0.05$).

The effect of mGluR III activation on GABA_A responses was tested by application of SOP at 100 μ M. Figure 4.21.A illustrates GABA_A responses of a neuron before and after application of SOP. The GABA_A response of this neuron was not affected by application of SOP. The average of the IPSC amplitudes (n=10) was not significantly changed by SOP (paired *t*-test, $p>0.05$, Fig. 4.21.B).

4.2.5. Presynaptic mechanism of mGluR II and GABA_A receptors-mediated IPSC

Paired-pulse stimulation of the LL was used to investigate the site of the mGluR II action on the GABA_A receptor-mediated IPSCs. For all cells tested, the 2nd IPSC was always smaller than the 1st one (n=10). LY379268 (20 nM) reduced both IPSCs but had a greater effect on the 1st one (Fig. 4.22.A). The 1st response in LY379268 solution was normalized (gray traces) to the 1st response in the control

solution (black traces), and two traces were then superimposed, further illustrating the smaller reduction of the 2nd IPSC during LY379268 application (Fig. 4.22.A, bottom traces). In 8/10 tested neurons, the ratio of the 2nd to 1st responses was increased in the LY379168 solution compared to that in the control solution (Fig. 4.22.B). The average ratios of the 2nd IPSC amplitude to the 1st one was significantly increased from 0.686 ± 0.022 in the control solution to 0.923 ± 0.095 in the LY379268 solution (paired *t*-test, $p < 0.01$, Fig. 4.22.C). The reduction of the 1st IPSC was highly correlated with the increase of the paired-pulse ratio during LY379268 application ($n=10$, $r = 0.93$, $p < 0.001$, Fig. 4.22.D).

The effects of the mGluR II agonist on the GABA_A receptor-mediated IPSCs ($n=10$) was also tested in a solution with a low extracellular Ca²⁺ concentration (1.0 mM). The IPSCs were always smaller in 1.0 mM Ca²⁺ solution than that in the normal Ca²⁺ solution (2.4 mM), however, when using paired-pulse protocol, the second response was less affected by lowering Ca²⁺ concentration (Fig. 4.23.A, top trace). The paired-pulse ratio in the low Ca²⁺ solution was significantly larger than that in the normal Ca²⁺ solution (student's *t*-test, $p < 0.01$, Fig. 4.23.B). The effect of LY379268 on the IPSCs in the low Ca²⁺ solution was similar to that in the normal Ca²⁺ solution. LY379268 (20 nM) reduced the IPSC amplitudes in response to the paired-pulse stimulation but suppressed the 1st IPSC more than the 2nd one (Fig. 4.23.A). For a better judgment, the 1st IPSC during LY379268 was normalized to the 1st response in the control solution, and then two traces were superimposed, demonstrating a less profound reduction in the 2nd IPSC by LY379268 (Fig. 4.23.A, lower traces). The ratio of the 2nd to 1st IPSCs was increased in 8/10 cells in the low

Ca^{2+} solution (Fig. 4.22.C). The average ratios of the 2nd to 1st IPSCs was significantly increased from 0.891 ± 0.022 to 0.981 ± 0.035 by LY379268 (paired *t*-test, $p < 0.05$) in the low Ca^{2+} solution (Fig. 4.22.D).

4.3. Effects of mGluR II activation on temporal integration of synaptic responses in ICC neurons

The above results showed that only the mGluR II were involved in modulation of synaptic responses of the ICC neurons. To test the effect of mGluR II activation on temporal integration of the synaptic responses, AMPA and GABA_A receptor-mediated responses were pharmacologically isolated. Then, three stimulation frequencies, low (5 Hz), medium (30 Hz) and high (100 Hz), were applied to the LL by 10 electrical pulses of the same intensity and duration. Finally, the AMPA and GABA_A receptors-mediated responses to repetitive stimulations were examined under LY379268 (20 nM) application.

4.3.1. Effects of LY379268 on temporal integration of AMPA receptor-mediated EPSCs

Ten pulses of stimulations at 5, 30 and 100 Hz could induce ten discernible AMPA receptor-mediated EPSCs in the ICC neurons ($n=13$). The EPSCs induced by the second and later pulses were always smaller than the EPSC induced by the first stimulation pulse. Figure 4.24.A shows EPSCs elicited in a neuron following stimulation of the LL at 5 Hz. Note that there was a big difference between the amplitudes of the 1st and the 2nd responses in the control solution. There was also a

notable difference between the amplitudes of the 2nd and 3rd ones. However, there were no big differences among the amplitudes of the 3rd response and later responses. By applying LY379268, the amplitude of all EPSCs became smaller than the EPSC amplitudes in the control solution. The averages of the EPSCs to 10 pulses of stimulations in the control and LY379268 solutions were plotted as a function of the pulse order in figure 4.24.B. RM-ANOVA revealed that there were significant differences among the averages of the EPSC amplitudes in the pulse order ($F_{(3.5, 42.2)}=29.1, p<0.001$). The treatment with LY379268 significantly reduced the EPSC amplitudes ($F_{(1, 12)}=13.3, p<0.01$). There was a significant interaction between the treatment with mGluR II agonist and the pulse order effects ($F_{(2.4, 32.7)}=6.4, p<0.01$). The ratio of each EPSC to the first one was calculated to estimate the amount of response suppressions in the 2nd and subsequent responses, and to compare the amount of suppressions in the control and LY379268 solutions. Then, the ratios (P_n/P_1) were plotted as a function of the pulse order (Fig. 4.24.C). It was found that there were significant differences between the ratios in the control and LY378268 solutions ($F_{(1, 12)}=11.8, p<0.01$), and among the ratios in the pulse order ($F_{(3.1, 37.5)}=29.3, p<0.001$). The interaction between the treatment and the pulse order effects was not significant ($F_{(3.5, 42.3)}=1.9, p>0.05$).

The EPSCs of a neuron to 10 pulses of stimulation at 30 Hz to the LL in the control and LY379268 solutions were depicted in figure 4.25.A. Compared to the EPSC amplitude in response to the first pulse, the EPSC amplitudes in responses to the 2nd and following pulses were decreased over the train in both solutions. However, the amplitude reductions of the 2nd and subsequent EPSCs were more

dramatic in the control solution. The averages of the EPSC amplitudes in the control and LY379268 solutions were plotted as a function of the pulse order in figure 4.25.B. Using RM-ANOVA, it was found that the averages of the EPSC amplitudes ($n=13$) in response to 30 Hz stimulation were significantly decreased by the application of LY379268 ($F_{(1, 12)}=19.4, p<0.01$). There were remarkable differences among the averages of the EPSC amplitudes in the pulse order ($F_{(3, 1, 37, 6)}=46.2, p<0.001$). The interaction between the treatment and pulse order effects was also significant ($F_{(3, 1, 37, 8)}=12.7, p<0.001$). The EPSC ratios in the control and LY379268 solutions were plotted as a function of the pulse order (Fig. 4.25.C). RM-ANOVA revealed that there was a significant raise in the EPSC ratios in the LY379268 solution compared to the ratios in the control solution ($F_{(1, 12)}=4.9, p<0.05$). There were also significant differences among the EPSC ratios in the pulse order ($F_{(1, 5, 16, 8)}=11.7, p<0.01$). The interaction between the treatment and pulse order effects was not significant ($F_{(1, 8, 19, 3)}=1.7, p>0.05$).

The EPSC amplitudes were progressively suppressed over a period of 10 stimulus pulses at 100 Hz (Fig. 4.26.A). At this high frequency, the 2nd and some of the subsequent EPSCs started before the previous one was returned to the baseline. It means that there was a temporal summation of the EPSCs at this frequency. Application of LY379268 depressed almost all of the EPSCs with the greatest effect on the 1st one, and the least effect on the last few ones. The averages of the EPSC amplitudes in the control and LY379268 solutions were plotted as a function of the pulse order in figure 4.26.B ($n=13$). RM-ANOVA showed that the treatment with the control and agonist solutions had a significant effect on the EPSC amplitudes in

response to 100 Hz stimulation ($F_{(1, 12)}=18.2$, $p<0.01$). There were significant differences among the EPSC amplitudes in the pulse order ($F_{(2.7, 29.6)}=62.2$, $p<0.001$). The interaction between the treatment and pulse order effects was significant ($F_{(3.1, 33.7)}=16.7$, $p<0.001$). The ratios of Pn/P1 in the control and drug solutions were plotted as a function of the pulse order in figure 4.26.C. The treatment with LY379268 had a significant effect on the increase of the ratios of the EPSCs for 10 pulses of stimulation (RM-ANOVA, $F_{(1, 12)}=19.9$, $p<0.01$). Unlike the interaction effects for 5 and 30 Hz stimulations, there were significant differences among the amplitude ratios in the pulse order for 100 Hz stimulation ($F_{(2.6, 30.8)}=38.4$, $p<0.001$). The interaction between the treatment and pulse order effects on the amplitude ratios at 100 Hz stimulation was significant ($F_{(4.3, 51.8)}=5.8$, $p<0.001$).

The averages of the EPSC amplitudes in response to 10 pulses at 5, 30 and 100 Hz stimulations in the control and LY379268 solutions are presented in figure 4.27.A ($n=13$). There were suppressive effects on the EPSC amplitudes of 10 consecutive pulses for all frequencies in the control solution. However, the suppressive effects on the last few EPSCs were more evident at the higher stimulation frequencies than at the lower stimulation frequencies. RM-ANOVA revealed that the suppression of the successive EPSC amplitudes depended on the stimulation frequency in the control solution ($F_{(2, 24)}=4.3$, $p<0.05$). Application of LY379268 depressed the EPSC amplitudes in responses to 10 pulses at all stimulation frequencies. However, the suppressive effects on the EPSC amplitudes over the stimulation train were almost equal for all stimulation frequencies. RM-ANOVA showed that the amount of the EPSC suppression over the 10 pulse train did not

depend on the stimulation frequency in the LY379268 solution ($F_{(2, 24)}=0.4$, $p>0.05$). The treatment with the control and LY379268 solutions had a significant effect on the frequency-dependency of the EPSC amplitudes ($F_{(1, 12)}=14.8$, $p<0.01$). The ratios of the EPSC amplitudes in responses to 5, 30 and 100 Hz stimulation frequencies were plotted as a function of the pulse order in figure 4.27.B. There were obvious differences among the EPSC ratios for the last few EPSCs at different frequencies in the control solution but there were not such differences in the LY379268 solution. RM-ANOVA showed that the EPSC ratios for successive pulses were dependent on the stimulation frequency in the control solution ($F_{(2, 24)}=10.1$, $p<0.01$) but not in the LY379268 solution ($F_{(2, 24)}=0.7$, $p>0.05$). The treatment with the control and agonist solutions had a significant effect on the frequency-dependency of the EPSC ratios ($F_{(1, 12)}=19.8$, $p<0.01$).

The integral of the EPSCs (i.e. AUC) was calculated to obtain the charge transfer across the membrane for the single pulse and the trains of 10 pulses at different frequencies (Fig. 4.28.A). To compare the AUC of the EPSC induced by single pulse stimulation with that induced by 10 repetitive stimulation pulses, the AUC with single pulse was multiplied by 10. The AUCs of the EPSCs before and after application of LY379268 were compared using paired *t*-test ($n=13$). It was found that LY379268 could significantly decrease the AUCs of the EPSCs with the single pulse ($\times 10$) ($p<0.01$) and with 10 pulses at 5 Hz ($p<0.05$), 30 Hz ($p<0.001$) and 100 Hz ($p<0.001$). The AUCs of the responses for the single pulse ($\times 10$) and 10 pulses at different frequencies in the control and LY379268 solutions were compared (Fig. 4.28.B). One can see that by increasing the stimulation frequency, the AUCs of the

responses gradually decreased in both solutions (note that the frequency of single stimulations could be considered as 0.01-0.05 Hz, because single stimulations were induced every 20-100 sec). RM-ANOVA revealed that the AUCs were significantly changed depending on the stimulation frequency ($F_{(2,1, 22,8)}=12.7, p<0.001$). Treatment with the control and LY379268 solutions had a significant effect on the AUCs induced by different stimulation frequencies ($F_{(1, 12)}=28.5, p<0.001$). The interaction between the treatment and stimulation frequency effects was not significant ($F_{(1,8, 20,5)}=2.1, p>0.05$).

4.3.2. *Effects of LY379268 on temporal integration of GABA_A receptor-mediated IPSCs*

The effect of LY379268 was examined on the GABA_A receptor-mediated IPSCs induced by a train of stimulation at different frequencies. Ten stimulus pulses at 5, 30 and 100 Hz could induce ten discernible IPSCs in the ICC neurons. The IPSC to the first stimulation pulse was usually (9 out of 10 cells) larger than the IPSCs to the second and later pulses at 5 Hz in the control solution. Figure 4.29.A illustrates IPSCs of a neuron induced by a train of 5 Hz stimulation. The amplitudes of all IPSCs were reduced by LY379268 in that neuron, with the strongest effect on the first IPSC. The averages of IPSC amplitudes to 10 pulses of stimulation in the control and LY379268 solutions were plotted as a function of the pulse order ($n=10$, Fig 4.29.B). RM-ANOVA revealed that there were significant differences among the average of the IPSC amplitudes in the pulse order ($F_{(2,6, 23,8)}=10.7, p<0.001$). The treatment with the mGluR II agonist, LY379268, significantly suppressed the consecutive IPSC

amplitudes ($F_{(1, 9)}=16.7, p<0.01$). There was also a significant interaction between the treatment and pulse order effects ($F_{(3.6, 32.1)}=3.9, p<0.05$). The ratio of each IPSC to the first one was calculated for each train in the control and LY379268 solutions. The ratios (P_n/P_1) were plotted as a function of the pulse order in figure 4.29.C. RM-ANOVA showed that there were significant differences among the IPSC ratios in the pulse order ($F_{(2.7, 24.3)}=10.0, p<0.001$). The treatment with the control and LY379268 solution significantly changed the consecutive IPSC ratios ($F_{(1, 9)}=6.5, p<0.05$). The interaction between the treatment and pulse order effects was not significant ($F_{(9, 81)}=1.2, p>0.05$).

The IPSCs of a neuron to 10 pulses of stimulation at 30 Hz to the LL in the control and LY379268 solutions were depicted in figure 4.30.A. Note that the amplitude of the 2nd IPSC is larger than the amplitude of the 1st one in both solutions. It was actually found that the 2nd IPSC amplitude were larger than the 1st IPSC amplitude in five out of 10 tested neurons. The 3rd and succeeding IPSC amplitudes were usually smaller than the 1st response amplitude in the control solution. Using RM-ANOVA, it was found that the averages of the IPSC amplitudes ($n=10$) in response to 30 Hz stimulation were significantly decreased by application of LY379268 ($F_{(1, 9)}=15.1, p<0.01$, Fig. 4.30.B). The pulse order effect on the average of the IPSC amplitudes was not significant ($F_{(1.7, 15.1)}=2.7, p>0.05$). However, the interaction between the treatment and pulse order effects was significant ($F_{(3.6, 32.5)}=11.1, p<0.001$). RM-ANOVA revealed that there was a significant increase in the IPSC ratios in the LY379268 solution compared to the ratios in the control solution ($F_{(1, 9)}=11.4, p<0.05$, Fig. 4.30.C). There was not any significant difference

among the ratios in the pulse order ($F_{(9, 81)}=1.5, p>0.05$). The interaction between the treatment and pulse order effects was significant ($F_{(9, 81)}=8.0, p<0.01$).

The IPSCs showed a temporal summation over a period of 10 stimulus pulses at 100 Hz. At this high frequency, the IPSC amplitudes to the 2nd and 3rd pulses were usually (8 out of 10 cells) greater than those to the 1st pulse. For some neurons (5/10), the 4th-10th IPSC amplitudes were also larger than the 1st one. Figure 4.31.A shows sample responses of a neuron before and after application of LY379268. The agonist depressed almost all of the IPSC amplitudes in responses to 10 stimulus pulses. There were greater effect on the first few responses (with the greatest effect on the 1st one) and less effect on the last few responses. The averages of the IPSC amplitudes in the control and LY379268 solutions were plotted as a function of the pulse order in figure 4.31.B (n=10). RM-ANOVA revealed that the agonist treatment had a significant effect on the IPSC amplitudes in response to 100 Hz stimulation ($F_{(1, 9)}=6.3, p<0.05$). There was not any significant difference among the IPSC amplitudes in the pulse order ($F_{(1.1, 10.3)}=3.1, p>0.05$). The interaction between the treatment effect and pulse order effect was significant ($F_{(2.5, 22.5)}=7.4, p<0.01$). The ratios of Pn/P1 in the control and LY379268 solutions were plotted as a function of the pulse order in figure 4.31.C. LY379268 significantly increased the IPSC ratios (RM-ANOVA, $F_{(1, 9)}=17.2, p<0.01$). There was not any significant difference among the IPSC ratios in the pulse order ($F_{(1.2, 10.9)}=3.9, p>0.05$). The interaction between the treatment and pulse order effects on the IPSC ratios was significant ($F_{(1.5, 13.5)}=9.9, p<0.01$).

The averages of the IPSC amplitudes in responses to 10 pulses at 5, 30 and 100 Hz stimulations in the control and LY379268 solutions are illustrated in figure 4.32.A (n=10). A train of stimulation at 5 Hz caused a suppressive effect on the succeeding IPSC amplitudes in the control solution. Such a suppressive effect was also observed for most of the IPSCs induced by a train of stimulation at 30 Hz. However, this suppression was changed to facilitation at 100 Hz stimulation. Bath application of LY379268 suppressed the IPSC amplitudes in responses to 10 pulses at all frequencies. Although the suppressive and facilitative effects for respective 5 and 100 Hz stimulations were still there in the LY379268 solution, the suppressive effect observed at 30 Hz stimulation in the control solution changed to a small facilitation in the LY379268 solution. Using RM-ANOVA, it was found that the modulation of the IPSC amplitudes over a train of stimulation depended on the stimulation frequency in both the control ($F_{(1.1, 9.4)}=5.4, p<0.05$) and LY379268 solutions ($F_{(1.2, 2)}=17, p<0.01$). The frequency-dependency of the IPSC amplitudes was significantly different in the control and LY379268 solutions ($F_{(1, 9)}=15.1, p<0.01$). The ratios of the IPSC amplitudes in responses to 5, 30 and 100 Hz stimulation frequencies were plotted as a function of the pulse order in figure 4.32.B. The ratios for the 2nd-10th IPSCs in 5 Hz stimulation, and 4th-10th ones in 30 Hz stimulation were smaller than one (<1, i.e. depression), and the ratios for the 2nd-10th IPSCs in 100 Hz stimulation were greater than one (>1, i.e. facilitation) in the control solution. Application of LY379268 increased almost all ratios in 3 stimulation frequencies compared to the respective stimulation frequencies in the control solution. However, the ratios in 100 Hz stimulation dramatically increased, and the ratios in 30 Hz became just greater than

one. RM-ANOVA showed that the IPSC ratios for the successive pulses were changed differently depending on the stimulation frequency in the control solution ($F_{(1.1, 9.6)}=8.1, p<0.05$). The changes in the IPSC ratios were also dependent on the stimulation frequency during application of LY379268 ($F_{(1, 8.1)}=14.7, p<0.01$). The treatment with the agonist significantly changed the frequency-dependency of the IPSC ratios ($F_{(1, 9)}=13.5, p<0.01$).

AUCs of the IPSCs for the single pulse ($\times 10$) and the train of 10 pulses at different frequencies before and after application of LY379268 were compared using paired *t*-test ($n=10$, Fig. 4.33.A). It was found that LY379268 significantly depressed the AUCs at the single pulse ($\times 10$) ($p<0.01$), and the train of 10 pulses at 5 Hz ($p<0.01$), 30 Hz ($p<0.05$) and 100 Hz ($p<0.05$). The AUCs of the IPSCs to the single pulse ($\times 10$) and 10 pulses at different frequencies in the control and LY379268 solutions were compared (Fig. 4.33.B). A train of 10 pulses of stimulation at 5 Hz produced less AUCs (so less charge transfer) than those at the single pulse ($\times 10$) and the trains at 30 and 100 HZ in both the control and LY379268 solutions. However, RM-ANOVA did not show any significant difference among the AUCs induced by different stimulation frequencies ($F_{(1.2, 10.4)}=2.4, p>0.05$). The LY379268 significantly decreased the AUCs induced by different stimulation frequencies ($F_{(1, 9)}=17.8, p<0.01$). The interaction between the treatment and stimulation frequency effects was not significant ($F_{(1.3, 11.6)}=2.2, p>0.05$).

4.4. Effects of glutamate transporter blocker on synaptic responses in ICC neurons

The mGluRs II are usually activated by spill-over of glutamate from synaptic clefts. However, glutamate transporters rapidly clear glutamate from the synaptic clefts, and limit the glutamate spill-over. Therefore, the glutamate transporters restrict the mGluRs activation in most synapses (Huang et al., 2004; Reichelt and Knopfel, 2002). In this experiment, glutamate transporters were blocked by TBOA (a non-transportable neuronal and glial glutamate transport blocker, 50 μ M) to test whether mGluR activation could be induced by intrinsic glutamate spill-over in the ICC neurons. TBOA itself did not affect the intrinsic membrane properties of the ICC neurons. Paired *t*-test showed that there was no significant difference in the RMP of the neurons ($n=10$, $p>0.05$) before (-60.4 ± 1.6 mV) and after application of TBOA (-58.9 ± 1.7 mV). R_{in} of the neurons ($n=10$, 335.6 ± 53.6 M Ω) did not change after TBOA application either (322.5 ± 39.5 M Ω , $p>0.05$). To test the effect of TBOA on AMPA and GABA_A receptors-mediated responses, in addition to single pulse stimulation, repetitive stimulation at 3 frequencies were used to increase the release of glutamate, and therefore to examine the effect of glutamate spill-over.

4.4.1. Effects of TBOA on AMPA receptor-mediated EPSC

Effect of blocking of glutamate transporters was studied on pharmacological isolated AMPA receptor-mediated EPSCs by application of TBOA. Figure 4.34.A shows AMPA responses of a neuron before and after application of TBOA. One can see that the EPSC amplitude was remarkably reduced by TBOA. To figure it out if this reduction was due to the mGluR II activation, mGluR II antagonist, LY341495

(10 nM), was added to the TBOA solution. By blocking the mGluR II, the EPSC amplitude returned to its control level. Washing out TBOA and LY341495 did not notably change the amplitude of the EPSC. RM-ANOVA showed that there were significant differences among the average of the EPSC amplitudes ($n=10$, Fig. 4.34.B) in the control, TBOA, TBOA+LY341495 and wash solutions ($F_{(3, 27)}=11.8$, $p<0.001$). It was found that the EPSC amplitude in the TBOA solution was significantly smaller than those in the control or TBOA+LY341495 solutions (*post-hoc* paired *t*-test, $p<0.001$). Rising time, decay time and half-width of the AMPA responses ($n=10$) were measured in the control, TBOA, TBOA+LY341495 and wash solutions (Fig. 4.34.C). RM-ANOVA did not show any significant difference among the rising time ($F_{(1.3, 11.9)}=0.3$, $p>0.05$), decay time ($F_{(3, 27)}=0.3$, $p>0.05$) and half-width ($F_{(3, 27)}=0.2$, $p>0.05$) of the EPSCs in the different solutions.

The effect of TBOA on the EPSCs of 10 neurons were tested over a train of 10 pulses at low (5 Hz), medium (30 Hz) and high (100 Hz) stimulation frequencies. Responses of a neuron in the control, TBOA, TBOA+LY341495 and wash solutions to a train of stimulation at 5 Hz are illustrated in figure 4.35.A. TBOA notably decreased the EPSC amplitudes in responses to all of 10 consecutive pulses. Adding LY341495 to the TBOA solution brought the EPSC amplitudes back almost to the control levels. The averages of 10 successive EPSC amplitudes (elicited by a train at 5 Hz) in the control, TBOA, TBOA+LY341495 and wash solutions are presented in figure 4.35.B. RM-ANOVA showed that there was a significant effect among the pulse order ($F_{(3, 27)}=9.5$, $p<0.001$). Treatment (with the control, TBOA, TBOA+LY341495 and wash solutions) significantly changed the average of the

EPSC amplitudes ($F_{(1.7, 15.3)}=70.9, p<0.001$). The interaction between the treatment and pulse order effects was also significant ($F_{(27, 243)}=3.2, p<0.05$). Ratios of ten EPSC amplitudes to the 1st EPSC amplitude (P_n/P_1) in all solutions were depicted in figure 4.35.C. RM-ANOVA revealed that the treatment with the different solutions had a significant effect on the EPSC ratios ($F_{(9, 72)}=136, p<0.001$). There was not any significant difference among the ratios in the pulse order ($F_{(3, 27)}=2.1, p>0.05$). The interaction between the treatment and pulse order effects was significant ($F_{(27, 243)}=1.8, p<0.05$).

Figure 4.36.A shows responses of a neuron to a train of 10 pulses at 30 Hz in the control, TBOA, TBOA+LY341495 and wash solutions. The EPSCs to each stimulus were started from the baseline in the control solution. TBOA decreased the EPSC amplitudes in responses to all 10 stimulus pulses. In addition, the 2nd -10th EPSCs (especially the last few ones) started before the previous one returned to the baseline level (there was a shift for those responses), and the decay time for the last response was prolonged in the TBOA solution. By adding LY341495 to the TBOA solution, the first few EPSC amplitudes nearly brought back to the control levels while the last few EPSC amplitudes were larger than the control responses. In addition, the shift of the 2nd -10th EPSCs, and the extension of the decay time after the 10th response became more prominent. The shift and the long decay time were disappeared by washing out TBOA and LY341495. The averages of the EPSC amplitudes in 4 different solutions were plotted as a function of the pulse order in figure 4.36.B ($n=10$). RM-ANOVA revealed that there were significant differences among the EPSC amplitudes in the pulse order ($F_{(3, 27)}=10.8, p<0.001$). The treatment

with the different solutions had a significant effect on the averages of the EPSC amplitudes ($F_{(9, 72)}=70.3, p<0.001$). The interaction between the treatment and pulse order effects was also significant ($F_{(27, 243)}=4.7, p<0.001$). The ratios of the EPSCs in the control, drugs and wash solutions were plotted as a function of the pulse order in figure 4.36.C. The treatment with the different solutions had a significant effect on the EPSC ratios for 10 pulses of stimulation (RM-ANOVA, $F_{(9, 72)}=139, p<0.001$). There were also significant differences among the EPSC ratios in the pulse order ($F_{(3, 27)}=6.5, p<0.01$). The interaction between the treatment and the pulse order effects for the EPSC ratios to 30 Hz stimulation was significant ($F_{(27, 243)}=2.6, p<0.001$).

The EPSCs of a neuron over a period of 10 stimulus pulses at 100 Hz in the control, TBOA, TBOA+LY341495 and wash solutions are presented in figure 4.37.A. At this frequency, the 2nd–10th EPSCs did start before the previous one was completed and returned to the baseline. TBOA depressed the amplitudes of the first few EPSCs with the greatest effect on the 1st one. In addition, the shift and the decay time for the last EPSC were increased by TBOA. Adding LY341495 to the TBOA solution increased the EPSC amplitudes. The amplitudes of the first few EPSCs returned back to the control levels while the amplitudes of the last few EPSCs were even larger than the amplitudes of those in the control solution. During TBOA and LY341495 application, the slow decay of the 10th EPSC became more extended. Washing out TBOA and LY341495 brought the amplitudes and other properties of the EPSCs back almost to the control levels. The averages of the EPSC amplitudes in four solutions were plotted as a function of the pulse order in figure 4.37.B (n=10). RM-ANOVA showed that the treatment with the different solutions had a significant

effect on the EPSC amplitudes in response to 100 Hz stimulation ($F_{(9, 72)}=56.5$, $p<0.001$). There were significant differences among the EPSC amplitudes in the pulse order ($F_{(3, 27)}=10.3$, $p<0.001$). The interaction between the treatment and pulse order effects was also significant ($F_{(27, 243)}=8.2$, $p<0.001$). The EPSC ratios in the control, TBOA, TBOA+LY341495 and wash solutions were plotted as a function of the pulse order in figure 4.37.C. RM-ANOVA showed that the treatment with the different solutions had a significant effect on the EPSC ratios ($F_{(9, 72)}=67.7$, $p<0.001$). There were significant differences among the ratios in the pulse order ($F_{(3, 27)}=9.24$, $p<0.001$). The interaction between the treatment and pulse order effects for EPSC ratios to 100 Hz stimulation was also significant ($F_{(27, 243)}=3.6$, $p<0.001$).

Averages of the EPSC amplitudes in responses to 10 pulses at 5, 30 and 100 Hz stimulations in the control, TBOA, TBOA+LY341495 and wash solutions are presented in figure 4.38 ($n=10$). There were suppressive effects on the EPSC amplitudes in response to 10 successive stimuli at all frequencies in the control solution (Fig. 4.38.A). However, the suppression was more prominent at 30 and 100 Hz stimulations than 5 Hz stimulation for the 5th-10th EPSCs. RM-ANOVA showed that the suppression of the EPSC amplitudes depended on the stimulation frequency in the control solution ($F_{(1.2, 6.9)}=8.3$, $p<0.05$). TBOA decreased the EPSC amplitudes in responses to the first few pulses at all frequencies (Fig. 4.38.B). There were suppressive effects on the last few pulses at 5 Hz stimulation while there were small facilitative effects on the last few pulses at 100 Hz stimulation in the TBOA solution compared to those in the control solution. RM-ANOVA revealed that the amount of the EPSC suppressions over the 10 pulse train did not rely on the stimulation

frequency in the TBOA solution ($F_{(1.1, 6.4)}=0.1$, $p>0.05$). Blocking the mGluR II by LY341495 returned the amplitudes of the first few EPSCs back to the control levels at all stimulation frequencies. The amplitudes of the last few EPSCs in response to 5 Hz stimulation returned back to the control levels but they were larger in responses to 100 Hz stimulation compared to the control levels (Fig. 4.38.C). RM-ANOVA did not show any significant difference among the EPSC amplitudes in responses to different stimulation frequencies in TBOA+LY341495 solution ($F_{(1.1, 6.6)}=0.4$, $p>0.05$). The EPSC amplitudes at 5, 30 and 100 Hz stimulation returned to the control levels by washing out the drugs (Fig. 4.38.D). RM-ANOVA showed significant differences among the EPSC amplitudes in responses to the different stimulation frequencies after washing out the drugs i.e. the amount of changes in the EPSC amplitudes over the train depended on the stimulation frequency ($F_{(1.5, 9.1)}=15.8$, $p<0.01$). RM-ANOVA revealed that the treatment with the different solutions had significantly different effects on the EPSC amplitudes depending on the stimulation frequency ($F_{(3, 27)}=11.7$, $p<0.001$), and there was a significant interaction between the treatment and stimulation frequency effects ($F_{(2.6, 15.4)}=6.8$, $p<0.01$).

The ratios of the EPSC amplitudes in responses to 5, 30 and 100 Hz stimulation frequencies were plotted as a function of the pulse order in figure 4.39. The ratios for the last few EPSCs among the different frequencies were different in the control solution (Fig. 4.39.A). RM-ANOVA showed that the ratios for successive EPSCs were changed differently depending on the stimulation frequency in the control solution ($F_{(1.2, 4.7)}=12.6$, $p<0.05$). There were not notable differences among the ratios in the different frequencies for each pulse number in the TBOA solution

(Fig. 4.39.B). RM-ANOVA showed that there was not any significant difference among the EPSC ratios in responses to the different stimulation frequencies ($F_{(1.5, 5.9)}=1.4$, $p>0.05$). Adding LY341495 to the TBOA solution decreased the ratios for the 2nd and 3rd pulses but slightly increased the ratios for the last few pulses in responses to all stimulations frequencies compared to those in the TBOA solution (Fig. 4.39.C). In spite of that, RM-ANOVA did not show any significant difference among the stimulation frequencies ($F_{(1.1, 6.5)}=0.2$, $p>0.05$). Washing out the drugs nearly returned the ratios back to the control levels (Fig. 4.39.D). RM-ANOVA showed that the ratios for successive EPSCs were changed differently depending on the stimulation frequency in the wash solution, like that in the control solution ($F_{(1.2, 6.9)}=12.0$, $p<0.01$). RM-ANOVA for all solutions revealed that the treatment with the different solutions significantly changed the EPSC ratios depending on the stimulation frequency ($F_{(3, 27)}=6.6$, $p<0.01$), and there was a significant interaction between the treatment and stimulation frequency effects ($F_{(2.5, 9.9)}=4.5$, $p<0.05$).

AUCs of the EPSCs in the control, TBOA, TBOA+LY341495 and wash solutions for the single pulse ($\times 10$) and 5, 30 and 100 Hz stimulation frequencies are presented in figure 4.40.A ($n=10$). RM-ANOVA showed that there were significant differences among the AUCs in the different solutions for the single pulse ($\times 10$) ($F_{(1.3, 11.7)}=10.3$, $p<0.01$), 5 Hz ($F_{(3, 27)}=11.3$, $p<0.001$), 30 Hz ($F_{(1.3, 7.6)}=17.9$, $p<0.01$) and 100 Hz ($F_{(1.2, 11.6)}=20.1$, $p<0.001$) stimulations. *Post-hoc* paired *t*-test showed that TBOA significantly decreased the AUC of the EPSCs in response to the single pulse ($\times 10$) ($p<0.01$). Adding LY341495 to TBOA solution significantly increased the AUC ($p<0.01$). When a train of stimuli at 5 Hz was used, there was no significant

difference between the AUCs in the control and that in the TBOA solutions (paired *t*-test, $p>0.05$) while there was a remarkable difference between AUCs in the TBOA solution and those in the TBOA+LY341495 solution (paired *t*-test, $p<0.001$). Unlike the effect of TBOA on the AUCs induced by the single pulse or 10 pulses at 5 Hz, TBOA significantly increased the AUCs when stimulations at 30 or 100 Hz were used (paired *t*-test, $p<0.01$, $p<0.001$, respectively). Adding LY341495 to the solution increased the AUCs even more, and the AUCs were significantly larger than the AUCs in the TBOA solution when either 30 or 100 Hz stimulation was applied (paired *t*-test, $p<0.001$, $p<0.01$, respectively). The AUCs of the EPSCs in the control, TBOA, TBOA+LY341495 and wash solutions were compared among the stimulation frequencies of single pulse ($\times 10$) (the frequency of the single pulse was 0.01-0.05 Hz), 5, 30 and 100 Hz (Fig. 4.40.B). In the control solution, the lowest frequency (single pulse $\times 10$) had the largest AUC. TBOA dramatically reduced the AUC for the single pulse ($\times 10$) but increased that for 30 and 100 Hz stimulations. In TBOA solution, 5 Hz stimulation had the smallest AUC. By blocking mGluR II, the AUCs for all stimulation frequencies increased with the highest increase at 100 Hz stimulation. Washing the drugs out brought the AUCs back to approximately their control levels. RM-ANOVA revealed that the changes in the AUCs significantly depended on the stimulation frequency ($F_{(1,3, 7.9)}=7.9$, $p<0.05$). The treatment with different solutions had a significant effect on the AUCs depending on the stimulation frequency ($F_{(1,2, 7.3)}=18.9$, $p<0.01$). The interaction between the treatment and the stimulation frequency effects was also significant ($F_{(1,4, 8.4)}=9.8$, $p<0.01$).

As it was mentioned earlier, application of TBOA and TBOA+LY341495 led to an extension of decay time for the 10th EPSC. Therefore, the decay times were measured for all frequencies in the control, TBOA, TBOA+LY341495 and wash solutions (n=10). The decay times in the solutions were compared for each stimulation frequency (Fig. 4.41.A). RM-ANOVA showed that there were significant differences among decay times in the different solutions for 5 Hz ($F_{(1.2, 11)}=10.4$, $p<0.01$), 30 Hz ($F_{(1.6, 6.1)}=7.9$, $p<0.05$) and 100 Hz ($F_{(1.6, 14.3)}=15.4$, $p<0.001$) stimulation frequencies. *Post-hoc* paired *t*-test showed that the decay time in the control solution was significantly longer than that in the TBOA solution for 5, 30 and 100 Hz stimulation frequencies ($p<0.05$, $p<0.05$ and $p<0.01$, respectively). Adding LY341495 to the TBOA solution prolonged the decay times even more, and they were significantly longer than those in the TBOA solution for all stimulation frequencies (paired *t*-test, 5 Hz; $p<0.05$, 30 Hz; $p<0.05$, 100 Hz; $p<0.01$). The decay times of the 10th EPSCs were compared among the different stimulation frequencies (Fig. 4.41.B). There were direct relationships between the stimulation frequency and the decay time of the 10th EPSC in the control, TBOA, TBOA+LY341495 and wash solutions, i.e. by increasing the stimulation frequency, the decay time increased. However, the slope of this increase was more in the TBOA solution than that in the control solution, and the slope in the TBOA+LY341495 solution had the biggest slope. RM-ANOVA showed that the changes in the decay times significantly depended on the stimulation frequency ($F_{(1.2, 7.2)}=10.5$, $p<0.05$). The treatment with the different solutions also had a significant effect on the decay times depending on the stimulation frequency ($F_{(1.2, 7)}=13.5$, $p<0.01$). The interaction between the treatment and stimulation frequency effects was significant ($F_{(2.2, 13)}=7.0$, $p<0.01$).

4.4.2. Effects of TBOA on GABA_A receptor-mediated IPSC

The effect of glutamate transporter blocker was studied on the GABA_A receptor-mediated IPSCs by application of TBOA (50 μ M). Figure 4.42.A shows IPSCs of a neuron in the control, TBOA, TBOA+LY341495 and wash solutions. The IPSC amplitude was remarkably reduced by TBOA. To figure it out if this reduction was due to the mGluR II activation, LY341495 (10 nM) was added to the TBOA solution. The IPSC amplitude was increased to its control level by LY341495. Washing out TBOA and LY341495 did not notably change the IPSC amplitude. The averages of the IPSC amplitudes (n=10) in the control, TBOA, TBOA+LY341495 and wash solutions are presented in figure 4.42.B. RM-ANOVA showed that there were significant differences among the averages of the IPSC amplitudes in the different solutions ($F_{(3, 27)}=15.3$, $p<0.001$). *Post-hoc* paired *t*-test showed that the IPSC amplitude in the TBOA solution was significantly smaller than that in the control or TBOA+LY341495 solution ($p<0.001$). Rising time, decay time and half-width of the IPSCs (n=10) were measured in the control, TBOA, TBOA+LY341495 and wash solutions (Fig. 4.42.C). RM-ANOVA did not show any significant difference among the rising time ($F_{(3, 27)}=2.1$, $p>0.05$), decay time ($F_{(3, 27)}=1.3$, $p>0.05$) and half-width ($F_{(3, 27)}=1.8$, $p>0.05$) of the IPSCs in the different solutions.

The effect of TBOA on the IPSCs of 9 neurons were tested over a train of 10 pulses at 5 Hz, 30 Hz and 100 Hz. Responses of a neuron in the control, TBOA, TBOA+LY341495 and wash solutions to a train of stimulation at 5 Hz are illustrated in figure 4.43.A. TBOA notably decreased the IPSC amplitudes in response to all of 10 consecutive pulses. Adding LY341495 to the TBOA solution remarkably

increased the IPSC amplitudes compared to that in the TBOA solution. Washing out TBOA and LY341495 did not notably change the IPSC amplitude. The averages of 10 successive IPSC amplitudes (elicited by a train at 5 Hz) in the control, TBOA, TBOA+LY341495 and wash solutions are presented in figure 4.43.B. RM-ANOVA showed that there was a significant effect among the pulse order ($F_{(3, 24)}=13, p<0.001$). Treatment (with the control, TBOA, TBOA+LY341495 and wash solutions) significantly changed the average of the IPSC amplitudes ($F_{(9, 72)}=48, p<0.001$). The interaction between the treatment and pulse order effects was also significant ($F_{(27, 216)}=1.7, p<0.05$). Ratios of ten succeeding IPSC amplitudes to the 1st IPSC amplitude were depicted for all solutions in figure 4.43.C. RM-ANOVA revealed that the treatment with the different solutions had a significant effect on the ratios of the responses ($F_{(9, 72)}=42, p<0.001$). There were significant differences among the ratios in the pulse order ($F_{(3, 24)}=5.1, p<0.05$). The interaction between the treatment and pulse order effects was also significant ($F_{(27, 216)}=1.7, p<0.05$).

Figure 4.44.A shows responses of a neuron to a train of 10 pulses at 30 Hz in the control, TBOA, TBOA+LY341495 and wash solutions. The amplitudes of the 2nd-10th IPSC were smaller than the amplitude of the 1st one in the control solution. TBOA decreased the IPSC amplitudes in responses to all 10 pulses but there were more reductions for the first few IPSCs. The IPSC amplitudes roughly brought back to the control levels by adding LY341495 to the TBOA solution. Washing out the drugs did not remarkably change the IPSC amplitudes. The averages of the IPSC amplitudes in four different solutions were plotted as a function of the pulse order in figure 4.44.B (n=9). RM-ANOVA showed that there were significant differences

among the IPSC amplitudes in the pulse order ($F_{(3, 24)}=6.8$, $p<0.01$). The treatment with the different solutions also had a significant effect on the averages of the IPSC amplitudes ($F_{(9, 72)}=53.3$, $p<0.001$). The interaction between the treatment and pulse order effects was significant ($F_{(27, 216)}=3.9$, $p<0.001$). The ratios of the IPSCs in the control, drugs and wash solutions were plotted as a function of the pulse order in figure 4.44.C. The treatment had a significant effect on the IPSC ratios (RM-ANOVA, $F_{(9, 72)}=62.4$, $p<0.001$). There were also significant differences among the IPSC ratios in the pulse order ($F_{(1.4, 10.1)}=3.8$, $p<0.05$). The interaction between the treatment and pulse order effects for the IPSC ratios for 30 Hz stimulation was significant ($F_{(27, 216)}=1.8$, $p<0.05$).

The IPSCs of a neuron over a period of 10 stimulus pulses at 100 Hz in the control, TBOA, TBOA+LY341495 and wash solutions are presented in figure 4.45.A. At this frequency, there was a great temporal integration of the IPSCs, and facilitation for the 2nd–5th IPSCs. TBOA depressed the amplitudes of the IPSCs especially those of the first few ones with the greatest effect on the 1st one. In TBOA solution, the amplitudes of the 2nd–10th IPSCs were larger than the amplitude of the first IPSC. Adding LY341495 to the TBOA solution increased the IPSC amplitudes, and returned them almost back to the control levels. Washing out TBOA and LY341495 did not have any remarkable effect on the IPSC amplitudes compared to those in the control or TBOA+LY341495 solutions. The averages of the IPSC amplitudes in four solutions were plotted as a function of the pulse order in figure 4.45.B (n=9). RM-ANOVA showed that the treatment with the different solutions had a significant effect on the IPSC amplitudes in response to 100 Hz stimulation ($F_{(9,$

$_{72})=32.7, p<0.001$). There were significant differences among the IPSC amplitudes in the pulse order ($F_{(3, 24)}=4.6, p<0.05$). The interaction between the treatment and pulse order effects was also significant ($F_{(27, 216)}=7.2, p<0.001$). The IPSC ratios in the control, TBOA, TBOA+LY341495 and wash solutions were plotted as a function of the pulse order in figure 4.45.C. Note that the ratios of the 2nd-10th IPSCs in TBOA solution were larger than one (i.e. facilitation) while the ratios of only the 2nd and 3rd IPSCs were larger than one (>1) in the other solutions. RM-ANOVA showed that the treatment with the different solutions had a significant effect on the IPSC ratios ($F_{(9, 72)}=25, p<0.001$). There were significant differences among the ratios in the pulse order ($F_{(3, 24)}=10.4, p<0.001$). The interaction between the treatment and pulse order effects for the IPSC ratios for 100 Hz stimulation was also significant ($F_{(27, 216)}=5.1, p<0.001$).

Averages of the IPSC amplitudes in responses to 10 stimulus pulses at 5, 30 and 100 Hz in the control, TBOA, TBOA+LY341495 and wash solutions are presented in figure 4.46 (n=9). There were suppressive effects on the IPSC amplitudes in response to 10 successive stimuli at 5 and 30 Hz, and to the last few stimuli at 100 Hz stimulation in the control solution (Fig. 4.46.A). RM-ANOVA showed that the changes of the IPSC amplitudes depended on the stimulation frequency in the control solution ($F_{(1,2, 9,8)}=14.1, p<0.01$). Application of TBOA decreased the amplitudes of all 10 succeeding IPSCs in responses to all stimulation frequencies except those of the last few IPSCs induced at 100 Hz stimulation frequency (Fig. 4.46.B). However, there were still suppressive effects on the IPSCs with all ten pulses at 5 Hz stimulation, and with most of pulses at 30 Hz stimulation.

There were facilitative effects with all pulses at 100 Hz stimulation, and a couple of pulses at 30 Hz stimulation in the TBOA solution. RM-ANOVA revealed that the changes of the IPSC amplitudes over the 10 pulse train relied on the stimulation frequency in the TBOA solution ($F_{(1.1, 8.9)}=32.3, p<0.001$). After adding LY341495 to the TBOA solution, the degree of the depression or facilitation as well as the IPSC amplitudes returned back to the control levels (Fig. 4.46.C). RM-ANOVA showed significant differences among the IPSC amplitudes in responses to the different stimulation frequencies in the TBOA+LY341495 solution, and the changes of the IPSC amplitudes over the train depended on the stimulation frequency ($F_{(1.1, 9.1)}=16.2, p<0.01$). The averages of the IPSC amplitudes in responses to the train of stimulations at 5, 30 and 100 Hz in the wash solution were almost the same as those in the control or TBOA+LY341495 solutions (Fig. 4.46.D). RM-ANOVA showed the changes of the IPSC amplitudes over the 10 pulse train depended on the stimulation frequency in the wash solution too ($F_{(1.2, 9.4)}=10.3, p<0.01$). RM-ANOVA for all solutions revealed that the treatment with the different solutions had a significant effect on the IPSC amplitudes depending on the stimulation frequency ($F_{(3, 24)}=8.6, p<0.001$), and there was a significant interaction between the treatment and stimulation frequency effects ($F_{(4, 31.7)}=8.1, p<0.05$).

The ratios of the IPSC amplitudes in responses to 5, 30 and 100 Hz stimulation frequencies were plotted as a function of the pulse order in figure 4.47. In the control solution, the ratios for the first few IPSCs among the different frequencies were different while those for the last few ones looked the same (Fig. 4.47.A). RM-ANOVA showed that the ratios of the responses for successive pulses were

differently changed depending on the stimulation frequency in the control solution ($F_{(1.1, 5.3)}=21.6, p<0.01$). TBOA increased the IPSC ratios for the different stimulation frequencies at different degree, and all ratios in one train stood away from the ratios in the other trains (Fig. 4.47.B). RM-ANOVA showed that in the TBOA solution, there were significant differences among the IPSC ratios in responses to the different stimulation frequencies, and the changes in the ratios depended on the stimulation frequency ($F_{(1.2, 5.3)}=21.7, p<0.01$). Adding LY341495 to the TBOA solution brought the ratios for each stimulation frequency back to those in the control solution (Fig. 4.47.C). RM-ANOVA did show significant differences among the IPSC ratios in responses to the different stimulation frequencies in the TBOA+LY341495 solution ($F_{(1.2, 9.4)}=15.5, p<0.01$). Washing out the drugs did not change the ratios from those in the control or TBOA+LY341495 solutions (Fig. 4.47.D). In the wash solution, the changes in the ratios over the stimulation train depended on the stimulation frequency ($F_{(1.3, 10.5)}=10.0, p<0.01$). RM-ANOVA for all solutions revealed that the treatment with the different solutions had a significant effect on the IPSC ratios depending on the stimulation frequency ($F_{(3, 24)}=9.7, p<0.01$), and there was a significant interaction between the treatment and stimulation frequency effects ($F_{(1.7, 8.7)}=5.6, p<0.05$).

AUCs of the IPSCs in the control, TBOA, TBOA+LY341495 and wash solutions for the single pulse ($\times 10$) and all tested frequencies are presented in figure 4.48.A ($n=9$). RM-ANOVA showed that there were significant differences among the AUCs in the different solutions for the single pulse ($\times 10$), ($F_{(3, 24)}=12.9, p<0.001$), 5 Hz ($F_{(1.7, 15.2)}=4.3, p<0.05$), 30 Hz ($F_{(3, 24)}=9.6, p<0.001$) and 100 Hz ($F_{(3, 24)}=6.7, p<0.01$) stimulations. For the single pulse ($\times 10$), *post-hoc* paired *t*-test showed that

TBOA significantly decreased the AUC of the IPSC ($p<0.001$), and adding LY341495 to the TBOA solution significantly increased the AUC ($p<0.001$). When a train of stimuli at 5 Hz was used, there was significant difference between the AUCs in the control and in the TBOA solutions (paired t -test, $p<0.01$), and adding LY341495 to the TBOA solution significantly increased the AUC compared to that in the TBOA solution (paired t -test, $p<0.01$). TBOA significantly reduced the AUCs when stimulations at 30 or 100 Hz were used (paired t -test, $p<0.001$, $p<0.01$, respectively). Adding LY341495 to the TBOA solution increased the AUCs, and the AUCs were significantly larger than those in the TBOA solution when 30 or 100 Hz stimulation was applied (paired t -test, $p<0.01$). The AUCs of the IPSCs in the control, TBOA, TBOA+LY341495 and wash solutions were compared among the stimulation frequencies of single pulse ($\times 10$), 5, 30 and 100 Hz (Fig. 4.48.B). In the control solution, the AUCs of the IPSCs in response to the single pulse stimulation ($\times 10$) were larger than those in response to a train of stimulation, regardless of its frequency. In addition, the AUC in response to 100 Hz stimulation frequency was larger than that in response to 5 or 30 Hz stimulation frequencies. Application of TBOA decreased the AUCs in response to all types of stimulations, with the most effect on the single pulse. Adding LY341495 to the TBOA solution increased the AUCs almost to their control levels. The trends and the amount of the AUCs in the control, TBOA+LY341495 and wash solutions were roughly the same. RM-ANOVA revealed that the changes in the AUCs significantly depended the stimulation frequency ($F_{(3, 24)}=96.7$, $p<0.001$). The treatment with the different solutions had a significant effect on the AUCs depending on the stimulation frequency ($F_{(3, 24)}=12.8$,

$p<0.001$). The interaction between the treatment and stimulation frequency effects was also significant ($F_{(9, 72)}=4.9, p<0.001$).

The decay times for the 10th IPSCs were measured for all stimulation frequencies in the control, TBOA, TBOA+LY341495 and wash solutions ($n=9$). The decay times in the solutions were compared for each stimulation frequency (Fig. 4.49.A). RM-ANOVA showed that there were significant differences among the decay times in the different solutions for stimulations at 5 Hz ($F_{(2, 18.3)}=5.3, p<0.05$), 30 Hz ($F_{(3, 24)}=5.7, p<0.01$) and 100 Hz ($F_{(3, 24)}=3.8, p<0.05$). *Post-hoc* paired *t*-test showed that the decay time in the control solution were significantly longer than the decay times in the TBOA solution for 5, 30 and 100 Hz stimulation frequencies ($p<0.01, p<0.05$ and $p<0.05$, respectively). Adding LY341495 to the TBOA solution prolonged the decay times for all frequencies. The decay times were significantly longer than those in the TBOA solution (paired *t*-test, 5 Hz; $p<0.01$, 30 Hz; $p<0.05$, 100 Hz; $p<0.05$). The decay times of the 10th IPSCs were compared among the different stimulation frequencies (Fig. 4.49.B). By increasing the stimulation frequency, the decay time increased in the control, TBOA, TBOA+LY341495 and wash solutions, and there were direct relationships between the stimulation frequency and the decay time of the 10th IPSC. RM-ANOVA revealed that the changes in the decay times significantly depended on the stimulation frequency ($F_{(1.1, 9.5)}=26.2, p<0.001$). The treatment also had a significant effect on the decay times ($F_{(3, 24)}=5.8, p<0.01$). The interaction between the treatment and the stimulation frequency effects was not significant ($F_{(2.3, 21)}=2.0, p>0.05$).

Table 4.1. Intrinsic membrane characteristic of the ICC neurons

Firing pattern	Rebound (ratio)	Number of cells	RMP (mV)	R _{in} (MΩ)	τ (ms)	AP amplitude (mV)	AP half-width (ms)	AP Threshold (mV)
Regular	35/47	n=10	-62.1±0.4	335±23 ^{*1}	21.3±2.1 ⁺⁷	52.6±3.4	1.03±0.09	-39.5±2.9
Adapting	19/25	n=10	-61.8±0.4	281±26 ^{*2}	14.3±2.1	48.3±3.7	1.07±0.08	-33.6±2.8
Build-up/ Pauser	2/14	n=10	-61.1±0.4	230±22 ^{*3,4}	15.6±1.4 ⁺⁸	50.3±2.6	0.85±0.06	-29.7±1.7
Onset/ Transient	19/19	n=10	-62.4±0.3	174±13 ^{*5}	8.9±0.9	46.7±2.3	0.81±0.07	-27.4±2.5
Bursting	8/8	n=8	-62.1±0.6	196±17 ^{*6}	11.9±2.2 ⁺⁹	45.7±2.7	0.88±0.10	-29.7±1.9

RMP: resting membrane potential, R_{in}: input resistance; τ: membrane time constant; AP: action potential. ANOVA followed by Tukey's *post-hoc* test revealed significant differences in the R_{in} and τ among the groups, *p*<0.001. *1: significant differences between the regular and onset/transient, *p*<0.001, *2: significant differences between the adapting and onset/transient, *p*<0.001, *3: significant differences between the build-up/pauser and onset/transient, *p*<0.01, *4 : significant differences between the build-up/pauser and adapting, *p*<0.001, *5: significant differences between the onset/transient and build-up/pauser, *p*<0.001, *6: significant difference between the bursting and regular, *p*<0.001, +7: significant difference between the regular and onset/transient, *p*<0.001, +8: significant differences between the build-up/pauser and onset/transient, *p*<0.05, +9: significant difference between the bursting and regular, *p*<0.05.

Table 4.2. Effects of ACPD and SOP on the intrinsic membrane properties of the ICC neurons

	<i>Number of cells</i>	<i>RMP (mV)</i>	<i>R_{in} (MΩ)</i>	<i>τ (ms)</i>	<i>AP amplitude (mV)</i>	<i>AP half-width (ms)</i>	<i>AP Threshold (mV)</i>
ACSF		-60.5±0.9	300±39	18.5±3.3	54.8±3.9	0.98±0.10	-40.7±2.6
ACP	n=8	-60.6±0.9	305±32	17.4±2.5	55.3±3.8	0.95±0.12	-41.1±2.5

ACSF		-62.0±0.6	288±42	15.1±2.2	58.6±3.6	0.89±0.16	-42.4±2.7
SOP	n=8	-62.4±0.3	290±40	14.9±2.8	59.7±3.5	0.86±0.15	-41.0±3.0

RMP: resting membrane potential, R_{in}: input resistance; τ: membrane time constant; AP: action potential.
Paired *t*-test revealed no significant difference between the responses in ACSF and mGluR agonists, ACPD or SOP, *p*>0.05.

Figure 4.1.

Figure 4.1. Firing pattern of ICC neurons; regular and adapting neurons. A₁: Voltage responses of a regular neuron to current injection of -110 and 110 pA. The interspike intervals were fairly constant during the positive current injection. With the negative current injection, the neuron showed an obvious sag and rebound depolarization (*). **A₂:** Rebound depolarization led to the generation of an AP when -130 pA was injected to the same neuron. The neuron produced more APs with higher level of positive current injection. **B₁:** Voltage responses of an adapting neuron to the current injections of -120 and 120 pA. The interspike intervals were gradually increased over the positive current injection. The negative current injection could not induce rebound depolarization in this neuron. **B₂:** At a higher level of positive current injection, the neuron produced more APs while keeping its adapting characteristic. **C:** Current-voltage relationship of the regular neurons (n=10) were measured at the peak (■) and steady state (Δ) hyperpolarization. **D:** The relationships of the voltage change at the peak (■) and steady state (Δ) hyperpolarization to the current levels in the adapting neurons (n=10).

Figure 4.2.

Figure 4.2. Firing pattern of ICC neurons; onset/transient neurons. **A₁:** Voltage responses of an onset neuron to the current injection of -140 and 140 pA. This neuron produced only one AP in response to the positive current injection. There were a prominent sag and rebound depolarization in response to the hyperpolarization current injection. **A₂:** The neuron could not generate more AP in response to a higher level of depolarization current injection. Rebound depolarization led to the generation of an AP when -220 pA was injected into the same neuron. **B₁:** Voltage responses of a transient neuron to the positive and negative current injections. This neuron could produce one spike in response to the depolarizing current at 130 pA level. **B₂:** The neuron could generate one full size AP followed by a few small spikes in response to a higher level of the depolarization current injection. **C:** The relationship of the voltage changes at the peak (■) and steady state (Δ) hyperpolarization to the current level in the onset/transient neurons (n=10).

Figure 4.3.

Figure 4.3. Firing pattern of ICC neurons; build-up/pauser neurons. A₁: Responses of a build-up neuron to the current injections of -140 and 140 pA. When the positive current injected, there was a notable delay before the neuron started to generate AP. There was no rebound in response to the hyperpolarizing current. **A₂:** The neuron showed a sustained firing pattern with higher level of depolarizing current injection. **A₃:** When the depolarizing current was preceded by hyperpolarizing current, there was a prominent delay before the neuron started to generate APs. The delay was there even with increasing positive current injection to 210 pA (lower trace). The expected places for generating spike are marked with arrows. **B₁:** Voltage responses of a pauser neuron to the positive and negative current injections. There was a pause between the first and second spikes. **B₂:** The duration of the pause was shorter with more positive current injection but it was still obvious. **B₃:** There was a clear pause between the first and second spikes when the depolarizing currents of 100 and 180 pA was preceded by a hyperpolarizing current. **C:** The relationship of the voltage changes at the peak (■) and steady state (Δ) hyperpolarization to the current level in the build-up/pauser neurons (n=10).

Figure 4.4.

Figure 4.4. Firing pattern of ICC neurons; bursting neurons. **A₁:** Responses of a bursting neuron to positive and negative current injections. This neuron generated a couple of bursting spikes on a depolarising hump with a long undershoot. There were bursting spikes on the depolarising rebound when the neuron was hyperpolarized with -140 pA. **A₂:** There were more bursting spikes when the neuron was injected with a higher level of depolarising current. **B₁:** When 80 pA depolarizing current was preceded by a hyperpolarizing current, there was bursting spike on the depolarizing hump. **B₂:** The neuron could produce more APs after the first bursting spikes when a higher level of depolarizing current was followed by the same hyperpolarizing current. **C:** The relationship of the voltage changes at the peak (■) and steady (Δ) hyperpolarization to the current level in the bursting neurons (n=8).

Figure 4.5.

Figure 4.5. Morphology of ICC neurons. **A₁:** A neuron with regular firing pattern was labelled with Lucifer Yellow, and classified as a bipolar cell. **A₂:** a bipolar neuron with sustained firing pattern. This neuron showed a rebound depolarization in response to hyperpolarization current injection. **B₁:** A sample neuron with regular firing pattern was classified as a multipolar neuron. **B₂:** A multipolar neuron with adapting firing pattern. Hyperpolarization current injection induced rebound depolarization in this neuron. The middle panels shows the locations of the neurons in the left (+) and right panels (*) in the ICC. Scale = 20 μm .

Figure 4.6.

Figure 4.6. Effect of ACPD (agonist for mGluR I and II) on intrinsic membrane properties of ICC neurons. A: Responses of a neuron to the positive (120 pA) and negative (0 to -200 pA in 40 pA/step) current injections in ACSF (left panel), following adding 100 μ M ACPD to the solution (middle panel) and washing out the agonist (right panel). This neuron showed a sag of the membrane potential during hyperpolarization, and a rebound depolarization (*) following hyperpolarization. ACPD did not change the responses of the neuron to the current injections. **B:** The current-voltage relationships of the neuron before, during and after application of ACPD did not change. **C:** The current-voltage relationships derived from responses of 8 neurons to hyperpolarizing current injections before and during application of ACPD, and after washing out ACPD.

Figure 4.7.

Figure 4.7. Effects of SOP (agonist for mGluR III) on membrane properties of ICC neurons. **A:** Responses of a neuron to the positive (120 pA) and negative (0 to -200 pA in 40 pA/step) current injections in ACSF (left panel), SOP (100 μ M) (middle panel) and after washing out SOP (right panel). This cell was a non-rebound cell. SOP did not affect the responses of the neuron to the current injections. **B:** The current-voltage relationships of the neuron before, during application of SOP and after washing it out. **C:** The current-voltage relationships obtained from responses of 8 neurons to hyperpolarizing current injections before and during application of SOP, and after washing out the drug.

Figure 4.8.

Figure 4.8. AMPA receptor-mediated EPSC in ICC neurons. **A:** AMPA receptor-mediated EPSCs of a neuron were recorded at different membrane holding potentials. The EPSCs were pharmacologically isolated by adding APV (NMDA antagonist, 100 μ M), bicuculline (GABA_A antagonist, 10 μ M) and strychnine (glycine antagonist, 0.5 μ M) into the normal ACSF. **B:** Averages of the EPSC amplitudes were plotted against membrane holding potentials (n=6). There was a linear relationship between the membrane potential and the EPSC amplitude. V_R : reversal potential for the EPSC.

Figure 4.9.

Figure 4.9. Effect of ACPD on AMPA receptor-mediated EPSC. **A:** AMPA receptor-mediated response of a neuron before (top traces), during application (middle traces) and after washing out (bottom traces) ACPD (50 μ M). ACPD reduced the EPSC amplitude reversibly. **B:** ACPD (50 μ M) significantly decreased the average of the EPSC amplitude ($p<0.01$). **C:** ACPD decreased the EPSCs in a dose-dependent manner.

Figure 4.10.

Figure 4.10. Effects of DHPG on AMPA receptor-mediated EPSC. **A:** AMPA receptor-mediated EPSCs before (top traces), during application (middle traces) and after washing out (bottom traces) DHPG (100 μ M) in a sample neuron. **B:** The average of the EPSC amplitudes was not significantly changed after administration of DHPG ($n=8$, $p>0.05$).

Figure 4.11.

Figure 4.11. Effects of LY379268 on AMPA receptor-mediated EPSC. **A:** AMPA receptor-mediated EPSCs of a neuron before (top trace), during application of 20 nM LY379268 (middle trace), and after washing out the agonist (bottom traces). LY379268 notably decreased the EPSC amplitude. Washing out the drug could to some extent return the EPSC amplitude back to the control level. **B:** Time course of the LY379268 (20 nM) application, and its effect on the average of the EPSC amplitudes of 7 neurons. **C:** LY379268 decreased the EPSC amplitudes in a dose-dependent manner (n=10). **D:** LY379268 did not have any significant effect on the rising time, decay time and half width of the EPSCs (n=10) ($p>0.05$). **E:** A neuron was labeled with Lucifer Yellow, and classified as a multipolar cell. The EPSC of this neuron was reduced by LY379268. * indicates the location of the neuron in the ICC (right panel). Scale = 20 μ m.

Figure 4.12.

Figure 4.12. Effect of LY379268 on AMPA receptor-mediated EPSC was reversible by mGluR II antagonist, LY341495. **A:** EPSC of a neuron in the control solution (top trace). Application of LY379268 (20 nM) considerably reduced the EPSC amplitude of the neuron (middle trace). The response was completely returned back to the control level when LY341495 (10 nM) was added to the bathing solution (bottom trace). **B:** The EPSC amplitudes of this neuron were plotted as a function of time. Five minutes after control response recording, LY379268 was applied for 10 min. The EPSC amplitude was greatly reduced during the LY379268 application, and the EPSC remained reduced even after 30 min wash with the control solution. Adding LY341495 to the control solution increased the EPSC amplitude back to the control level. However, further adding LY379268 to the solution containing LY341495 did not change the EPSC amplitude. **C:** The average of the EPSC amplitude ($n=10$) was significantly reduced following administration of 20 nM LY379268 ($p<0.01$). Adding LY341495 (10 nM) returned the EPSC amplitude back to the control level.

Figure 4.13.

Figure 4.13. Effect of SOP on AMPA receptor-mediated EPSC. **A:** AMPA receptor-mediated EPSCs of a neuron before (top trace), during application (middle trace) and after washing out SOP (100 μ M) (bottom trace). Adding SOP did not change EPSC amplitude. **B:** The average of the EPSC amplitude was not significantly changed by SOP ($p>0.05$).

Figure 4.14.

Figure 4.14. Effects of LY379268 on AMPA receptor-mediated EPSCs elicited by paired-pulse stimulation of the LL. **A:** AMPA receptor-mediated EPSCs were evoked by paired-stimulus pulses before (top traces) and during LY379268 (20 nM) administration (middle traces) in a neuron in 2.4 mM extracellular Ca^{2+} medium. Bottom traces: the normalized EPSCs of the neuron before (black) and during LY379268 administration (gray) were superimposed. Note the smaller reduction of the 2nd response in the LY379268 solution. **B:** The ratios of the 2nd to 1st EPSCs in the control and LY379268 solutions with 2.4 mM Ca^{2+} are illustrated for 10 individual neurons. **C:** The average ratios of the 2nd to 1st EPSCs for the neurons was significantly increased by LY379268 ($p < 0.01$). **D:** There was a good correlation between the reduction in the amplitude of the 1st EPSC and the increase in the ratio of the 2nd to 1st EPSCs during application of LY379268 ($p < 0.01$).

Figure 4.15.

Figure 4.15. Effects of LY379268 on AMPA receptor-mediated EPSCs elicited by paired-pulse stimulation in low Ca^{2+} medium. **A:** With 1.0 mM extracellular Ca^{2+} , AMPA receptor-mediated EPSCs were evoked by paired-stimulus pulses before (top traces) and during LY379268 (20 nM) administration in a neuron (middle traces). Bottom traces: normalized EPSCs of the neuron before (black) and during LY379268 (gray) were superimposed. The smaller reduction of the 2nd response in the LY379268 solution is obvious. **B:** Comparison of the ratios of the 2nd to 1st EPSCs in 2.4 and 1.0 mM Ca^{2+} solutions (n=10, student's *t*-test, $p<0.05$). **C:** The ratios of the 2nd to 1st EPSCs in the control and LY379268 solutions in 1.0 mM Ca^{2+} medium are illustrated for 10 individual neurons. **D:** The average ratios of the 2nd to 1st EPSCs for the neurons was significantly increased by LY379268 ($p<0.05$).

Figure 4.16

Figure 4.16. GABA_A receptor-mediated IPSC in ICC neurons. **A:** GABA_A receptor-mediated IPSC was pharmacologically isolated in a neuron, and recorded at different membrane holding potentials. The GABA_A was isolated by adding APV (NMDA antagonist, 100 μ M), CNQX (AMPA antagonist, 10 μ M) and strychnine (glycine antagonist, 0.5 μ M) into the normal ACSF. **B:** The averages of the IPSC amplitudes were plotted against membrane holding potentials (n=6). There was a linear relationship between the membrane potential and the IPSC amplitude. V_R : reversal potential for the GABA_A receptor-mediated IPSC.

Figure 4.17.

Figure 4.17. Effect of ACPD on GABA_A receptor-mediated IPSC. **A:** GABA_A receptor-mediated response of a neuron before (top traces), during application (middle traces) and after washing out (bottom traces) ACPD. Administration of ACPD (50 μ M) reversibly reduced the IPSC amplitude of the neuron. **B:** The average of IPSC amplitudes was significantly decreased by 50 μ M ACPD ($p < 0.001$). **C:** The effect of ACPD on the IPSC amplitude was dose-dependent.

Figure 4.18.

Figure 4.18. Effects of DHPG on GABA_A receptor-mediated IPSC. **A:** GABA_A receptor-mediated IPSCs before (top traces), during application (middle traces) and after washing out (bottom traces) DHPG (100 μ M) in a sample neuron. **B:** The average of the IPSC amplitude was not significantly changed after administration of DHPG (n=9, $p>0.05$).

Figure 4.19.

Figure 4.19. Effects of LY379268 on GABA_A receptor-mediated IPSC. **A:** GABA_A receptor-mediated IPSCs before (top traces), during application (middle traces) and after washing out (bottom traces) LY379268 (20 nM) in a neuron. LY379268 remarkably decreased the IPSC amplitude. Washing out LY379268 could partially return the IPSC amplitude back to the control level. **B:** Time course of application of LY379268 (20 nM), and its effect on the average of the IPSC amplitudes of 6 neurons. **C:** LY379268 decreased the IPSC amplitude in a dose-dependent manner (n=8). **D:** LY379268 did not have any significant effect on the rising time, decay time and half width of the IPSCs (n=10). **E:** A neuron was labeled with Lucifer Yellow, and classified as a multipolar cell. The IPSCs of this neuron was reduced by LY379268. * indicates the location of the neuron in the ICC (right panel). Scale = 20 μ m.

Figure 4.20.

Figure 4.20. Effect of LY379268 on GABA_A receptor-mediated IPSC was reversible by mGluR II antagonist, LY341495. A: IPSC of a neuron in the control solution (top trace). Application of LY379268 (20 nM) substantially decreased the IPSC amplitude (middle traces). The IPSC was completely returned back to the control level when LY341495 (10 nM) was added to the bath solution (bottom trace). **B:** The IPSC amplitudes of this neuron were plotted as a function of time. The IPSC amplitudes were greatly reduced during the LY379268 application, and the IPSC amplitudes remained reduced even after 30 min wash with the control solution. Adding LY341495 to the control solution increased the IPSC amplitude to the control level. Re-application of LY379268 to the solution containing LY341495 did not have any effect on the IPSC amplitude. **C:** The average of IPSC amplitudes (n=10) was significantly reduced by LY379268 ($p<0.001$). Adding LY341495 (10 nM) returned the IPSC amplitude back to the control level.

Figure 4.21.

Figure 4.21. Effect of SOP on GABA_A receptor-mediated IPSC. **A:** GABA_A receptor-mediated IPSCs of a neuron before (top trace), during application (middle trace) and after washing out SOP (100 μ M) (bottom trace). Adding SOP did not change the IPSC amplitude. **B:** The average of the IPSC amplitudes was not significantly changed by SOP ($p>0.05$).

Figure 4.22.

Figure 4.22. Effects of LY379268 on GABA_A receptor-mediated IPSCs elicited by paired-pulse stimulation of the LL. **A:** GABA_A receptor-mediated IPSCs were evoked by paired-stimulus pulses before (top traces) and during LY379268 (20 nM) (middle traces) in a neuron in 2.4 mM extracellular Ca²⁺ medium. Bottom traces: normalized IPSCs of the neuron before (black) and during LY379268 application (gray) were superimposed. Note the smaller reduction of the 2nd response in the LY379268 solution. **B:** The ratios of the 2nd to 1st IPSCs in the control and LY379268 solutions with 2.4 mM Ca²⁺ are illustrated for 10 individual neurons. **C:** The average ratios of the 2nd to 1st IPSCs for the neurons was significantly increased by LY379268 ($p<0.01$). **D:** The reduction in the amplitude of the 1st IPSC and the increase in the ratio of the 2nd to 1st IPSCs during application of LY379268 were highly correlated ($p<0.001$).

Figure 4.23.

Figure 4.23. Effects of LY379268 on GABA_A receptor-mediated IPSCs elicited by paired-pulse stimulation in low Ca²⁺ medium. **A:** With 1.0 mM extracellular Ca²⁺, GABA_A receptor-mediated IPSCs were evoked by paired-stimulus pulses before (top traces) and during LY379268 (20 nM) application in a neuron (middle traces). Bottom traces: normalized IPSCs of this neuron before (black) and during LY379268 application (gray) were superimposed. The smaller reduction of the 2nd response in the LY379268 solution is clear. **B:** Comparison of the ratios of the 2nd to 1st IPSCs in 2.4 and 1.0 mM Ca²⁺ solutions (n=10, student's *t*-test, *p*<0.01). **C:** The ratios of the 2nd to 1st IPSCs in the control and LY379268 solutions with 1.0 mM Ca²⁺ are illustrated for 10 individual neurons. **D:** The average ratios of the 2nd to 1st IPSCs for the neurons was significantly increased by LY379268 (*p*<0.05).

Figure 4.24.

Figure 4.24. Effect of LY379268 on EPSC amplitudes elicited by 10 stimulus pulses at 5 Hz. **A:** AMPA receptor-mediated EPSCs of a neuron to 10 consecutive stimuli at 5 Hz in control solution (top trace) and after application of 20 nM LY379268 (bottom trace). **B:** Averages of the EPSC amplitudes before and after application of LY379268 were plotted as a function of the pulse order ($n=13$). There was a significant effect among the pulse order ($p<0.01$). The LY379268 treatment significantly reduced the EPSC amplitudes ($p<0.01$). There was a significant interaction between the pulse order and treatment effects ($p<0.001$). **C:** Ratios of the EPSC amplitudes (P_n) to the first EPSC amplitude (P_1) before and after application of LY379268 were plotted as a function of the pulse order ($n=13$). There was a significant effect among the pulse order ($p<0.001$). The treatment with LY379268 significantly enhanced the EPSC ratios ($p<0.01$). The interaction between the pulse order and the treatment effects was not significant ($p>0.05$).

Figure 4.25.

Figure 4.25. Effect of LY379268 on EPSC amplitudes elicited by 10 stimulus pulses at 30 Hz. **A:** APMA receptor-mediated EPSCs of a neuron to 10 consecutive stimuli at 30 Hz in control solution (top trace) and after application of 20 nM LY379268 (bottom trace). **B:** Averages of the EPSC amplitudes before and after application of LY379268 were plotted as a function of the pulse order ($n=13$). There was a significant effect among the pulse order ($p<0.001$). The treatment with LY379268 significantly reduced the EPSC amplitudes ($p<0.01$). There was a significant interaction between the pulse order and treatment effects ($p<0.001$). **C:** Ratios of the EPSC amplitudes (P_n) to the first EPSC amplitude (P_1) before and after application of LY379268 were plotted as a function of the pulse order ($n=13$). There was a significant effect among the pulse order ($p<0.01$). The LY379268 treatment significantly increased the EPSC ratios ($p<0.05$). The interaction between the pulse order and treatment effects was not significant ($p>0.05$).

Figure 4.26.

Figure 4.26. Effect of LY379268 on EPSC amplitudes elicited by 10 stimulus pulses at 100 Hz. **A:** APMA receptor-mediated EPSCs of a neuron to 10 consecutive stimuli at 100 Hz in control solution (top trace) and after application of 20 nM LY379268 (bottom trace). **B:** Averages of the EPSC amplitudes before and after application of LY379268 were plotted as a function of the pulse order ($n=13$). There was a significant effect among the pulse order ($p<0.001$). LY379268 treatment significantly reduced the EPSC amplitudes ($p<0.01$). There was a significant interaction between the pulse order and treatment effects ($p<0.001$). **C:** Ratios of the EPSC amplitudes (P_n) to the first EPSC amplitude (P_1) before and after application of LY379268 were plotted as a function of the pulse order ($n=13$). There was a significant effect among the pulse order ($p<0.001$). The treatment with LY379268 significantly increased the EPSC ratios ($p<0.01$). The interaction between the pulse order and treatment effects was significant ($p<0.001$).

Figure 4.27.

Figure 4.27. Effect of LY379268 on EPSC amplitudes elicited by 10 stimulus pulses at different frequencies. A: Averages of the EPSC amplitudes for 5, 30 and 100 Hz stimulation frequencies before (left panel) and after application of LY379268 (right panel) were plotted as a function of the pulse order ($n=13$). The EPSC amplitudes in responses to 10 consecutive stimulations depended on the stimulation frequency in the control solution ($p<0.05$) while they did not depend on the stimulation frequency in the LY379268 solution ($p>0.05$). The treatment had a significant effect on the dependency of the EPSC amplitudes to the stimulation frequency ($p<0.01$). **B:** Ratios of the EPSC amplitudes (P_n) to the first EPSC amplitude (P_1) before and after application of LY379268 were plotted as a function of the pulse order for 3 different stimulation frequencies ($n=13$). The ratios of 10 consecutive EPSCs depended on the stimulation frequency in the control solution ($p<0.01$) while they did not depend on the stimulation frequency in the LY379268 solution ($p>0.05$). The treatment significantly changed the dependency of the EPSC ratios to the stimulation frequency ($p<0.01$).

Figure 4.28.

Figure 4.28. Effect of LY379268 on area under curve (AUC) of EPSCs elicited by 10 stimulus pulses at different frequencies. **A:** Comparison of the averages of AUCs before and after application of LY379268 for single pulse ($\times 10$) and trains of 10 pulses at 5, 30 and 100 Hz. There was significant difference between the AUC before and the AUC after application of LY379268 for the single pulse ($\times 10$) ($p < 0.01$) and the trains at 5 ($p < 0.05$), 30 ($p < 0.001$) and 100 Hz ($p < 0.001$). **B:** Comparison of the AUCs among the single pulse ($\times 10$) and the trains of 10 pulses at 5, 30 and 100 Hz in the control and LY379268 solutions. RM-ANOVA revealed that the amount of the AUCs significantly depended on the stimulation frequency ($p < 0.001$). The treatment with LY379268 had a significant effect on the reduction of the AUCs ($p < 0.001$). There was not any significant interaction between the stimulation frequency and treatment effects ($p > 0.05$).

Figure 4.29.

Figure 4.29. Effect of LY379268 on IPSC amplitudes elicited by 10 stimulus pulses at 5 Hz. **A:** GABA_A receptor-mediated IPSCs of a neuron to 10 consecutive stimuli at 5 Hz in control solution (top trace) and after application of 20 nM LY379268 (bottom trace). **B:** Averages of the IPSC amplitudes before and after application of LY379268 were plotted as a function of the pulse order (n=10). There was a significant effect among the pulse order ($p<0.001$). LY379268 treatment significantly reduced the IPSC amplitudes ($p<0.01$). There was a significant interaction between the pulse order and treatment effects ($p<0.05$). **C:** Ratios of the IPSC amplitudes (P_n) to the first IPSC amplitude (P₁) before and after application of LY379268 were plotted as a function of the pulse order (n=10). There was a significant effect among the pulse order ($p<0.001$). The treatment with LY379268 significantly raised the IPSC ratios ($p<0.05$). The interaction between the pulse order and treatment effects was not significant ($p>0.05$).

Figure 4.30.

Figure 4.30. Effect of LY379268 on IPSC amplitudes elicited by 10 stimulus pulses at 30 Hz. **A:** GABA_A receptor-mediated IPSCs of a neuron to 10 consecutive stimuli at 30 Hz in control solution (top trace) and after application of 20 nM LY379268 (bottom trace). **B:** Averages of the IPSC amplitudes before and after application of LY379268 were plotted as a function of the pulse order (n=10). There was not any significant effect among the pulse order ($p>0.05$). The treatment with LY379268 significantly reduced the IPSC amplitudes ($p<0.01$). There was a significant interaction between the pulse order and treatment effects ($p<0.001$). **C:** Ratios of the IPSC amplitudes (P_n) to the first IPSC amplitude (P₁) before and after application of LY379268 were plotted as a function of the pulse order (n=10). There was not any significant effect among the pulse order ($p<0.05$). LY379268 treatment significantly enhanced the IPSC ratios ($p<0.05$). The interaction between the pulse order and treatment effects was significant ($p<0.01$).

Figure 4.31.

Figure 4.31. Effect of LY379268 on IPSC amplitudes elicited by 10 stimulus pulses at 100 Hz. **A:** GABA_A receptor-mediated IPSCs of a neuron to 10 consecutive stimuli at 100 Hz in control solution (top trace) and after application of 20 nM LY379268 (bottom trace). **B:** Averages of the IPSC amplitudes before and after application of LY379268 were plotted as a function of the pulse order (n=10). There was not any significant effect among the pulse order ($p < 0.05$). LY379268 treatment significantly reduced the IPSC amplitudes ($p < 0.05$). There was a significant interaction between the pulse order and treatment effects ($p < 0.01$). **C:** Ratios of the IPSC amplitudes (P_n) to the first IPSC amplitude (P₁) before and after application of LY379268 were plotted as a function of the pulse order (n=10). There was not any significant effect among the pulse order ($p < 0.05$). The treatment with LY379268 significantly increased the IPSC ratios ($p < 0.01$). The interaction between the pulse order and treatment effects was significant ($p < 0.01$).

Figure 4.32.

Figure 4.32. Effect of LY379268 on IPSC amplitudes elicited by 10 stimulus pulses at different frequencies. **A:** Averages of the IPSC amplitudes for 5, 30 and 100 Hz stimulation frequencies before (left panel) and after application of LY379268 (right panel) were plotted as a function of the pulse order ($n=10$). The IPSC amplitudes in response to 10 consecutive stimulations were dependent on the stimulation frequency in both the control ($p<0.05$) and LY379268 solutions ($p<0.01$). The treatment significantly affected the dependency of the IPSC amplitudes to the stimulation frequency ($p<0.01$). **B:** Ratios of the IPSC amplitudes (P_n) to the first IPSC amplitude (P_1) before and after application of LY379268 were plotted as a function of the pulse order for 3 different stimulation frequencies ($n=10$). The IPSC ratios were dependent to the stimulation frequency in both the control ($p<0.05$) and LY379268 solutions ($p<0.01$). The treatment significantly changed the dependency of the ratios to the stimulation frequency ($p<0.01$).

Figure 4.33.

Figure 4.33. Effect of LY379268 on area under curve (AUC) of IPSCs elicited by 10 stimulus pulses at different frequencies. **A:** Comparison of the AUC averages before and after application of LY379268 for single pulse ($\times 10$) and trains of 10 pulses at 5, 30 and 100 Hz. There was significant difference between the AUC before and the AUC after application of LY379268 for the single pulse ($\times 10$) ($p < 0.01$) and the trains at 5 ($p < 0.01$), 30 ($p < 0.05$) and 100 Hz ($p < 0.05$). **B:** Comparison of the AUCs among the single pulse ($\times 10$) and trains of 10 pulses at 5, 30 and 100 Hz in the control and LY379268 solutions. RM-ANOVA revealed that the amount of the AUCs did not rely on the stimulation frequency ($p > 0.05$). The treatment with LY379268 had a significant effect on the reduction of the AUCs ($p < 0.01$). There was not any significant interaction between the stimulation frequency and treatment effects ($p > 0.05$).

Figure 4.34.

Figure 4.34. Effect of TBOA on AMPA receptor-mediated EPSC. **A:** AMPA receptor-mediated EPSCs of a neuron are illustrated in control solution, after applying TBOA (50 μ M), after adding LY341495 (10 nM) to the solution and after washing out TBOA and LY341495. TBOA notably decreased the EPSC amplitude. Adding LY341495 to the solution returned the EPSC amplitude back to the control level. **B:** RM-ANOVA followed by paired *t*-test revealed that the average of the EPSC amplitudes (n=10) was significantly reduced by TBOA ($p<0.001$). **C:** TBOA did not have any significant effect on the rising time, decay time and half width of the EPSCs (n=10) ($p>0.05$).

Figure 4.35.

Figure 4.35. Effect of TBOA on EPSC amplitudes elicited by 10 stimulus pulses at 5 Hz. **A:** APMA receptor-mediated EPSCs of a neuron to 10 consecutive stimuli at 5 Hz in control solution, after applying TBOA (50 μ M), after adding LY341495 (10 nM) to the solution and after washing out TBOA and LY341495. **B:** Averages of the EPSC amplitudes in the control solution and after the treatment with TBOA, TBOA+LY341495 and washing out the drugs were plotted as a function of the pulse order ($n=10$). There was a significant effect for the EPSC amplitudes among the pulse order ($p<0.001$). The treatment with the different solutions had a significant effect on the EPSC amplitudes ($p<0.001$). There was a significant interaction between the pulse order and treatment effects ($p<0.05$). **C:** Ratios of the EPSC amplitudes (P_n) to the first EPSC amplitude (P_1) before and after the treatment with TBOA, TBOA+LY341495 and after washing out the drugs were plotted as a function of the pulse order ($n=10$). There was not any significant effect for the ratios among the pulse order ($p>0.05$). The treatment with the solutions had a significant effect on the EPSC ratios ($p<0.001$). The interaction between the pulse order and treatment effects was also significant ($p<0.05$).

Figure 4.36.

Figure 4.36. Effect of TBOA on EPSC amplitudes elicited by 10 stimulus pulses at 30 Hz. **A:** APMA receptor-mediated EPSCs of a neuron to 10 consecutive stimuli at 30 Hz in control solution, after applying TBOA (50 μ M), after adding LY341495 (10 nM) to the solution and after washing out TBOA and LY341495. **B:** Averages of the EPSC amplitudes in the control solution and after the treatment with TBOA, TBOA+LY341495 and washing out the drugs were plotted as a function of the pulse order ($n=10$). There was a significant effect for the EPSC amplitude among the pulse order ($p<0.001$). The treatment with the different solutions had a significant effect on the EPSC amplitudes ($p<0.001$). There was a significant interaction between the pulse order and treatment effects ($p<0.001$). **C:** Ratios of the EPSC amplitudes (P_n) to the first EPSC amplitude (P_1) before and after the treatment with TBOA, TBOA+LY341495 and after washing out the drugs were plotted as a function of the pulse order ($n=10$). There was a significant effect for the EPSC ratios among the pulse order ($p<0.01$). The treatment with the solutions had a significant effect on the EPSC ratios ($p<0.001$). The interaction between the pulse order and treatment effects was also significant ($p<0.001$).

Figure 4.37.

Figure 4.37. Effect of TBOA on EPSC amplitudes elicited by 10 stimulus pulses at 100 Hz. **A:** APMA receptor-mediated EPSCs of a neuron to 10 consecutive stimuli at 100 Hz in control solution, after applying TBOA (50 μ M), after adding LY341495 (10 nM) to the solution and after washing out TBOA and LY341495. **B:** Averages of the EPSC amplitudes in the control solution and after the treatment with TBOA, TBOA+LY341495 and washing out the drugs were plotted as a function of the pulse order ($n=10$). There was a significant effect for the EPSC amplitudes among the pulse order ($p<0.001$). The treatment with the different solutions had a significant effect on the EPSC amplitudes ($p<0.001$). There was a significant interaction between the pulse order and the treatment effects ($p<0.001$). **C:** Ratios of the EPSC amplitudes (P_n) to the first EPSC amplitude (P_1) before and after the treatment with TBOA, TBOA+ LY341495 and after washing out the drug were plotted as a function of the pulse order ($n=10$). There was a significant effect for the EPSC ratios among the pulse order ($p<0.001$). The treatment with the solutions had a significant effect on the EPSC ratios ($p<0.001$). The interaction between the pulse order and treatment effects was also significant ($p<0.001$).

Figure 4.38.

Figure 4.38. Effect of TBOA on EPSC amplitudes elicited by 10 stimulus pulses at different frequencies. Averages of the EPSC amplitudes for 5, 30 and 100 Hz stimulation frequencies in **(A)** control solution, **(B)** after applying TBOA (50 μ M), **(C)** in TBOA+LY341495 (10 nM) solution and **(D)** after washing out TBOA and LY341495 were plotted as a function of the pulse order ($n=10$). The EPSC amplitudes in response to 10 consecutive stimulations were dependent on the stimulation frequency in the control ($p<0.05$) and wash solution ($p<0.01$) while they were not dependent on the stimulation frequency in the TBOA and TBOA+LY341495 solutions ($p>0.05$). The treatment with the different solutions had a significant effect on the dependency of the EPSC amplitudes to the stimulation frequency ($p<0.001$). The interaction between the treatment and stimulation frequency effects was significant ($p<0.01$).

Figure 4.39.

Figure 4.39. Effect of TBOA on ratios of EPSC amplitudes elicited by 10 stimulus pulses at different frequencies. Ratios of the EPSC amplitudes (P_n) to the first EPSC amplitude (P_1) in **(A)** control solution, **(B)** after applying TBOA (50 μ M), **(C)** in TBOA+LY341495 (10 nM) solution and **(D)** after washing out TBOA and LY341495 were plotted as a function of the pulse order for 3 different stimulation frequencies ($n=10$). The ratios of 10 consecutive EPSCs depended on the stimulation frequency in the control ($p<0.05$) and wash solutions ($p<0.01$) while they did not depend on the stimulation frequency in the TBOA and TBOA+LY341495 solutions ($p>0.05$). The treatment with the different solutions significantly changed the dependency of the EPSC ratios to the stimulation frequency ($p<0.01$). The interaction between the treatment and stimulation frequency effects was significant ($p<0.05$).

Figure 4.40.

Figure 4.40. Effect of TBOA on area under curve (AUC) of EPSCs elicited by 10 stimulus pulses at different frequencies. **A:** Comparison of the AUC averages in control solution, after applying TBOA (50 μ M), in TBOA+LY341495 (10 nM) solution and after washing out TBOA and LY341495 for single pulse ($\times 10$) and trains of 10 pulses at 5, 30 and 100 Hz. RM-ANOVA revealed that the treatment with the different solutions significantly changed the AUCs for single pulse ($\times 10$) ($p < 0.01$), 5 Hz ($p < 0.001$), 30 Hz ($p < 0.01$) and 100 Hz ($p < 0.001$) stimulation frequencies ($n = 10$). The significant difference between the AUCs in the control and TBOA solutions are shown by +, and the difference between the AUCs in the TBOA and TBOA+LY341495 solutions are shown by * (paired t -test, $(+ / ** \ p < 0.01, + + + / *** \ p < 0.001)$). **B:** Comparison of the AUCs among the single pulse ($\times 10$) and the trains of 10 pulses at 5, 30 and 100 Hz in the control, TBOA, TBOA+ LY341495 and wash solutions. RM-ANOVA revealed that the amount of the AUCs depended on the stimulation frequency ($p < 0.05$). The treatment with the different solutions had a significant effect on the AUCs ($p < 0.01$). There was a significant interaction between the stimulation frequency and treatment effects ($p < 0.01$).

Figure 4.41.

Figure 4.41. Effect of TBOA on the decay time of the 10th EPSC elicited by different stimulation frequencies. **A:** Comparison of the averages of the decay times of the 10th EPSC in control solution, after applying TBOA (50 μ M), in TBOA+LY341495 (10 nM) solution and after washing out TBOA and LY341495 for stimulations at 5, 30 and 100 Hz. RM-ANOVA (n=10) revealed that the treatment with the different solutions significantly changed the decay times at 5 Hz ($p<0.01$), 30 Hz ($p<0.05$) and 100 Hz ($p<0.001$). The significant difference between the decay times in the control and TBOA solutions are shown by +, and the difference between the decay times in the TBOA and TBOA+LY341495 solutions are shown by * (paired t -test, (+/* $p<0.05$, ++ $p<0.01$). **B:** Comparison of the decay times of the 10th EPSCs among the stimulation frequencies of 5, 30 and 100 Hz in the control, TBOA, TBOA+ LY341495 and wash solutions. RM-ANOVA revealed that the EPSC decay times depended on the stimulation frequency ($p<0.05$). The treatment had a significant effect on the decay times of the 10th EPSC ($p<0.01$). There was significant interaction between the stimulation frequency and treatment effects ($p<0.01$).

Figure 4.42.

Figure 4.42. Effect of TBOA on GABA_A receptor-mediated IPSC. **A:** GABA_A receptor-mediated IPSCs of a neuron are illustrated in control solution, after applying TBOA (50 μ M), after adding LY341495 (10 nM) to the solution and after washing out TBOA and LY341495. TBOA remarkably decreased the IPSC amplitude. Adding LY341495 to the solution returned the IPSC amplitude back to the control level. **B:** RM-ANOVA followed by paired *t*-test revealed that the average of the IPSC amplitudes (n=10) was significantly reduced by TBOA ($p<0.001$). **C:** TBOA did not have any significant effect on the rising time, decay time and half width of the IPSCs (n=10) ($p>0.05$).

Figure 4.43.

Figure 4.43. Effect of TBOA on IPSC amplitudes elicited by 10 stimulus pulses at 5 Hz. **A:** GABA_A receptor-mediated IPSCs of a neuron to 10 consecutive stimuli at 5 Hz in control solution, after applying TBOA (50 μ M), after adding LY341495 (10 nM) to the solution and after washing out TBOA and LY341495. **B:** Averages of the IPSC amplitudes in the control solution and after the treatment with TBOA, TBOA+LY341495 and washing out the drugs were plotted as a function of the pulse order (n=9). There was a significant effect for the IPSC amplitude among the pulse order ($p<0.001$). The treatment with the different solutions had a significant effect on the IPSC amplitudes ($p<0.001$). There was a significant interaction between the pulse order and treatment effects ($p<0.05$). **C:** Ratios of the IPSC amplitudes (P_n) to the first IPSC amplitude (P₁) before and after the treatment with TBOA, TBOA+LY341495 and after washing out the drugs were plotted as a function of the pulse order (n=9). There was a significant effect for the IPSC ratios among the pulse order ($p<0.001$). The treatment with the solutions had a significant effect on the IPSC ratios ($p<0.05$). The interaction between the pulse order and treatment effects was also significant ($p<0.05$).

Figure 4.44.

Figure 4.44. Effect of TBOA on IPSC amplitudes elicited by 10 stimulus pulses at 30 Hz. **A:** GABA_A receptor-mediated IPSCs of a neuron to 10 consecutive stimuli at 30 Hz in control solution, after applying TBOA (50 μ M), after adding LY341495 (10 nM) to the solution and after washing out TBOA and LY341495. **B:** Averages of the IPSC amplitudes in the control solution and after the treatment with TBOA, TBOA+LY341495 and washing out the drugs were plotted as a function of the pulse order (n=9). There was a significant effect for IPSC amplitudes among the pulse order ($p<0.001$). The treatment with the different solutions had a significant effect on the IPSC amplitudes ($p<0.01$). There was a significant interaction between the pulse order and treatment effects ($p<0.001$). **C:** Ratios of the IPSC amplitudes (P_n) to the first IPSC amplitude (P₁) before and after the treatment with TBOA, TBOA+LY341495 and after washing out the drugs were plotted as a function of the pulse order (n=9). There was a significant effect for the IPSC ratios among the pulse order ($p<0.001$). The treatment with the solutions had a significant effect on the IPSC ratios ($p<0.05$). The interaction between the pulse order and treatment effects was also significant ($p<0.05$).

Figure 4.45.

Figure 4.45. Effect of TBOA on IPSC amplitudes elicited by 10 stimulus pulses at 100 Hz. **A:** GABA_A receptor-mediated IPSCs of a neuron to 10 consecutive stimuli at 100 Hz in control solution, after applying TBOA (50 μ M), after adding LY341495 (10 nM) to the solution and after washing out TBOA and LY341495. **B:** Averages of the IPSC amplitudes in the control solution and after the treatment with TBOA, TBOA+LY341495 and washing out the drugs were plotted as a function of the pulse order (n=9). There was a significant effect for the IPSC amplitudes among the pulse order ($p<0.001$). The treatment with the different solutions had a significant effect on the IPSC amplitudes ($p<0.05$). There was a significant interaction between the pulse order and treatment effects ($p<0.001$). **C:** Ratios of the IPSC amplitudes (P_n) to the first IPSC amplitude (P₁) before and after the treatment with TBOA, TBOA+ LY341495 and after washing out the drugs were plotted as a function of the pulse order (n=9). There was a significant effect for the IPSC ratios among the pulse order ($p<0.001$). The treatment with the solutions had a significant effect on the IPSC ratios ($p<0.001$). The interaction between the pulse order and treatment effects was also significant ($p<0.001$).

Figure 4.46.

Figure 4.46. Effect of TBOA on IPSC amplitudes elicited by 10 stimulus pulses at different frequencies. Averages of the IPSC amplitudes for 5, 30 and 100 Hz stimulation frequencies in **(A)** control solution, **(B)** after applying TBOA (50 μ M), **(C)** in TBOA+LY341495 (10 nM) solution and **(D)** after washing out TBOA and LY341495 were plotted as a function of the pulse order (n=9). The IPSC amplitudes in response to 10 consecutive stimulations were dependent on the stimulation frequency in the control ($p<0.01$), TBOA ($p<0.001$), TBOA+LY341495 ($p<0.01$) and wash solutions ($p<0.01$). The treatment with the different solutions significantly affected the dependency of the IPSC amplitude to the stimulation frequency ($p<0.001$). The interaction between the treatment and stimulation frequency effects was significant ($p<0.05$).

Figure 4.47.

Figure 4.47. Effect of TBOA on ratios of IPSC amplitudes elicited by 10 stimulus pulses at different frequencies. Ratios of the IPSC amplitudes (P_n) to the first IPSC amplitude (P_1) in **(A)** control solution, **(B)** after applying TBOA (50 μ M), **(C)** in TBOA+LY341495 (10 nM) solution and **(D)** after washing out TBOA and LY341495 were plotted as a function of the pulse order for 3 different frequencies ($n=9$). The ratios of 10 consecutive IPSCs depended on the stimulation frequency in the control, TBOA, TBOA+LY341495 and wash solutions ($p<0.01$). The treatment with the different solutions significantly changed the dependency of the IPSC ratios to the stimulation frequency ($p<0.01$). The interaction between the treatment and stimulation frequency effects was significant ($p<0.05$).

Figure 4.48.

Figure 4.48. Effect of TBOA on area under curve (AUC) of IPSCs elicited by 10 stimulus pulses at different frequencies. **A:** Comparison of the AUC averages in control solution, after applying TBOA (50 μ M), in TBOA+LY341495 (10 nM) solution and after washing out TBOA and LY341495 for single pulse ($\times 10$) and trains of 10 pulses at 5, 30 and 100 Hz. RM-ANOVA ($n=10$) revealed that the treatment with the different solutions significantly changed the AUCs for single pulse ($\times 10$) ($p<0.001$), 5 Hz ($p<0.05$), 30 Hz ($p<0.001$) and 100 Hz ($p<0.01$) stimulation frequencies. The significant difference between the AUCs in the control and TBOA solutions are shown by +, and the difference between the AUCs in the TBOA and TBOA+LY341495 solutions are shown by * (paired t -test, (++)/** $p<0.01$, (+++)/*** $p<0.001$). **B:** Comparison of the AUCs among the single pulse ($\times 10$) and the trains of 10 pulses at 5, 30 and 100 Hz in the control, TBOA, TBOA+ LY341495 and wash solutions. RM-ANOVA revealed that the amount of the AUCs were dependent on the stimulation frequency ($p<0.001$). The treatment with the different solutions had a significant effect on the AUCs ($p<0.001$). There was a significant interaction between the stimulation frequency and treatment effects ($p<0.001$).

Figure 4.49.

Figure 4.49. Effect of TBOA on the decay time of the 10th IPSC elicited by different stimulation frequencies. **A:** Comparison of the averages of the decay times of the 10th IPSC in control solution, after applying TBOA (50 μ M), in TBOA+LY341495 (10 nM) solution and after washing out TBOA and LY341495 for stimulations at 5, 30 and 100 Hz. RM-ANOVA (n=10) revealed that the treatment with the different solutions significantly changed the decay times at the stimulation frequency of 5 Hz ($p<0.05$), 30 Hz ($p<0.01$) and 100 Hz ($p<0.05$). The significant difference between the decay times in the control and TBOA solutions are shown by +, and the difference between the decay times in the TBOA and TBOA+LY341495 solutions are shown by * (paired t -test, (+/* $p<0.05$, ++ $p<0.01$). **B:** Comparison of the decay times for the 10th IPSC among the stimulation frequencies of 5, 30 and 100 Hz in the control, TBOA, TBOA+ LY341495 and wash solutions. RM-ANOVA revealed that the IPSC decay time depended on the stimulation frequency. The treatment with the different solutions had a significant effect on the decay times of the 10th IPSC ($p<0.01$). There was not any significant interaction between the stimulation frequency and treatment effects ($p>0.05$).

5. Discussion

5.1. Membrane properties of ICC neurons

In this study, ICC neurons were categorized according to their firing patterns in responses to depolarization as well as hyperpolarization current injections. The neurons were divided into five different groups, i.e., regular, adapting, onset/transient, build-up/pauser and bursting types. The regular and bursting neurons were respectively the most and least commonly encountered cell types in the ICC. Some ICC neurons (73%) showed sags and rebound depolarization in response to negative current injection while others did not (27%). There were some differences in the R_{in} and time constant but no any difference in the RMP and AP amplitude, half-width and threshold among the different groups of the ICC neurons. The ICC neurons were morphologically categorized into two groups, bipolar and multipolar cells. The bipolar neurons had their dendritic branches parallel to the fibro-dendritic laminae while multipolar neurons had their dendritic branches across to the fibro-dendritic laminae,

These results are in agreement with those of previous studies of IC neurons (Bal et al., 2002; Koch and Grothe, 2003; Sivaramakrishnan and Oliver, 2001; Tan et al., 2007; Xie et al., 2008). In these studies, the IC neurons were categorized into sustained or onset discharge pattern under depolarization current injection. The exact percentages of neurons with a sustained firing pattern differ in a wide range among studies from 55% (Reetz and Ehret, 1999) to 82% (Bal et al., 2002). A small group of IC neurons show onset discharges, which have one spike or a burst of spikes at the beginning of the depolarizing current injection (Reetz and Ehret, 1999; Tan et al.,

2007; Xia et al., 2008). The sag and rebound were seen in both the sustained and onset neurons in the present study, and have also been frequently reported in previous *in vitro* (Koch and Grothe, 2003; Sivaramkrishnan and Oliver, 2001) and *in vivo* studies of the IC (Tan et al., 2007; Xia et al., 2008). The sag has been detected more frequently in burst and in adapting neurons than in the build-up/pauser neurons. Sivaramkrishnan and Oliver (2001) have shown the presence of six distinct firing patterns in the IC; sustained-regular, buildup/pauser, onset, rebound regular, rebound adapting and rebound transient. Interestingly, they showed a correlation between most of these firing patterns to the presence of unique potassium conductance. They reported that the build-up/pauser neurons have an A-type K^+ current, the onset neurons show a special high-threshold tetraethylammonium-sensitive current, rebound neurons express different types of calcium-dependent potassium currents, and neurons with a sustained-regular firing pattern mainly express delayed-rectifier-type potassium currents. Sivaramkrishnan and Oliver (2001) did not report bursting firing pattern, which is the only difference between the present study and their study. However, bursting neurons have also been reported in both *in vitro* (Ahuja and Wu, 2007; Bal et al., 2002) and *in vivo* studies (Tan et al., 2007; Xie et al., 2008) in the IC. Bursting neurons have been categorized into onset- and sustained- bursting groups (Tan et al., 2007). The sustained-bursting neurons which show bursting activity over a stimulation period have been reported in ICX (Ahuja and Wu, 2007), ICC (Bal et al., 2002) and whole IC (Tan et al., 2007; Xie et al., 2008). The onset-bursting neurons generate bursting activity on the beginning of the stimulation period, and like onset neurons, there is no more action potentials after that (Tan et al., 2007; Xie et al.,

2008). The bursting neurons reported in this study had an onset bursting activity which could be followed by a few action potentials, and were slightly different from the onset- or sustained- bursting neurons reported elsewhere. Bal et al. (2002) reported a group of neurons in the ICC (14%) with a bursting like activity on the beginning of the positive current injection followed by a few spikes, and a burst of APs on the rebound following negative current injection. The firing pattern of those neurons is very similar to the firing pattern of the bursting neuron in this study, however, those neurons were categorized as irregular adapting. It is believed that burst activity in the IC neurons is related to the after depolarization that is probably a manifestation of calcium spikes (Xie et al., 2008).

5.2. Morphology of ICC neurons

In this study, the recordings were made from ICC neurons regardless of their sizes and shapes. Patch clamp recording from large neurons is generally easier than from small ones. The regular neurons were the most popular cell type in this study. They had larger R_{in} compared to the other types of neurons. Because of a reverse relationship between the R_{in} and the size of a cell, the regular neurons may be relatively smaller than the other neurons. To test if there was any relationship between the electrophysiological responses and the morphology of ICC neurons, a few neurons whose firing patterns were identified, were stained with Lucifer Yellow. The morphology of these neurons was either bipolar or multipolar type. The cell morphology in three-dimensional axes was not further analyzed because the recordings were made from neurons near the surface of a thin slice, and part of their

dendritic tree in Z-axis (rostral to caudal) might have been sectioned. Therefore, it is difficult to conclude that the bipolar and multipolar cells found in this study correspond to “flat” or “non-flat” cells that are classified according to the orientation and thickness of the dendritic tree (Malmierca et al., 1993; Oliver and Morest, 1984; Peruzzi et al., 2000). Nevertheless, no correspondence between the intrinsic membrane properties and two morphological types (i.e. bipolar and multipolar types) of the neurons could be found in this study. Very similar results were also obtained in previous studies (Bal et al., 2002; Wagner, 1994).

5.3. Synaptic responses in ICC

In the present study, both AMPA and GABA_A receptor-mediated responses could be elicited by stimulation of the LL. The current-voltage relationships for AMPA receptor-mediated EPSC and GABA_A receptor-mediated IPSCs were linear, which suggest that AMPA and GABA_A receptors are not voltage dependent. These results are in agreement with previous studies in ICC (Ma et al., 2002 a and b; Sun et al., 2006; Wu et al., 2004).

The results of this study showed that paired-pulse stimulation (with 200 ms interval) of LL could produce two individual EPSCs or IPSCs, with the first response mostly larger than the second one. In my pilot study (data not shown), the results showed that there was paired-pulse depression (PPD) for the EPSC of most neurons in response to paired-pulse stimulation at 10, 20, 33, 50, 100, 200 and 400 ms intervals while PPD was found for the IPSC of most neurons to stimulations

separated by >50 ms. With paired-pulse stimulation at 10-50 ms intervals, the ICC neurons mostly showed paired-pulse facilitation (PPF) for the IPSC.

In general, the generation of PPF is likely due to residual calcium in the presynaptic terminals, which leads to release of more neurotransmitter in response to the second pulse while multiple mechanisms can contribute to PPD (Zucker and Regehr, 2002). These mechanisms involve postsynaptic and presynaptic events. The common presynaptic mechanism is the decrease in neurotransmitter release due to depletion of a release-ready pool of vesicles (von Gersdorff et al., 1997), modification of calcium current (Bellingham and Walmsley, 1999; Forsythe et al., 1998) and activation of presynaptic metabotropic receptors (Barnes-Davies and Forsythe, 1995; von Gersdorff et al., 1997) as well as presynaptic ionotropic receptors (Zucker and Regehr, 2002).

One possibility for the PPD is inactivation of Ca^{2+} channels. Presynaptic Ca^{2+} channels can go to inactivation state after the first stimulus pulse. When the second pulse arrives, Ca^{2+} channels would be inactive, and consequently less glutamate (Forsythe et al., 1998) or GABA (Kirischuk et al., 2002) would be released. The results in this study showed PPD for the EPSC and IPSC in both normal and low Ca^{2+} mediums, however, the degree of PPD was less obvious in the low Ca^{2+} medium. This result suggests that the PPD is Ca^{2+} dependent. Lowering extracellular Ca^{2+} concentration, which is known to decrease Ca^{2+} influx to presynaptic terminals and reduces transmitter release, caused reduction of the EPSC and IPSC amplitudes, and significantly reduced the extent of the PPD measured as the ratio of the 2nd to the first response amplitudes. It has been suggested that Ca^{2+} entering into the presynaptic

terminals is involved in two different processes. It first triggers the transmitter release, and then leads to activation of a process which transiently inhibits the vesicle release. Therefore, when the second AP arrives at a terminal in a specific time period, less transmitter would be released, and the second response would be smaller than the first one (i.e. PPD) (Bellingham and Walmsley, 1999). In the low Ca^{2+} medium, it was likely that less Ca^{2+} entered into the terminal. Therefore, Ca^{2+} had less power to inhibit the vesicle release for the second pulse. Therefore, the decrease in the second response was less than that in the first one, and consequently there was less PPD. Therefore, it is very possible that observed PPD in this experiment was related to the modulation of Ca^{2+} current.

The other possible mechanism of PPD is activation of metabotropic receptors (Zucker and Regehr, 2002). Evidence from previous studies in the ICC suggests that activation of presynaptic GABA_B receptors can suppress the IPSC (Ma et al., 2002a) and EPSC (Wu et al., 2004) by reducing transmitter release, but there is not any postsynaptic GABA_B receptor involved (Ma et al., 2002a; Wu et al., 2004). In the present study, both AMPA and GABA_A receptor-mediated responses could be elicited by stimulation of the LL. Thus, GABA molecules were probably released while recording EPSCs even though the GABA_A receptors were blocked. If GABAergic and glutamatergic terminals were located in close proximity to each other on the neurons from which recordings were made, it was possible that GABA molecules reached glutamate terminals to activate presynaptic GABA_B receptors, thus reducing glutamate release. Also, during recordings of GABAergic IPSCs, GABA molecules could probably affect presynaptic GABA_B receptors on GABAergic

terminals. However, it is unlikely that the PPD observed in this study was due to the presynaptic GABA_B receptor activation because a single pulse of stimulation (i.e. the first pulse) may not be able to release many of GABA molecules to reach GABA_B receptors and lead to depression of the second responses. Actually, it has been proposed that GABA_B receptors are located in extrasynaptic sites, and their activation depends on GABA spill-over, which can be induced by high frequency stimulation (Kulik et al., 2003). It was found that the properties of the IPSC and EPSC induced in the ICC neurons by single pulse of stimulation or paired- or multi-pulses of stimulations at 5-200 Hz did not change by blocking of the mGluR (data not shown). Therefore, it seems that mGluR did not have any remarkable action on the PPD observed in this experiment. Since there is no study about the effect of activation of the other kind of metabotropic receptors (such as adenosine and serotonin) or presynaptic ionotropic receptors on synaptic responses in the ICC, the effect of those receptors on the PPD or even the PPF can not be completely ruled out.

The postsynaptic mechanism for PPD is usually receptor desensitization (Jones and Westbrook, 1996). Although AMPA receptor desensitization has been reported to play a role in synaptic depression in auditory neurons (Zhang and Trussell, 1994), this type of postsynaptic modification would not likely explain the PPD of the EPSCs observed in this study. AMPA receptors recover very fast from the deactivation and desensitization states. For example; Trussell et al. (1993) reported that AMPA receptor desensitization in auditory chick nucleus magnocellularis was almost fully recovered 60 ms after exposure to glutamate of 3 mM for 5 ms. The stimulus parameters used in this study were 200 ms interpulse interval and 0.1 ms for

each pulse. Therefore, the possibility that smaller EPSC to the 2nd pulse was due to desensitization of AMPA receptors is very low. GABA_A receptor desensitization has also been shown to modulate GABAergic responses (Jones and Westbrook, 1996), however, the possible desensitization of GABA_A receptor also unlikely accounts for the PPD observed in this study (the reason is explained in the next part). Taking above discussion into account, it is suggested that the generation of PPD is due to modulation of Ca²⁺ influx at presynaptic terminals in the ICC.

5.4. Temporal integration of synaptic responses

The results showed that in the control solution, the EPSC and IPSC could follow the individual pulses of repetitive stimulation over the range of 5, 30 and 100 Hz. The temporal summation of the EPSC could be seen at 100 but not at 5 and 30 Hz stimulation frequencies. The temporal summation for the IPSC could be found at both 30 and 100 Hz stimulation frequencies. The EPSCs showed depression during repetitive stimulation of 5, 30 and 100 Hz. The IPSCs also showed depression with 5 and 30 Hz stimulation frequencies but facilitation with 100 Hz stimulation frequency. In this study, GABA_A receptors-mediated responses had longer rise time, decay time and half-width than AMPA receptors-mediated responses (Fig. 4.34.C and 4.42.C). These differences probably explain the facilitation of the IPSC at high frequency stimulation (Wu et al., 2004).

The amplitudes and ratios of both the EPSC and IPSC over a ten pulse train were dependent on the stimulation frequency. The charge transfer across the membrane (measured as AUC) for the EPSC was gradually decreased by increasing

the stimulation frequency. The charge transfer across the membrane for the IPSC at the single ($\times 10$), 30 and 100 Hz stimulation frequencies was more than that at 5 Hz stimulation frequency. The AMPA receptor-mediated EPSC showed frequency-dependent suppression during repetitive stimulation in this study. The dependency of AMPA response depression on the stimulation frequency has been reported throughout the CNS (Andresen and Yang, 1995; Brenowitz et al., 1998; Trussell, et al., 1993; von Gersdorff et al., 1997) and well documented in the ICC *in vitro* (Wu et al., 2004). The modulation (depression or facilitation) of GABA_A receptor-mediated responses over the repetitive stimulation has also been shown to depend on the stimulation frequency in the ICC (Wu et al., 2004) as well as in other region of the brains (Andresen and Yang, 1995; Grabauskas and Bradley, 2003; Pugh and Raman, 2005). As it was discussed in the previous part, the synaptic depression can be due to the presynaptic and/or post synaptic mechanisms. One possible reason of the synaptic depression is the depletion of the readily-releasable vesicles pool in consequence of the repetitive stimulation. A considerable amount of the ready-releasable vesicles discharge in response to the first AP. As the second AP arrives at the terminal in a certain time course, there are not many ready-releasable-vesicles to discharge, producing the smaller size of synaptic response in the postsynaptic neuron. The degree of the depression depends on the amount of the vesicles in the reserve pool and the release-ready pools, and the transition rates between these pools (Zucker and Regehr, 2002). Repetitive stimulation can lead to a decrease in the number of readily-releasable vesicles for subsequent stimuli and consequently depression of the successive responses. Changes in Ca^{2+} influx in presynaptic terminals can also

account for the depression of the synaptic responses during the repetitive stimulation (Forsythe et al., 1998; Kirischuk et al., 2002). Another possibility of the depression is the fatigue of the axons during the repetitive high frequency stimulation. This fatigue is a result of K^+ build-up or Na^+ channel inactivation during the stimulation (Fedchyshyn and Wang, 2007). The activation of presynaptic metabotropic receptors such as mGluRs, GABA_B and serotonin was also suggested to contribute to synaptic depression induced by repetitive high frequency stimulation (Zucker and Regehr, 2002). It was found that application of LY341495 at 100 nM did not have any significant effect on the EPSCs and IPSCs induced by repetitive stimulation (at 5-200 Hz) in the ICC neurons (data not shown), suggesting that the synaptic depression is unlikely due to the activation of the mGluRs II.

AMPA and GABA_A receptor desensitization can lead to depression of the synaptic responses with high frequency stimulation (Kraushaar and Backus, 2002; Trussell et al., 1993). It has been shown that complete recovery of GABA_A receptor desensitization in the IC takes a few seconds and the recovery is almost 50% after 200 ms when the receptors are exposed to 1 mM GABA for 1 ms (Kraushaar and Backus, 2002). However, Pugh and Raman (2005) showed that if the GABA concentration transients are at 100 μ M or less, synaptic currents may not show obvious desensitization. In this study, IPSCs showed depression at 5 Hz but temporal summation and facilitation at higher frequencies. The type of modification of GABAergic IPSCs in this study was very similar to the changes in the responses induced by repetitive administration of 30 μ M GABA at the same frequency range in Pugh and Raman's study (2005). Thus, each of the electrical pulse probably induced a

release of GABA of a concentration of about 30 μM at synaptic cleft. Therefore, it is unlikely that multi pulse depression or PPD of the GABA_A receptor-mediated IPSCs in this study was attributed to desensitization of GABA_A receptors.

GABA_A receptor-mediated responses showed facilitation at medium and high frequency stimulations in the ICC neurons. There are a couple of main mechanisms that could explain the facilitation of IPSCs observed in this experiment. Facilitation is usually caused by the residual Ca^{2+} remains in the nerve terminals after the arrival of the first pulse. The residual Ca^{2+} adds to the Ca^{2+} entering the terminal following the subsequent stimuli, and leads to more transmitter release, and consequently a larger response (Zucker and Regehr, 2002). Another possibility is formation of “cloud” of neurotransmitter in the extra-synaptic space by the repetitive stimulation of presynaptic terminals. This “cloud” can lead to the activation of extra-synaptic receptors and induce a larger response. The cloud of neurotransmitter induced by repetitive stimulation has a grater gradient in the synaptic cleft than when it is induced by single stimulation. The cloud can activate the receptors for a longer time because it takes more time for the cloud to be removed from the cleft. Longer activation of the receptors can lead to temporal integration and facilitation by increasing the decay time of the postsynaptic responses for subsequent stimuli (Roepstorff and Lambert, 1994).

5.5. Effect of mGluRs activation on synaptic responses

With application of the mGluR agonists and antagonist, the role of metabotropic glutamate receptors in synaptic transmission in the ICC was explored

for the first time. The data from this study showed that mGluRs of only group II, not group I or III, have physiological actions on the ICC neurons. The mGluR II activation did not affect intrinsic membrane properties of the ICC neurons but substantially suppressed synaptic responses mediated by AMPA and GABA_A receptors. With morphological data, it was found that fast glutamatergic and GABAergic synaptic transmission can be modulated by mGluR II in both the bipolar and multipolar ICC neurons.

The results demonstrated that ACPD and LY379268 significantly reduced the EPSCs and IPSCs evoked by stimulation of the LL. LY379268 is an agonist for mGluRs II, which produced comparable reduction in the amplitudes of the EPSCs and IPSCs by ACPD, the agonist for both the group I and II mGluRs. The group I agonist, DHPG, had no effect on the EPSCs and IPSCs. Thus, the depression produced by ACPD appears to result from activation of group II, not group I mGluRs. Similar suppressive effects on glutamatergic and GABAergic responses by activation of mGluR II have been observed in a number of brain tissues including striatum (Pisani et al., 2002), hippocampus (Price et al., 2005), thalamus (Alexander and Godwin, 2005 and 2006), superior colliculus (Neal and Salt, 2006), and amygdala (Muly et al., 2007). The results of the present study suggest that the fast excitatory glutamatergic and inhibitory GABAergic transmission in the ICC can be modulated by mGluR II because 1) the reductions of EPSC and IPSC by ACPD and LY379268 were dose dependents. 2) the reduction of the responses was reversible (although the recovery from LY379268 was much slower than the changes of the response by LY379268). 3) the reduction by LY379268 was blocked by the mGluR II antagonist,

LY341495 at 10 nM (the concentration with which only mGluR II, not I and III, receptors can be blocked (Johnson et al., 1999)).

To test the effect of the mGluR I and III activations on the synaptic responses in the ICC, DHPG and SOP at 100 μ M were used, respectively. This concentration for DHPG is at least 2-5 times its EC₅₀ for mGluR I. The concentration for SOP is high enough for activation of most receptor types in mGluR III (Cartmell and Schoepp, 2000). At these concentrations neither drugs had a significant effect on the EPSCs and IPSCs of the ICC neurons. Therefore, it is concluded that group I and III mGluRs play little or no role in modulation of synaptic transmission in the ICC.

The physiological effect of mGluRs found in this study may be explained by the histological studies on the presence of these receptors in the ICC. Immunoreactivity and mRNA for all 3 groups of mGluRs have been detected in the IC. But the expression of 3 groups of mGluRs and different types of mGluRs in each group in the IC varies. There is also a different distribution of each type of mGluRs in different regions of the IC. Strong expression of mGluR5 was observed only in the shell region of the IC, perhaps the dorsal and external nuclei of the IC (Ferraguti and Shigemoto, 2006; Romano et al., 1995; Shigemoto et al., 1993). There was scattered distribution of the mRNA for mGluR1 in the IC (Shigemoto et al., 1992). The results in this study did not show any effect of the agonist of mGluRs I (mGluR 1 and 5) on membrane properties and synaptic responses of ICC neurons, which is consistent with the scattered distribution of the mRNA for mGluR I in the ICC.

Low to moderate immunolabeling and [³H] LY341495 binding for mGluR2/3 were found in the IC (Petrálie et al., 1996; Wright et al., 2001). The moderate

labeling of mGluR2/3 may correspond to presynaptic mGluRs II on glutamatergic and GABAergic terminals. However, several immunohistochemical and *in situ* hybridization studies detected only mGluR3 but not mGluR2 in the IC (Ohishi et al., 1993a, 1993b and 1998; Tamaru et al., 2001). In the present study, it could not be distinguished which type of mGluRs II influenced the synaptic responses. More specific agonist or antagonists are required to determine the type of mGluRs in group II involving synaptic regulation in the IC in the future. Amongst group III mGluRs, distribution of mGluR7 is the most extensive in the central nervous system (Ferraguti and Shigemoto, 2006). Kinzie et al. (1995) detected low levels of mGluR7 mRNA in the IC. However, immunoreactivity for mGluR7a and mGluR7b (two subgroup of mGluR7) in the IC was negative (Kinoshita et al., 1998). The results in the present study did not detect any notable effect of the agonist of mGluR III on membrane properties or synaptic responses of ICC neurons, suggesting little or no physiological role of mGluR III in the ICC.

5.6. Presynaptic mechanism of mGluR II

The results in this study showed that activation of the mGluR II decreased the PPD for both EPSC and IPSC induced by paired-pulse stimulation at 200 ms interval in both the normal and low Ca^{2+} mediums. Similar results have been shown in other studies in various regions of the brain (nucleus tractus solitarii: Chen and Bonham, 2005; dorsolateral bed nucleus: Grueter and Winder, 2005; somatosensory cortex: Mateo and Porter, 2007; superior colliculus: Neale and Salt, 2006; hippocampus: O'Leary et al., 1997; Price et al., 2005). Many of those studies have shown that

inhibitory action of mGluR II on AMPA and GABA_A receptor-mediated responses is through presynaptic mechanism (e.g., Neal and Salt, 2006; Price et al., 2005). One of the most popular mechanism by which mGluR II has been shown to have their effects on neurotransmitter release is through suppression of high-threshold Ca^{2+} conductance (Anwyl, 1999). Recordings from giant synaptic terminals, the calyx of Held synapses, in the medial nucleus of the trapezoid body have provided direct evidence of suppression of the P/Q type of Ca^{2+} conductance by activation of mGluRs on presynaptic site (Takahashi et al., 1996). In the present study, similar to lowering Ca^{2+} concentration, the mGluR II agonist, LY379268, also reduced the degree of the PPD while depressed both the first and second responses in paired-pulse protocols. It seems that this effect was mediated by modulating Ca^{2+} current. As it is discussed earlier, Ca^{2+} has two actions on neurotransmitter release. Ca^{2+} induces the transmitter release for the first pulse, and then triggers the process that transiently inhibits synaptic vesicle release in response to the 2nd presynaptic action potential, and consequently lessens the PPD (Bellingham and Walmsley, 1999). Activation of mGluR II probably decreased the influx of Ca^{2+} , and consequently decreased the amplitudes of paired-synaptic responses induced by paired-pulse stimulations. Because of less Ca^{2+} influx induced by the first pulse, there was less inhibition of the vesicle release for the second pulse, and consequently increase in the P2/P1 ratio. The high correlation between the reduction in the amplitude of the 1st EPSC or IPSC and the increase in the ratio of the 2nd to 1st EPSC or IPSC during application of LY379268 is another piece of evidence that suggests the role of Ca^{2+} in this phenomenon. This correlation means that when there was more suppression of the

first response because of less entering of Ca^{2+} , there was less inhibition of the second response due to less inhibitory action of Ca^{2+} on vesicle release for the second response. In low Ca^{2+} medium, there was the same scenario. Low Ca^{2+} medium led to decrease in Ca^{2+} influx, and mGluR activation further decreased Ca^{2+} influx, and consequently reduced not only the neurotransmitter release for the first pulse but also the vesicle release inhibition for the second pulse, and consequently increased the P2/P1 ratio.

Taking above discussion into account, this study suggests the presynaptic origin of mGluR II-mediated response inhibition. First, there was no indication of direct effects of mGluR II on the postsynaptic membrane; viz., ACPD did not affect any intrinsic membrane properties of the ICC neurons. Second, comparable decrease in the PPD in conjunction with the decrease in the single AMPA- and GABA_A receptor-mediated responses by the mGluR II agonist. Third, the effects of lowering extracellular Ca^{2+} were similar to the effects of LY379268 on paired-pulse depression of AMPA receptor-mediated EPSCs and GABA_A receptor-mediated IPSCs. All of these results support the idea that mGluRs II serve as autoreceptors on glutamatergic terminals and heteroreceptors on GABA_A terminals to regulate transmitter release in the IC.

5.7. Effects of mGluR II activation on temporal integration of synaptic responses

The role of mGluR II activation in temporal characteristics and short-term plasticity of AMPA and GABA_A receptor-mediated responses in the ICC was examined in this study. The results showed that the mGluR II agonist, LY379268,

significantly reduced the amplitudes of both the EPSCs and IPSCs at 3 different frequencies, however, there was more reduction for the first few responses compared to the last few ones at higher frequencies. The temporal summation of responses was still obvious for the EPSC and IPSC after application of LY379268 though the summation was attenuated. LY379268 reduced the depression of the EPSCs induced by stimulation at 5, 30 and 100 Hz, and the depression of the IPSCs induced by stimulation frequency of 5 Hz. LY379268 increased the facilitation of the IPSCs induced by stimulation frequency of 100 Hz, and turned the depression to facilitation of the IPSCs induced by stimulation frequency of 30 Hz. The EPSC amplitudes and ratios (P_n/P_1) over the ten pulse train were not dependent on the stimulation frequency any more after application of LY379268 while the dependency of the IPSC amplitudes and ratios (P_n/P_1) to the stimulation frequency was dramatically modulated. The charge transfer across the membrane for both the EPSC and IPSC reduced following the application of LY379268 at the single pulse ($\times 10$), 5, 30 and 100 Hz stimulation frequencies, however, there was less reduction for higher than lower frequency stimulations.

These findings suggest that mGluR II activation had different effects on GABA and AMPA receptor-mediated responses, depending on the stimulation frequency. This finding is in agreement with previous studies. For example, it has been found that mGluR II activation can depress EPSPs induced by repetitive stimulation in dentate gyrus but the degree of the depression of the consecutive EPSPs depends on stimulation frequency. The mGluR II activation equally depresses all consecutive EPSPs in low frequency stimulation while there is less depression on

the last few EPSPs in high frequency stimulation (Kilbride et al., 2001). Frequency-dependent modulation of synaptic responses has also been reported following activation of other kinds of metabotropic receptors such as GABA_B (Brenowitz et al., 1998) and muscarinic ACh receptors (Pennartz and Lopes Da Silva, 1994). The modulation of the EPSCs and IPSCs over a train of stimulation by mGluR II activation in the ICC was probably due to a presynaptic mechanism (comprehensively explained in the previous part). One of the most accepted mechanism by which mGluR II have been shown to have their effects on neurotransmitter release, is through modulation of calcium influx (Anwyl, 1999). Barnes-Davies and Forsythe (1995) found that activation of mGluR, like decreasing extracellular Ca²⁺, leads to depression of the 1st EPSCs in MNTB with less depression or even facilitation on the subsequent EPSCs in a train of stimulation. The similar result has also been reported in dentate gyrus. Kilbride et al. (2001) found that the effect of lowering extracellular Ca²⁺ on EPSPs induced by a train of stimulation is very similar to the effect of mGluR II activation on the EPSPs in the dentate gyrus. They concluded that the modulation of responses over the train of stimulation by the mGluR II is likely mediated by inhibition of Ca²⁺ influx. Therefore, it is very possible that frequency-dependency of the EPSC and IPSC modulations observed in this experiment was due to depression of Ca²⁺ influx triggered by the mGluR II activation.

The question is why at different stimulation frequencies, the effect of the mGluR II activation on the EPSCs was different from the effect on the IPSCs. In other words, why the mGluR II activation removed the frequency dependency of the EPSC modulations but changed the frequency dependency of the IPSC modulations.

This difference is probably related to the nature of AMPA and GABA_A receptor-mediated responses by repetitive stimulation. In control condition, there was always depression ($P_n/P_1 < 1$) for the EPSCs over a stimulation train (less depression for low frequency and more depression for high frequency stimulations). The mGluR II activation had the most effect on the first EPSC at all stimulation frequencies, and almost evenly suppressed following EPSCs at the low frequency stimulation but had very small effect on the last few EPSCs at the high frequency stimulation. Therefore, there was a small decrease in the depression (i.e. increase in P_n/P_1) of the EPSCs at the low frequency stimulation and a big decrease in the depression (i.e. a remarkable increase in P_n/P_1) of the last few EPSCs at the high frequency stimulation. As a result, the depression effect at the low frequency stimulation became comparable to the depression effect at the high frequency stimulation following the mGluR II activation, thus the depression effect was not frequency-dependent any more.

In control solution, the IPSC changes over a stimulation train were bidirectional, depressive or facilitative, depending on the stimulation frequency. The effect of the mGluR II activation on the IPSCs was the same as that on the EPSCs (i.e. the most effect on the first response, a medium effect on the subsequent IPSCs at the low frequency stimulation, and very small effect on the last few responses at the high frequency stimulation). Therefore, there was a small decrease in the depression (i.e. increase in P_n/P_1) of the IPSCs at the low frequency stimulation but a big increase in the facilitation (i.e. a huge increase in P_n/P_1) of the IPSCs (especially the last few ones) at the high frequency stimulation. Therefore, the modulation of the IPSCs over a train was still bidirectional and frequency-dependent after the mGluR II activation.

5.8. Effects of glutamate transporter blocker on synaptic responses

The mGluRs II are located more distantly from synaptic active zone. Therefore, they require glutamate spill-over for physiological activation (Petrálie et al., 1996; Scanziani et al., 1997). My pilot study tried to activate mGluR II receptors by endogenous glutamate in the ICC. It is believed that the spill-over can be produced by sustained repetitive activity at a synapse (Knopfel and Uusisaari, 2007). Therefore, the LL was stimulated with repetitive stimulations (up to 20 pulses at 30-200 Hz), and synaptic responses were recorded. Then, mGluR II antagonist, LY341495 (100 nM) was applied, and the responses before and during application of the antagonist were compared. Statistical analysis showed no significant difference between the responses before and during LY341495 application (data not shown). Several reports also showed little or no endogenous activation of mGluR II in several brain areas including midbrain periaqueduct gray (Drew et al., 2008), olfactory bulb (Ennis et al., 2006) entorhinal cortex (Iserhot et al., 2004) and calyx of Held synapses (Renden et al., 2005). However, Voytenko and Galazyuk (2008) found endogenous activation of mGluRs in IC. In the present study, the failure of activating the mGluRs by endogenous glutamate might be caused by the use of inappropriate stimulation for inducing glutamate spill-over. Another possibility is sectioning of many afferents to the ICC neurons when cutting thin brain slices, thus the stimulation could not lead to the release of enough glutamate to spill over and activate the mGluR II. The failure could also be due to the temperature of bathing solution (29-30°C), which was used in this experiment, and was lower than physiological temperature of about 37°C. Since it has been shown that lowering the temperature can reduce the extent of

neurotransmitter release from terminals (Yang et al., 2005), thereby reducing the possibility of transmitter spill-over. Nevertheless, mGluR II can be activated by endogenous glutamate after inhibition of glutamate transporters. It is believed that without blocking of the glutamate transporters, glutamate spill-over from one single synapse after a single stimulus pulse can not activate mGluRs, (Knopfel and Unsisaari, 2007). To test if mGluR II could be activated by endogenous glutamate in the ICC, glutamate transporters were blocked by TBOA, and synaptic responses were then recorded. It was found that TBOA led to suppression of the EPSC and IPSC amplitudes with no effect on their kinetics. The suppression of the synaptic responses could be removed by blocking the mGluR II. It seems that TBOA led to spill over of glutamate from glutamatergic terminals. The glutamate spilled-over could reach and activate the mGluRs II on glutamatergic and adjacent GABAergic terminals, and consequently inhibited neurotransmitter releases through presynaptic mechanism. These results are in agreement with the finding in calyx of Held synapses (Renden et al., 2005), periaqueductal gray (Drew et al., 2008), hippocampus (Fitzsimonds and Dichter, 1996) and entorhinal cortex (Iserhot et al., 2004). The question is how TBOA could affect a single pulse response. The answer might be that there are spontaneous releases of glutamate from glutamatergic terminals in the ICC (Tan et al., 2007). It seems that in the absence of glutamate transporters, the spontaneous release of glutamate could provide enough glutamate molecules to spill over and reach the mGluRs II on both the glutamatergic and GABAergic terminals, and activate them, inhibiting the neurotransmitter release and consequently reducing synaptic responses.

5.9. Effects of glutamate transporter blocker on temporal integration of synaptic responses

Using repetitive stimulation, it was found that TBOA could reduce the amplitudes of the consecutive EPSCs and IPSCs at different stimulation frequencies. The reduction of the amplitudes was more obvious for the first few responses in a train. During the repetitive stimulations, the modulation of the EPSCs and IPSCs by TBOA was comparable to the modulation of the responses by applying mGluR II agonist, LY379268, though there were some differences at medium and high frequencies for the EPSCs. In control solution, temporal summation of the EPSCs could be found only at the high frequency stimulation (i.e. 100 Hz). But summation of the responses was observed not only at high frequency but also at the medium frequency (i.e. 30 Hz) stimulations when glutamate transporters were blocked. The EPSC amplitudes and ratios of P_n/P_1 over a ten pulse train were not dependent on the stimulation frequency after application of TBOA, no matter the mGluRs II were blocked or not. The modulation of the charge transfer across the membrane for the EPSCs after TBOA depended on the stimulation frequency. The charge transfers were decreased, not changed and increased by TBOA depending on the stimulation frequency (for the single pulse, 5 Hz and 30 and 100 Hz stimulations, respectively). While blocking the mGluR II, TBOA dramatically increased the charge transfer for all stimulation frequencies compared to when the mGluRs II were active. TBOA also produced long EPSCs when evoked by medium to high frequency stimulations in the ICC neurons. But the EPSC extension (measured as the decay time for the 10th

response) was reduced and limited by activation of the mGluRs II. The extension of the IPSCs was not observed after TBOA application.

These results imply that inhibition of glutamate uptake caused a large “cloud” of glutamate. This cloud could repeatedly rebind to AMPA receptors and lead to extension of the EPSCs while had no effect on the duration of GABA_A receptors-mediated IPSC induced by high frequency stimulations. It has been shown that glutamate transporter blocker can extend the EPSC induced by 30-100 Hz stimulation frequencies in auditory avian nucleus magnocellularis (Turecek and Trussell, 2000). It has been suggested that glutamate transporters are responsible to limit the activation of postsynaptic receptors, and sustain glutamate concentration at low level in synaptic clefts (Palmer et al., 2003). The glutamate concentration in the synaptic cleft can reach millimolar levels by physiological activation of glutamatergic terminals. Glutamate is then quickly cleared within a millisecond (or less) to concentrations far below the binding constant of glutamate receptors. The clearance of glutamate is mainly resulted from diffusion into the surrounding extracellular space and reuptake by the glutamate transporters (Knopfel and Unsisaari, 2007). It is believed that the spill-over takes place when a neurotransmitter diffuses and activates those receptors (such as mGluR II) which are not located in the active zone (Pugh and Raman, 2005). Renden et al. (2005) recorded Ca²⁺ current from presynaptic terminal in MNTB while blocking glutamate transporters. They found that Ca²⁺ current was significantly reduced by TBOA. This reduction was reversed by application of the mGluR II antagonist, LY341495. They suggested that activation of presynaptic

mGluR II, induced by glutamate spill-over, causes inhibition of the Ca^{2+} influx and consequently reduction of neurotransmitter release.

The effect of TBOA found in this experiment could not be related to a postsynaptic mechanism. First, no change in the membrane potential or input resistance of the ICC neurons was observed following TBOA application. Second, the effect of TBOA on GABA_A receptor-mediated IPSC in responses to single and repetitive stimulations was completely comparable to the effect of mGluR II agonist, LY379268 (discussed in previous parts). Therefore, TBOA modulated the synaptic responses of the ICC neurons through the activation of presynaptic mGluR II.

It was shown that there was a direct relationship between the stimulation frequency and the duration of the EPSC induced by repetitive stimulation. By increasing the stimulation frequency, the duration of the EPSC increased, and this increase was enormous in the absence of active glutamate transporters. This result suggests that the transporter activity is critical for removing of glutamate. In addition, physiological activation of the mGluRs depends on the efficiency of the transporters and the stimulation frequency. In other words, the mGluRs can be activated when there is ambient extrasynaptic glutamate concentration due to high frequency stimulations and/or limited activity of the transporters.

5.10. Function of mGluRs in ICC

Physiological function of mGluRs has been investigated in neurons of various structures along the auditory pathway. A variety of effects of activation of the mGluRs has been observed in different auditory areas. For example, mGluRs act only

presynaptically, modulating transmitter release in principal cells of the MNTB and LSO (Barnes-Davies and Forsythe, 1995, Wu and Fu, 1998). However, in the DCN, activation of group I mGluRs leads to synaptic depression between parallel fiber and cartwheel cells, suggesting postsynaptic action (Molitor and Manis, 1997). Postsynaptic mGluRs are also found to depolarize neurons in the developing LSO (Kotak and Sanes, 1995) and the ventral division of the MGB, and to enhance the neuron's membrane excitability (Schwarz et al., 2000). In the primary auditory cortex, mGluRs not only regulate transmitter release presynaptically, suppressing glutamatergic and GABAergic transmission but also influence synaptic transmission by acting postsynaptically (Bandrowski et al., 2002). The results from the present study strongly suggest that in the ICC, only presynaptic mGluRs contributes to regulating release of glutamate and GABA, and there might be little expression of functional postsynaptic mGluRs. It is also suggested that the mGluR II are involved in temporal summation and short term plasticity in the ICC neurons.

It appears that in the ICC the physiological role of mGluRs is very similar to that of the metabotropic type of GABA receptors, i.e., GABA_B receptors. Previous studies suggest that GABA_B receptors exert their action presynaptically in the ICC. Activation of GABA_B receptors suppresses EPSCs mediated by AMPA and NMDA receptors, and suppresses IPSCs mediated by GABA_A receptors but does not affect intrinsic membrane properties of the ICC neurons (Ma et al., 2002a, Sun et al., 2006). Therefore, it is suggested that mGluR II along with GABA_B receptors are present presynaptically on both the GABAergic and glutamatergic terminals in the ICC (Fig. 5.1). The fact that both the excitatory and inhibitory synaptic transmission can be

modulated by either GABA_B receptors or mGluRs indicates that both the excitatory and inhibitory synaptic transmission in the ICC may require multiple mechanisms in order to balance the level of excitation and inhibition. Interestingly, in avian cochlear nucleus magnocellular neurons, glutamatergic transmission is not subject to modulation by mGluRs but is regulated by GABA_B receptors; whereas GABAergic transmission is regulated by mGluRs (Brenowitz and Trussell, 2001; Brenowitz et al., 1998; Lu, 2007). The significance of differential distribution of functional mGluRs and GABA_B receptors in different neurons (or in different regions or different species) is not well understood. Nevertheless, in the ICC, the two types of metabotropic receptors play similar roles in synaptic modulation. The ICC is thought to be an integration centre, and it receives excitatory and inhibitory inputs from various structures of the lower auditory brainstem (Kelly and Caspary, 2005). The balanced excitation and inhibition in the ICC is critical not only for appropriate processing of incoming auditory information but also for preventing pathological activity, such as audiogenic seizures (Faingold, 2005). To maintain the balance between excitation and inhibition in an ICC neuron, presynaptic mGluR II and GABA_B receptors on both the glutamatergic and GABAergic terminals may work synergically, or act in different extent. With the regulation by both the metabotropic glutamate and GABA receptors on glutamatergic and GABAergic terminals, the neuron can have a balanced level of excitation and inhibition to process auditory information.

Synaptic modulation (depression or facilitation) is a kind of short-term synaptic plasticity that can be induced by repetitive stimulation in the CNS (Buonomano and Merzenich, 1998). Modulation of synaptic responses can change

coding of information between neuronal networks (Tsodyks and Markram, 1997). In this experiment, the mGluR II activation affected basic aspects of short-term plasticity such as changing the neurotransmitter release, modulation of summation and depression of the synaptic responses and changing the dependency of EPSCs and IPSCs on the stimulation frequency. Therefore, it can be suggested that mGluRs II not only play a key role in regulating glutamate and GABA releases but also are important in short-term plasticity in the ICC.

Many questions about physiological function of mGluRs in the ICC remain to be answered. Studies of mGluRs in auditory nuclei have explored other functional roles of mGluRs, such as regulation of neuronal survival in avian cochlear nucleus during development (Lachica et al., 1995; Diaz et al., 2009), contribution to development of auditory circuits in the synapses between the MNTB and LSO in rats (Nishimaki et al., 2007), down regulation of NMDA receptors at the synapses of calyx of Held during development in mice (Joshi et al., 2007), and protection from sound-induced seizures in genetically epilepsy-prone rats (Chapman et al., 2001; Tang et al., 1997; Yip et al., 2001). Certainly, mGluRs appears to be involved in many aspects of cellular and pathological mechanisms in the auditory system.

5.11. Function of glutamate transporters in ICC

There is very little information about the role of glutamate transporters in the auditory system. Shimizu et al. (2005) have suggested that the glutamate transporters are involved in preventing inner hair cell degeneration induced by ototoxicity. In addition, involvement of glutamate transporter in hearing loss and aging has been

reported in the IC (Tadros et al., 2007). The results of the present study suggest that glutamate transporters are highly active and regulate the synaptic transmissions as well as short term plasticity in the ICC. The glutamate transporters are located mostly on glial cells and postsynaptic terminals in the brain (Danbolt, 2001; Torres and Amara, 2007), perhaps including the ICC (Fig. 5.1). The glutamate transporters control the rate and extent of the glutamate clearance. As a result, the transporters can affect the degree of postsynaptic glutamate receptor activation and desensitization, and further influence the synaptic responses. They also indirectly control activation of presynaptic mGluR II, and consequently lead to modulation of neuronal responses, and synaptic plasticity. Therefore, it can be inferred that the transporters can be involved in those mechanisms that mGluRs II are involved. The author believes that glutamate transporters play a significant role in neurotransmitter clearance and consequently modulation of neuronal responses in the ICC.

Figure 5.1.

Figure 5.1. Schematic view of the sites and action of presynaptic mGluRs II, GABA_B receptors and EAAT (glutamate transporter) in the ICC. The location of GABA_B receptors on glutamatergic and GABAergic terminals was suggested by previous studies (Ma et al., 2002; Sun et al., 2006). ●: GABA molecules. ○: glutamate molecules.

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