Transition to Seizure in the CA3 Hippocampal Network: Predominant Preictal GABAergic Potentials, followed by Predominant Ictal Glutamatergic Potentials

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Physiology

University of Toronto

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Degree of Master of Science, 2010
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ABSTRACT

The mechanisms underlying the transition to seizure are still unresolved. Proposed mechanisms include excitatory GABAergic drive, loss of interneuron-mediated inhibition, and glutamatergic input potentiation. The objective of this thesis was to investigate the relative contributions of synchronized glutamatergic and GABAergic inputs and their functional roles during ictogenesis in the epileptic neonatal (postnatal days 6-12) mouse hippocampus, induced with 0.25mM Mg^{2+}/5mM K^+ perfusion. Simultaneous field and whole-cell patch-clamp recordings were obtained from CA3 stratum-oriens interneurons and pyramidal cells.

The antagonists for GABA_A and glutamate receptors abolished the preictal and ictal discharges, respectively, suggesting that the preictal state is mediated by the coherent discharges of GABAergic inhibitory interneurons, whereas the recurrent excitatory inputs are required for ictogenesis. Synaptic charge transfers underlying the synchronized discharges showed a dynamic change in the balance between the inputs: GABAergic currents markedly diminished by ictal onset whereas glutamatergic currents dominated at ictal onset and throughout the ictus.
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<tbody>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate</td>
</tr>
<tr>
<td>APV</td>
<td>(2R)-amino-5-phosphonovaleric acid</td>
</tr>
<tr>
<td>fAHP</td>
<td>fast spike afterhypopolarization</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BMI</td>
<td>bicuculline methiodide</td>
</tr>
<tr>
<td>[Ca(^{2+})]_i</td>
<td>intracellular calcium ion concentration</td>
</tr>
<tr>
<td>CA1</td>
<td>coronu ammonis area 1</td>
</tr>
<tr>
<td>CA3</td>
<td>coronu ammonis area 3</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalography, electroencephalogram</td>
</tr>
<tr>
<td>E(_{GABA_A})</td>
<td>reversal potential of GABA(_A) receptor mediated current</td>
</tr>
<tr>
<td>E(_{Cl})</td>
<td>reversal potential of chloride</td>
</tr>
<tr>
<td>EPSP/C</td>
<td>excitatory postsynaptic potential/current</td>
</tr>
<tr>
<td>FS</td>
<td>fast spiking</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GDPs</td>
<td>giant depolarizing potentials</td>
</tr>
<tr>
<td>HFOs</td>
<td>high frequency oscillations</td>
</tr>
<tr>
<td>Ih</td>
<td>hyperpolarization-activated cation current</td>
</tr>
<tr>
<td>IPSP/C</td>
<td>inhibitory postsynaptic potential/current</td>
</tr>
<tr>
<td>KCC</td>
<td>K-Cl cotransporter</td>
</tr>
<tr>
<td>LIA</td>
<td>large amplitude irregular activity</td>
</tr>
<tr>
<td>NKCC</td>
<td>Na-K-Cl cotransporter</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PSC</td>
<td>postsynaptic current</td>
</tr>
<tr>
<td>RSA</td>
<td>rhythmic slow activite</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
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<tr>
<td>SLEs</td>
<td>seizure-like events</td>
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<tr>
<td>TLE</td>
<td>Temporal lobe epilepsy</td>
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<td>VIP</td>
<td>vasoactive intestinal polypeptide</td>
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1. INTRODUCTION

1.1 Brief overview of the clinical characteristics of epilepsy

Epilepsy is a common neurological disorder affecting approximately 1% of adults and 2% of children (Hauser and Hesdorffer, 1990, Sander and Shorvon, 1996). Two-thirds of the patients achieve sufficient seizure control from anticonvulsive medication, and another 8–10% could benefit from surgery. For the remaining 25% of patients, no sufficient treatment is currently available (Kwan and Brodie, 2005). A better understanding of the mechanisms of ictogenesis would help developing novel effective strategies to cure these treatment-resistant forms of epilepsy.

The epileptic seizure is characterized by sustained periods of hyperexcitation and/or hypersynchrony associated with brain dysfunction (Jasper 1954). Normal brain functions are interrupted by abnormal activity consisting of rhythmic, coordinated neuronal discharges. The characteristics of epilepsy vary widely depend on the seizure types, electroencephalogram (EEG) abnormalities, and concomitant neurological deficits.

Two types of seizures are characterized and distinguished by their clinical features: generalized seizures arise simultaneously throughout the cortex and partial seizures arise from one part of the brain (Somjen 2004). Generalized seizures significantly affect both cerebral hemispheres and are often accompanied by a loss of consciousness (Somjen 2004). These seizures are associated with an initial tonic phase involving simultaneous spasms, and then followed by the clonic phase, where the body undergoes rhythmic jerking movements (Engel 1989). On the other hand, partial seizures are defined by abnormal electrical activity originating...
from a seizure focus, where a small group of neurons triggers the enhanced excitability (Engel 1989). Enhanced excitability may result from factors such as altered cellular activities or altered synaptic connections caused by local scar, blood clot, or tumours (Ottman et al., 1996). The phases in the seizure development can be divided into the interictal period, followed by neuronal synchronization, seizure spread, and finally secondary generalization (Litt and Lehnertz, 2002). Partial seizures can be further divided into 1) simple partial seizures, which have a discrete focus such as in the primary motor cortex cause twitching of a finger or jerking of limb, without impairment of consciousness; 2) complex partial seizures, where a focus often in the limbic system, and associated with unusual behaviour or an alteration of consciousness (Somjen 2004). In summary, seizure disorders are classified according to their different behaviours and electrical features. In this study, our focus is to investigate the transition to seizure in an \textit{in vitro} model of temporal lobe epilepsy (TLE), which represents approximately 60% of all partial epilepsies.

1.2 An overview of the experimental models utilized to study ictogenesis

1.2.1 Introduction

One of the strategies to study the mechanisms of epileptogenesis is to reproduce this seizure-like event (SLE) in simplified animal models and then examine the network and cellular changes responsible for its generation. The generation of experimental seizures can be induced either in the whole animal with both acute and chronic models of epilepsy \textit{in vivo} or in brain slices obtained from both naive and epileptic animals \textit{in vitro}. Different types of treatments would induce different types of seizure-like patterns.
1.2.2 *In vivo* seizure models

Several *in vivo* seizure models have been used to study the spontaneous discharge patterns, which also mimic the features observed in patients with mesial TLE. The ictal electrical activities in rodent models of mesial TLE were initiated by focal cortical penicillin (Matsumoto and Marsan, 1964), and injection of γ-aminobutyric acid A (GABA\(_A\)) receptor antagonist bicuculline methiodide (BMI) and glutamate agonist kainic acid, into the coronu ammonis area 3 (CA3) area of hippocampus (Bragin et al. 2009). During the period of ictal onset, two main patterns of activities observed are the hypersynchronous high frequency oscillations (HFOs) characterized by large spikes with high discharge frequency and the low voltage fast firing pattern in the beta-gamma range (Bragin et al., 2007).

In addition to drug application, *in vivo* SLEs can also be generated by high-frequency stimulation. In contrast to the spontaneous seizure patterns previously described, stimulus-evoked seizures are initiated with an afterdischarge (Bertram, 1997; McIntyre and Gilby, 2008). Therefore, studies using *in vivo* models of spontaneous seizures provide useful details on mechanisms involved in the spontaneous transition from the interictal state into seizure, whereas seizures evoked by stimulation are only informative about the network dynamics during a seizure (de Curtis and Gnatkovsky 2009).

1.2.3 *In vitro* seizure models

Simplified *in vitro* models have also used in a large majority of experimental studies. Similar to *in vivo* seizure models, the ictal pattern varies depending on the seizure inducing method and the region considered. Spontaneous recurrent SLEs can be generated by perfusing
the brain tissue with convulsant agents such as cobalt (He et al. 2009), low Mg (0.25 mM)/high K (5mM) (Derchansky et al. 2004), 4-aminopyridine (Ziburkus et al. 2006), or GABA \(_A\) receptor antagonists such as penicillin, bicuculline, and picrotoxin (Uva et al., 2008, 2009). In these seizure models, ictal discharges are typically characterized by a progressive recruiting activity that gets larger and more synchronous with time, followed by a decremental discharge during postictal depression in which the amplitude and rate of firing progressively decrease (de Curtis and Gnatkovsky 2009). Factors involved in inducing SLEs include the altered intrinsic membrane properties, shift in synaptic efficacy, as well as possible neuromodulators that may affect the currents through second messenger pathways (Dichter and Ayala 1987).

Other studies have used high-frequency stimulation to generate SLEs in hippocampal slices \textit{in vitro}. Epileptiform activity can be induced by a strong synaptic stimulation of the coronu ammonis area 1 (CA1) pyramidal cells (Fujiwara-Tsukamoto et al. 2004). Jahromi et al. (2002) also showed that evoked seizures were elicited by repeated (10 min intervals) tetanization of relevant afferents at 100Hz for 2 seconds. Stimulus-induced afterdischarges are usually coupled with large and abrupt membrane depolarization of neurons, burst firing, recurrent excitation, and large changes in extracellular potassium concentration (Fujiwara-Tsukamoto et al. 2004). However, as mentioned earlier, afterdischarges observed in slices \textit{in vitro} are neither ideal nor appropriate to study the mechanisms of initiation of spontaneously occurring focal seizures.

Because the generation of seizures not only involves the excessive activity of a neuronal focus but also the entrainment of a pathologic network of epileptogenic structures, spontaneous seizure patterns characteristic of hippocampal EEG activity \textit{in vivo} do not occur in \textit{in vitro} hippocampal or neocortical slice preparations (Bragin et al., 2000). This is because many long-
range neuronal connections in the three-dimensional architecture of the hippocampus are removed by the slicing procedure. Thus it is advantageous to use animal models that contain the intact and more distant local circuitry, while maintaining a healthy environment for the tissue.

Studies have developed seizure models using intact embryonic and neonatal rat hippocampal formation and surrounding limbic structures perfused with 4-aminopyridine (4-AP), kainate, or low Mg$^{2+}$ artificial cerebrospinal fluid (ACSF) (Khalilov et al. 1997; Quilichini et al. 2002; Derchansky et al. 2004, 2008). This model preserves the hippocampal network activity, and at the same time permits electrophysiological assessment of the hippocampus at both single cell and network levels. However, there is the issue of anoxic damage due to the thickness of the intact hippocampus which limits the oxygen perfusion. In order to maintain a healthy intact hippocampus, Wu et al. (2002) removed the dentate gyrus area beyond the hippocampal fissure, while preserving the functional integrity of the extensive CA3 to CA1 connections in vitro, and at the same time permitting adequate perfusion of oxygen. They were able to isolate the healthy hippocampal tissue from mice up to postnatal day 28. In our study, the intact neonatal hippocampus was isolated from neonatal mice. Because of the small tissue volume, dentate gyrus removal was not necessary. To optimize the tissue viability, a nylon mesh was placed below the intact hippocampus, allowing rapid top and bottom perfusion of oxygenated ACSF in the submerged chamber (Derchansky et al. 2004). This type of tissue preparation has been previously used in our lab for seizure studies, where persistent recurrent spontaneous SLEs, along with interictal epileptiform discharges were recorded in intact hippocampi from mice up to age P25 days, while perfusing the structure with ACSF containing 0.25 mM Mg$^{2+}$ and 5 mM K$^+$ (Derchansky et al. 2004, 2006, 2008).
1.3 Previous studies on the mechanisms underlying focal ictogenesis

1.3.1 Introduction

In principle, a seizure can evolve by two different scenarios: a sudden and abrupt transition or a cascade of gradual changes (Lopes da Silva et al. 2003). Generalized epilepsy is primarily initiated by the abrupt transition, where no prior dynamical changes in EEG are detected. Alternatively, the initiation of focal epilepsies involves a gradual change in dynamics, which is detectable in EEG. Several mechanisms underlying epileptic seizure generation have been proposed. Traditionally an epileptic seizure was believed to be manifested from decreased inhibition along with excessive excitation (Jasper 1954).

On the neuronal network level, focal seizures are assumed to be initiated by abnormally discharging principal neurons, that are progressively recruit and entrain neighbouring neurons with synchronized discharges (Yaari and Beck, 2002). This build-up might be mediated by an increasing synchronization of neuronal activity that is accompanied by a loss of inhibition, or by processes that facilitate seizures by lowering the threshold for excitation or synchronization. The transition to seizure remains a big mystery (Le Van Quyen et al. 2003). Several researches have conducted studies both in vivo and in vitro to improve our understanding of the mechanisms underlying the focal ictogenesis. This chapter will attempt to describe what is currently known regarding the characteristic activity patterns observed during the transition to seizure and the proposed mechanisms responsible for the seizure generation.
1.3.2 *In vivo* studies: EEG patterns during the seizure transition

Clinically, an epileptic seizure is characterized by high-amplitude EEG signals with complex dynamics, which are the result of synchronized spontaneous rhythmic activities of large neuronal populations (Jirsh et al. 2006). These activities can be experimentally detected by recording either from large neuronal assemblies (as in EEG) or from a neuronal cluster of a cell population (Buzsaki et al., 1983). The EEG represents a set of field potentials recorded by multiple electrodes on the surface of the scalp. It measures the rhythmic oscillations in voltage as a result of the synchronized firing of postsynaptic potentials from neurons around the electrode positions. EEGs can be split into a number of frequency ranges: large amplitude irregular activity (LIA, 0.5-20Hz), rhythmic slow activity (RSA, theta, 4-10 Hz), and fast oscillatory activity (gamma, 30-100 Hz) (Niedermeyer 1972). LIA is associated with slow-wave sleep and in-wake immobility, whereas theta rhythms occur during exploration and rapid-eye movement sleep (Buzaki et al. 1983; Wu et al. 2002). Studies have suggested that these hippocampal rhythms play important roles in synaptic plasticity, sensory-motor behavior, and learning and memory processing.

Presurgical intracranial recordings have been used to localize the epileptogenic zone in patients. In patients and animal models of mesial temporal lobe epilepsy, two predominant depth-recorded EEG patterns were observed at the seizure transition period: low –voltage fast activity and HFOs (Bragin et al. 2005). The generation of these different EEG patterns depends on specific disruptions to the existing balance between excitatory and inhibitory components of the neuronal network (Bragin et al. 2009). Preictal patterns, which precede the fast activities at seizure onset, may be mediated by either reinforcement or reduction of interictal/preictal
discharges (de Curtis and Avanzini 2001; Avoli et al. 2006). Several network mechanisms have been proposed for ictogenesis, but the exact underlying mechanism remains unclear.

With intracranial EEG studies, seizure onset is shown to be consisted of a buildup of low-voltage fast activity of 15-40 Hz, which often begins regionally and then involves extrahippocampal structures in seizure generation (Spencer et al. 1992). Because of the temporal correlation with seizure onset, the recorded low-voltage fast activity could be used to localize the zone of seizure onset (Fisher et al. 1992; Gotman et al. 1995). The underlying neuronal mechanisms of the observed low-voltage fast activity involve disinhibition, in addition to enhanced excitation, to produce hypersynchrony (Engel, 1989). But a more recent study by de Curtis and Gnatkovsky (2009) suggested that low-voltage fast activity at the seizure onset is associated with reinforcement and synchronization of inhibitory networks. Other studies have also observed a spatial correlation of signals during ictal low-voltage fast discharges, suggesting that these discharges was mediated by a synchronization of cortical networks established by the recruitment of inhibition (Bartolomei et al. 2001; Wendling et al. 2003).

Another interictal pattern observed during the seizure transition is characterized by brief runs of ultrafast activity (200–600 Hz), termed HFOs or fast ripples (Buzsaki et al., 1992; Engel et al., 2009). Because HFOs are restricted to the epileptogenic focus, they are considered as markers of eileptogenesis and also suggest that the seizure is driven by the emergence of a hypersynchronous neuronal subnetwork (Bragin et al. 2005; Jirsch et al. 2006; Lasztoczi et al. 2004). According to the in vivo studies in both chronic animal models and human temporal lobe epilepsy, the underlying mechanism of the HFOs generation has been proposed to be the
synchronous activation of clusters of highly interconnected principal neurons that overpowers feedback inhibition (Bragin et al. 2004, 2005; Le Van Quyen et al. 2008).

1.3.3 In vitro studies: the role of inhibitory network in seizure generation

1.3.3.1 Human tissue studies

Studies performed in patients with TLE have demonstrated that recurrent inhibition is functionally retained in the hippocampi during the interictal spiking (Engel and Wilson, 1986; Isokawa-Akesson et al. 1989). Cohen et al. (2002) have also recorded interictal epileptiform patterns and functionally preserved inhibition in the subiculum from postsurgical human TLE tissue maintained in vitro. They suggested that the shift in the reversal potential of GABA_A current in the subpopulation of subicular neurons was possibly due to an overexpression of K-Cl cotransporter 1 (KCC1). These findings support the concept that inhibition is not lost in human focal epilepsies. This contradicts other findings where abundant loss of subpopulations of inhibitory interneurons was found in human and experimental epileptic tissue samples (Magloczky and Freund, 2005; Dudek and Sutula, 2007). Whether inhibition can result in enhanced or reduced local cortical network excitability is area- and pathway-specific (Bernard et al., 2000). Thus, depending on the local alterations, the function of inhibitory inputs in epileptogenesis can be variable.
1.3.3.2 Animal tissue studies

In addition to the studies using pathologic human tissue, a number of animal studies have also suggested the involvement of inhibitory networks in the transition to seizure initiation. Several authors hypothesized that the preictal spikes are sustained by the synchronous reinforcement of inhibition associated with the generation of large population spikes (Bragin et al. 2007, Lasztoczi et al. 2009). GABAergic mechanisms were also shown underlying the generation of fast ripples (Ylinen et al. 1995), neonatal network oscillations (Palva et al. 2000), and oscillations associated with epileptiform activity (Khalilov et al. 2005). Intracellular in vivo studies by Timofeev et al. (2002) showed that putative inhibitory interneurons are not silent during ictal discharges, but contribute to the seizure onset with increased inhibitory firing.

Brief arterial perfusion with the BMI induced a low-amplitude fast activity discharge pattern at the seizure onset in the entorhinal cortex of the isolated guinea pig brain in vitro (Gnatkovsky et al. 2008). Gnatkovsky et al. (2008) showed that the GABAergic inhibition is only transiently and partially reduced (to 60–70%) and inhibitory networks are paradoxically reinforced. The onset of seizure was also found to be correlated with a blockade of activity in the deep layer principal neurons and a marked increase in action potential firing in the interneurons. Thus they suggested that seizure onset is mediated by the inhibitory circuits and blocking of neuronal firing in principal neurons, such that a transient enhancement of interneuronal network activity is responsible for excitability changes that precede seizure onset.

Using the low Mg$^{2+}$ induced epilepsy model in juvenile rat hippocampal slices, Lasztoczi et al. (2004) observed two components of the HFOs in the CA3 region: a lower frequency component between 50 and 400 Hz occurring throughout SLEs and a higher frequency
component in the range of 400-800 Hz found mainly preictally (5 ms prior to the onset) and at the SLE onset (first 10 ms of the ictus). Based on the temporal correlation with the seizure onset, a role for HFO in seizure initiation has been proposed (Bragin et al. 2000; Traub et al. 2001; Dzhala and Staley 2003; Lasztoczi et al. 2004; Khosravani et al. 2005). In addition, the synchrony of GABAergic currents and pyramidal cell firing emerged gradually with preictal discharges and peaked with the HFOs at the ictal onset, whereas the glutamatergic excitation is most significant for the ictal discharges (Lasztoczi et al. 2009). Thus they suggested that during the period leading to ictal onset, the functional properties of GABAergic inputs undergo dynamic changes, which are mediated by the activity-dependent shift in GABA reversal potential during the preictal phase. These excitatory GABAergic inputs would contribute to the synchronization and recruitment of pyramidal cells, leading to the hypersynchronous and hyperactive network state. Pyramidal cell firing is also associated with HFOs, since Dzhala and Staley (2004) suggested that fast ripples are initiated and synchronized by excitatory interactions between pyramidal cells.

Several studies have also demonstrated that seizure-like events can occur spontaneously in the area CA1 of the hippocampal slices bathed in 4-AP and low-Mg²⁺ solution (Ziburkus et al. 2006; Derchansky et al. 2008). Ziburkus et al. (2006) observed the enhanced firing of the GABAergic interneuron prior to the ictal onset, which is then followed by a depolarization block at the onset. They then suggested that during the seizure transition period, the transient decrease in inhibition due to the depolarization block in interneurons is responsible for the enhanced excitatory firing and synchronization of principal cells, thereby promoting the progression of the seizure into the higher bursting ictal phase.
The seizure transition study by Derchansky et al. (2008) performed on the isolated immature mouse hippocampus in vitro demonstrated that at the ‘receiving’ end, the CA1 region, the intracellularly measured postsynaptic potentials switch from dominant phasic inhibition to dominant phasic excitation during the preictal period of the low Mg^{2+} SLEs. Inhibition is maintained throughout the seizure transition period, but just prior to the ictal onset, the synaptic excitability is enhanced and overwhelms the prominent inhibitory postsynaptic potentials (IPSPs). Thus they suggested both the persistent inhibition and enhanced excitation contribute to the ictogenesis.

In conclusion, regardless of the patterns observed at the onset of a seizure, several studies performed on brain slices demonstrated the involvement of inhibitory networks in seizure initiation. During the transition to seizure, there are dynamic changes in the functional properties of GABAergic input contributed to the ictogenesis. The synchronized hyperexcitable state observed in the neuronal network may result from the disruption of balance between inhibition and excitation mediated by the GABAergic and glutamatergic inputs (Lasztoczi et al., 2009).

1.4 Synaptic mechanisms underlying the transition to seizure

1.4.1 Introduction

As previously discussed, GABA_A receptor–mediated mechanisms play a pivotal role in initiating ictal discharges determined by 4-aminopyridine and low-magnesium solutions in the entorhinal cortex and in the hippocampus of the rat studied in vitro (Perreault and Avoli, 1992; Avoli et al., 1996; Velazquez and Carlen, 1999; Kohling et al., 2000). Many previous studies
have investigated the increased seizure susceptibility of the immature brain. Both excitatory (Rivera et al., 1999; Dhzala et al., 2005; Marty and Llano 2005; Lasztoczi et al., 2009) and inhibitory /shunting (Banke and McBain, 2006) actions of GABAergic transmission were reported. This chapter will attempt to briefly describe what is currently known regarding the functions of GABAergic transmission in epilepsy.

1.4.2 Integration of postsynaptic excitatory and inhibitory inputs

The excitatory or inhibitory effect of the synaptic terminal is determined not only by the type of neurotransmitter released from the presynaptic neuron but also the type of ion channels gated by the transmitter in the postsynaptic cell. Such that neurons release glutamate act on receptors that produce excitation and neurons release GABA or glycine act on receptors that produce inhibition.

Excitatory synaptic action is mediated by glutamate-gated channels that conduct sodium and potassium. Na⁺ and K⁺ flows through the glutamate-gated channels with nearly equal permeability, thus the reversal potential for the current flow through these channels lies around 0 mV (Hollmann and Heinemann 1994). Two major categories of glutamate receptors are the ionotropic receptors that directly gate channels and the metabotropic receptors that indirectly gate channels through second messengers. α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA), kainate, and N-methyl-D-aspartate (NMDA) are the three major subtypes of the ionotropic glutamate receptors. The action of glutamate on the ionotropic receptors is always excitatory, while activation of the metabotropic receptors can produce either excitation or inhibition (Hollmann and Heinemann 1994). Non-NMDA ionotropic receptors gate cation
channels with relatively low conductance that are permeable to both Na\(^+\) and K\(^+\) but are usually not permeable to Ca\(^{2+}\). NMDA receptor-channels are permeable to Ca\(^{2+}\) as well as to Na\(^+\) and K\(^+\). The opening of the channel is voltage-dependent and also requires extracellular glycine as a cofactor. Extracellular Mg\(^{2+}\) binds to a site in the pore of the NMDA-activated open channel blocking current flow.

IPSPs recorded from most central neurons are usually mediated by GABA- and glycine-gated channels that conduct Cl\(^-\) (Kuhse et al. 1995). Inhibitory IPSP results from an increase in conductance to Cl\(^-\). At resting potential, the electrochemical driving force on Cl\(^-\) is positive. The opening of Cl\(^-\) channels leads to an outward current, which corresponds to an influx of Cl\(^-\) down its electrochemical gradient. This causes a net increase in the total negative charge on the inside of the membrane’s capacitance so the membrane hyperpolarizes (Kuhse et al. 1995).

However in some conditions, usually in the immature neurons, the opening of GABA-gated Cl channels in brain cells can cause excitation. With the enhanced influx of Cl\(^-\) into the cell after intense stimulation, the intracellular Cl\(^-\) concentration increases resulting a more positive Cl\(^-\) equilibrium potential than the resting potential (Ben-Ari 2002). Under these conditions opening of Cl\(^-\) channels will depolarize the neuron. Depolarizing GABA-gated responses play a role in generating an oscillatory activity in brain. These large and synchronized depolarizing GABA-gated responses may also contribute to the epileptic discharges.

The location of the synapses on the postsynaptic cell is important for their functional effectiveness. Axosomatic synapses generate a stronger current signal to influence the outcome at the trigger zone than those current signals generated from the more remote axodendritic contacts (Kandel et al. 2000). In the brain significant inhibitory input frequently occurs on the
cell body of neuron. This is because increasing Cl⁻ conductance at the cell body will effectively shunt much of the depolarization produced by the spreading excitatory current. In contrast, excitatory synapses often occur on the dendritic spines, where contain both non-NMDA and NMDA types of glutamate receptors. Voltage-gated Na⁺ and Ca⁺ channels in the dendrites serve as a local trigger zone to amplify the small excitatory postsynaptic potential (EPSP). Thus action potentials that are generated in the dendrites propagate electrotonically to the cell body and axon hillock, where they are integrated with all other input signals in the cell (Kandel et al. 2000). The excitatory input’s drive for the membrane toward threshold depends on the conductance of the excitatory synaptic channels and their chemical driving force, as well as the conductance of all other ion channels in the postsynaptic membrane and the driving force of these channels (Kandel et al. 2000). The opening of inhibitory channels increases the total resting conductance of the membrane, thus the size of the depolarization during the EPSP will be decreased. This consequence of synaptic inhibition is called the short-circuiting or shunting effect of an increased conductance IPSP.

1.4.3 The functions of GABA_A receptors in immature neurons

There is a differential distribution of ions across the cell membrane, in which chloride is the principal anion in the extracellular fluid. The direction and magnitude of chloride diffusion is determined by both the concentration gradient and the membrane potential (Kandel et al. 2000). GABA_A receptors are anion-permeable channels for HCO₃⁻ and Cl⁻ ions. Whether the action of GABA is excitatory or inhibitory depends on the intracellular Cl concentration ([Cl⁻]ᵢ), the Cl⁻ reversal potential (E_Cl), the resting membrane potential, and the action potential threshold. The
$E_{Cl}$ must be above the threshold of action potential generation for the GABA action to be excitatory. The driving force for Cl ion is the difference between the $E_{Cl}$ and the resting membrane potential of the neuron, which determines the amplitude of the depolarization.

The immature brain is more prone to seizure than adult brain due to their enhanced excitability through the depolarizing action of GABA (Ben-Ari et al., 2007). This is shown by the age-dependent differences in the efficiency of GABA$\_A$ acting anticonvulsive drugs. Studies comparing the seizure susceptibility in immature and adult have shown that the immature CA3 have a higher propensity of generating prolonged epileptiform burst when blocking GABA$\_A$ receptors pharmacologically (Shao and Dudek, 2009). In the immature brain, the enhanced excitability is contributed by the depolarizing and excitatory action of GABA. Thus GABA-enhancing drugs have a limited anticonvulsive effect. In the immature CA3 hippocampal neurons (from birth to two weeks old), the reversal potential for chloride was more depolarized than the resting membrane potential, indicating a higher $[Cl^-]_i$ (Ben-Ari et al. 1989). The strength and polarity of GABA-mediated neurotransmission is largely determined by the $[Cl^-]_i$. When $[Cl^-]_i$ is high, $E_{Cl}$ is positive relative to the neuron’s membrane potential. The opening of Cl channels by GABA-receptor activation results in chloride efflux, which consequently depolarizes the neuron (Ben-Ari et al., 2007). On the other hand, when $[Cl^-]_i$ is low, $E_{Cl}$ is negative relative to the neuron’s resting membrane potential, activation of the GABA$\_A$ receptor results in chloride influx, leading to hyperpolarization of the neuron. Altering the $[Cl^-]_i$ homeostasis is determined in part by the activities of cation-chloride cotransporters: Na-K-Cl cotransporter 1 (NKCC1) for Cl$^-$ influx and KCC2 and for Cl$^-$ efflux.
Postsynaptic GABAergic response can be excitatory depending on the local internal Cl⁻ concentration. Perforated-patch recordings from hippocampal neurons indicated that the [Cl⁻]i is higher in immature neurons than in adult neurons by 20-40mM (Staley et al. 2001). They have also noted that during postnatal week two, small changes in [Cl⁻]i are sufficient to cause the GABA reversal potential to be either below or above the resting Membrane potential and the threshold for action potential generation. This high [Cl⁻]i is sufficient to shift the action of GABA from inhibition to excitation. Thus in the immature neuron, the net movement of Cl⁻ is inward through NKCC1 (Ben Ari et al. 2002). When GABA_\text{A} receptors are activated, Cl⁻ flow out of the neuron and depolarize the cell. This especially happens during embryonic and early postnatal hippocampal development. In addition to the hippocampal neurons, the developmental shift in the actions of GABA has been observed also in cultured chick neurons (Obata et al. 1978), spinal neurons of Xenopus larvae (Rohrbough et al. 1996), embryonic zebrafish (Saint-Amant et al. 2000), and turtle embryonic retina (Sernagor et al. 1999). Thus there is enhanced seizure susceptibility early in development.

The depolarizing actions of GABA can generate sodium and calcium action potentials by directly activating these voltage-dependent channels and also remove the voltage-dependent magnesium block from NMDA channels inducing an increase in the intracellular calcium (Leinekugel et al., 1995). This depolarizing activation of GABA_\text{A} receptors can also lead to an increase in the concentration of intracellular calcium in immature neurons in a wide range of brain structures, including the hippocampus and neocortex, the hypothalamus, the spinal cord and the cerebellum (Luhmann and Prince, 1991; Chen et al. 1996; Ben-Ari et al. 2002). GABA actions are mediated by a positive loop with brain-derived neurotrophic factor (BDNF). The
calcium currents activated by excitatory GABA lead to the activation of c-fos, BDNF mRNA and KCC2 protein expression, which promotes the molecular shift in GABA actions during development (Ben-Ari 2002).

However, many studies have also documented the shunting effect of GABA receptors in immature neurons (Rivera et al. 1999; Tyzio et al. 2007). GABA-induced depolarizing currents can inhibit neuronal activity if they concomitantly decrease the effectiveness of glutamatergic excitatory postsynaptic currents (EPSCs), by clamping the resting membrane potential to $E_{Cl}$. The GABA depolarization can also lead to the inactivation of sodium conductance through a shunting mechanism that raises the spike threshold (Ben-Ari et al., 2007). Thus, GABA synapses initially excite their targets, but they can also shunt and reduce the excitation by glutamatergic EPSCs. The net function of GABA depends on the levels of ongoing activity, the density of GABA receptors, and the excitability of the network. Banke and McBain (2006) has shown the shunting effect of GABAergic input onto the CA3 hippocampal stratum lucidum inhibitory interneurons throughout development, whereas the pyramidal cells demonstrated the developmental shift from depolarizing to hyperpolarizing GABAergic transmission.

In the rodent hippocampus, the intracellular Cl concentration and $E_{Cl}$ decreases with age during the postnatal period. In adult cells, Cl$^{-}$ ions usually move into the cell to produce strong inhibitory hyperpolarization, since the reversal potential for Cl$^{-}$ is 15 to 20 mV more negative than the resting membrane potential (Kandel et al. 2000). HCO$^{3-}$ ions move out of the cells and mildly depolarize the cell, since HCO$^{3-}$ has a reversal potential of -12 mV. However the depolarization through HCO$^{3-}$ is normally balanced out by the Cl hyperpolarization (Kandel et al. 2000). The HCO$^{3-}$ efflux gradient is maintained by the actions of extracellular carbonic
anhydrase. When the HCO$_3^-$ / Cl$^-$ permeability ratio changed, the outward HCO$_3^-$ flux would depolarize the membrane at the resting membrane potential.

**1.4.4 Developmental expression and function of cation-chloride cotransporters**

As previously discussed, two families of Cl$^-$ transporters control the intracellular accumulation of chloride in immature neurons: NKCCs and KCCs (Ben-Ari 2002). Studies have shown that NKCC1 is expressed at early developmental stages and is responsible for the high [Cl$^-$]i, but KCC2 co-transporter is a key player in the developmental switch from GABA-mediated excitation to inhibition (Rivera et al. 1999). The modification of GABA actions in hippocampal neurons is associated with the changes in the levels of KCC2 messenger RNA and the transfection of KCC2 into neurons.

The function of KCC2 was studied by Ganguly et al. (2001) using calcium imaging to measure the actions of GABA, perforated-patch recordings to measure E$_{Cl}$, and RNase protection assay to estimate KCC2 activity. Their study showed that the action of GABA was depolarizing and the expression level of KCC2 was low in the neonatal rodent hippocampal neurons in culture. During development, the GABA action shifted from excitation to inhibition, as indicated by the abolishment of the depolarizing potentials with GABA$_A$ receptor antagonist only. Thus the ongoing release of GABA$_A$ receptor-mediated miniature postsynaptic currents (PSCs) is sufficient to trigger the expression of KCC2 and a reduction in intracellular Cl$^-$, which promotes the shift in the GABA action from excitatory to inhibition (Staley and Smith, 2001).

In the immature mice, the two cation-chloride cotransporters are found to be expressed differently between hippocampal CA1 and CA3 regions. mRNA and protein expression study
have found that in mice, CA3 region has a minimal expression of KCC2 until P17 whereas its expression in CA1 increased gradually from P1 to P7 (Zhu et al., 2008). On the other hand, immunohistochemistry study found that for CA3 region, NKCC1 is highly expressed in the pyramidal cell soma starting at P1 (Marty et al. 2004). At P21, NKCC1 was not expressed in the soma, but dendritic processes are labeled in the CA3. For CA1 region before P7, very few pyramidal cells’ soma are immunolabeled with NKCC1. Similar to the CA3 neurons at P21, NKCC1 was prominently labeled in the dendrite, not the soma of CA1 PCs (Marty et al., 2004). Because of the different expression levels of the two cation-chloride cotransporters, CA3 neurons are hypothesized to have a weaker Cl⁻ extrusion ability and retains a higher [Cl⁻]i than CA1 neurons at the early developmental stage (before P7). This developmental variation is important because Cl⁻ homeostasis determines whether GABA mediated conductance hyperpolarizes or depolarizes a neuron, especially under pathological conditions such as epilepsy.

1.5 The local circuits in the CA3 region generating the pathological oscillations

1.5.1 Introduction

The brain function involves the coordination of activities of large populations of neurons. Local circuit connections between homogeneous and heterogeneous cell types in the hippocampus are critical in determining the functional output patterns of the discrete brain region. The brain is capable of integrating the processing signals from difference sources (Buzaski 2006).
Population discharges such as the theta and gamma rhythms are associated with distinct behavioural states. Several fundamental processes in the brain (e.g. memory consolidation and spatial navigation) are thought to be mediated by the synchronized firings among population of neurons. However under pathological conditions, the normal neural circuits are altered to generate abnormal oscillatory network activity. In this section, I describe first the normal local circuits mediating neural network oscillation, and then the aberrant synchronized hyperexcitable oscillatory networks underlying the seizure generation.

1.5.2 Normal local circuits mediating the neural network oscillation

The hippocampus is characterized by robust oscillatory network activity. Under normal conditions, information enters the hippocampus via the dentate gyrus and proceeds to CA3, then to CA1, and finally to the subiculum. Synchronized bursting of many CA3 pyramidal cells can generate the sharp wave (Ylinen et al. 1995). This excitation mediated by CA3 pyramidal cells is then transmitted to CA1 where subsequent ripple activity (~200 Hz) is generated. Together, this oscillation is known as the sharp-wave ripple complex, which is associated with both normal and epileptic brain (Ylinen et al. 1995).

In the hippocampus, complex interconnections between GABAergic interneurons and pyramidal cells have been found to underlie the cellular bases for establishing and maintaining of large scale network oscillations (Buzsaki et al. 1992). In an ‘acute’ model of epileptiform activity, horizontal interneurones of the CA1 and CA3 region have been shown to burst synchronously with pyramidal cell (Aradi and Maccaferri, 2004). Hippocampal interneurons could synchronize
large neuronal populations via their abundant connectivity to pyramidal neurons and other interneurons (Buhl et al. 1994; Sik et al. 1995).

A diverse population of CA3 interneurons have been characterized. Here, we focused on the horizontal stratum oriens interneurons, which have been described by Freund and Buzsaki (1991) to have similar horizontal dendritic architecture with different axonal projections. A variety of intracellular labelling studies have shown that GABAergic interneurons in the CA3 regions are morphologically diverse and can be classified with respect to their laminar position, somatodendritic morphology, and their efferent connectivity (Gulya et al., 2003; Buhl et al. 1994). The organization of interneuron microcircuitry depends on their efferent connectivity to surrounding neurons. Interneurons in the CA3 subfield can be divided into perisomatic targeting (e.g., axo-axonic and basket cells) (Maccaferri et al. 2000; Losonczy et al. 2002; Ganter et al. 2004) and dendrite targeting (e.g., bistratified and O-LM) (Maccaferri et al. 2000; Sik et al. 1995; Gloveli et al. 2005). Interneurons may be preferentially involved in either feedforward or recurrent inhibitory microcircuits according to their efferent target profile. For example, O-LM cells, selectively innervate the perforant path termination zone in the stratum lacunosum-moleculare, are shown to exclusively mediate recurrent inhibition onto pyramidal cells; whereas bistratified cells, receive associational affects, are predominately involved in feedforward inhibitory circuits (Maccaferri and McBain, 1995). Other criteria have also been used for classification purposes like, for example, the expression of a variety of peptides (somatostatin, vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), etc.) and/or calcium binding proteins. However, no exclusive marker for specific cell-types has been found yet (Maccaferri
and Lacaille, 2003). For example, somatostatin is expressed by more than a specific neuronal type and is found at least in O-LM and O-bistratified cells.

*In vivo* recordings from awake rats have demonstrated that interneuronal firings are temporally correlated with both gamma and theta activity (Bragin et al. 1995). Fast EPSPs in interneurons are important for the network activity during inhibition-based brain rhythms by improving the precision of spike timing. Studies have shown that EPSPs on hippocampal interneurons have faster kinetic properties than the excitatory inputs on principal neurons (Miles 1990). This is because interneurons have low membrane time constants, active dendritic conductance, and fast EPSP kinetics mediated by the molecular composition of interneuronal AMPA receptors. Together, these factors accelerate spiking in interneurons. Recurrent inhibition is also effective in curtailing EPSPs evoked using electrical stimulation of afferent fibers. Perisomatic-targeting interneurons with widespread divergence of axons are sufficient to synchronize hippocampal population activity by phase-locking the subthreshold oscillations. Repetitive IPSPs originating from a few presynaptic interneurons are effective in pacing and synchronize the spontaneous action potential generation in the postsynaptic pyramidal neurons (Cobb et al. 1997).

In addition, the pyramidal cells and GABAergic interneurons in the CA3 subfield are also interconnected, increasing the complexity of local information processing (Figure 1). Interconnected neurons allow rapid and reliable synchronization of network activity. Many interneurons can innervate either pyramidal cells or interneurons with no apparent specificity (Freund and Buzaki, 1996). A small proportion of unitary EPSPs are sufficiently strong enough to trigger action potentials in a postsynaptic neuron. Reciprocally connected GABAergic...
interneurons are important in the generation of gamma frequency network oscillations. Cobb et al. (1997) have shown that basket cells are synaptically interconnected and also coupled with other interneuronal classes. Therefore, the output from basket cells onto the target neurons is likely to occur synchronously, which is important in hippocampal rhythmogenesis.

A recent review by Beenhakker and Huguenard (2009) hypothesized that the local inhibitory networks in CA1 are the rhythm generator for ripple oscillations. Excitation from CA3 activates CA1 inhibitory neurons. At the trough of the rhythmic local field potential, the activity of CA1 inhibitory neurons is low and increases progressively in response to the CA3 excitation. At the peak of the rhythmic local field potential, inhibitory neuron activity is strengthened through recurrent inhibition and overpowers the CA3 excitatory drive. This powerful surge of inhibition results an overall decrease in the population firing rate. The rhythmic oscillation observed in local field potential is defined by the synaptic delays and rise times associated with inhibitory events (Beenhakker and Huguenard 2009).

The highly synchronized discharge of CA3 pyramidal cells has been shown to initiate sharp waves (Traub et al. 2001; Buzsaki et al. 1992). This is supported by findings that blocking adenosine receptors induces the spontaneously occurring CA3 sharp waves in the hippocampal slices (Wu et al. 2009). In addition, the local circuitry in the hippocampal CA3 region is capable of exhibiting spontaneous synchronous GABAergic activities in vitro (Wong et al. 2005; Wu et al. 2002). They recorded spontaneous rhythmic field potentials from whole hippocampal isolates and thick slices prepared from young and adult mice, which are intracellularly correlated with the synchronous GABA<sub>A</sub>-IPSPs in pyramidal neurons and repeated discharges in inhibitory interneurons. Thus, Wu et al. (2002) hypothesized that a population glutamatergic drive arose
from the hippocampal CA3 recurrent circuitry in vitro are capable of triggering coherent discharges in a group of GABAergic inhibitory interneurons, which in turn generate the IPSP-based spontaneous rhythmic field potentials. In addition to the GABA<sub>A</sub>-mediated oscillations, synergistic excitatory activities of the depolarizing GABA and glutamate NMDA receptors generate giant depolarizing potentials (GDPs) at CA3 region in postnatal day 0-10 mice (Sipila and Kaila, 2007). There is extensive branching of the dendrites and axons of the CA3 neurons during the second postnatal week (Gomez-Di Cesare et al. 1997).

Figure 1. Schematic diagram of the CA3 hippocampal circuitry.
This schematic diagram shows the synaptic interactions of pyramidal cells (black) and several classes of interneurons: bistratified cells (blue), basket cells (red), and O-LM cells (green). Note that pyramidal cells (PCs) are mutually interconnected and also innervated by perisomatic/somatic-targeting interneurons (ie. basket cells) and dendritic-targeting interneurons (ie. bistratified cells and O-LM cells). Basket cells are also interconnected via multiple synapses. Perisomatic/somatic-targeting interneurons are likely to play key roles in rhythm generation, because the synaptic inputs from these cells can synchronize the firing of postsynaptic pyramidal cells. PC, pyramidal cells; O-LM cells, oriens – lacunosum molecular cells; s.l.m, stratum lacunosum molecular; s.rad, stratum radiatum; p.c.l, pyramidal cell layer; s.o., stratum oriens.
1.5.3 Synchronized hyperexcitable oscillatory networks underlying the seizure generation

Under pathological epileptic conditions, fast ripples were recorded near the seizure onset, reflecting the hypersynchronous discharges of pyramidal neuron within in the epileptogenic region (Bragin et al. 1999). The CA3 region is the key structure for driving epileptogenesis and hyperexcitability in the low Mg²⁺ seizure model, leading the CA1 region during the preictal and ictal states. Neurons found in the CA3a region have more complex dendritic arbors, acts as the pacemaker cells by firing early and recruiting other cells to fire (Wittner and Miles, 2007).

However, the exact mechanisms underlying the fast ripple oscillation still remain unknown. Because many studies have shown that ripples and fast ripples appear to be coupled, Beenhakker and Huguenard (2009) hypothesized that these oscillations reply on similar brain states for expression. Dzhala and Staley (2004) and Foffani et al. (2007) have studied the mechanisms underlying the fast ripples in CA3 region using the high K⁺ bath perfusion model and the pilocarpine-treated epileptic animal model, respectively. They concluded that fast ripple generation is promoted by the elevated synaptic activity, initiated by a hypersynchronous burst among CA3 pyramidal neurons, and its oscillation frequency is dependent on the precision of spike-timing.
2. RESEARCH RATIONALE, OBJECTIVES, HYPOTHESES

Research Rational and Objectives

Epileptic seizures are often described as “hypersynchronous” events, but the underlying dynamical cellular mechanisms leading to the ictogenesis are poorly understood. Many previous studies have investigated the increased seizure susceptibility of immature hippocampus. Both excitatory (Rivera et al., 1999; Dzhala et al., 2005; Lasztoczi et al., 2009) and inhibitory /shunting (Banke and McBain, 2006) actions of GABAergic transmission were reported. But there are only few studies focusing on the short-term dynamics of glutamatergic and GABAergic synaptic drive contributing to ictogenesis.

The brain is composed of many rhythmic microcircuits. The synchronized firings among population of neurons are thought to mediate several fundamental processes in the brain, including memory consolidation and spatial navigation (Buzsaki, 2006). Because epileptic seizures are characterized by neuronal hyperexcitability, the underlying mechanism should be associated with the altered oscillatory activity patterns (Beenhakker and Huguenard 2009). The existing circuitry in the normal brain is the template upon which pathological changes are taken place. Rather than utilizing completely different circuit mechanisms, epileptic seizure is derived from similar but altered hippocampal circuit mechanism (Beenhakker and Huguenard 2009). Thus alterations in the intrinsic and/or synaptic excitability would account for the pathological changes that promote network hyperexcitability.

The main objective of this thesis is to examine the cellular synaptic activities underlying the period of seizure transition in the CA3 region, which is the key structure for driving epileptogenesis and hyperexcitability in the low Mg\textsuperscript{2+} seizure model in the hippocampus. Here I
characterize the relative contributions and time course of the synchronized glutamatergic and GABAergic inputs into the hippocampal CA3 region during the seizure-like event at the population level, and then quantify the intracellular dynamic changes in the balance between excitatory and inhibitory drive of CA3 non fast-spiking (non-FS) interneurons and pyramidal cells during the preictal period. Also I examine the possible mechanisms underlying these dynamic changes in GABAergic and glutamatergic inputs during the seizure transition.
Hypotheses:

The overall hypothesis is that during the period of transition to seizure, the synchronized hyperexcitable state observed in the CA3 neuronal network results from the disruption of balance between inhibition and excitation mediated by the GABAergic and glutamatergic inputs, with markedly decreased inhibition and increased excitation.

Specific hypotheses:

More specifically, the experiments of this thesis will aim to test the following hypotheses:

i) The rhythmic recurrent discharges during the interictal and early preictal states are mediated by increased synchronous GABAergic activity.

This hypothesis is based upon previous observations that the reinforcement and synchronization of inhibitory networks underlie the generation of fast ripples (Ylinen et al. 1995) and the oscillations associated with preictal spikes (Khalilov et al. 2005, Bragin et al. 2007, Lasztoczi et al. 2009) (Figure 1). In addition, experimental and modeling data suggest that the ripples observed in the CA1 region are initiated when the CA3 sharp wave provides powerful excitation to the inhibitory networks in the CA1 region (Beenhakker and Huguenard 2009). Similarly, in the CA3 region, repetitive IPSPs originating from presynaptic interneurons could be effective in pacing and synchronizing the spontaneous action potential generated in the postsynaptic pyramidal neurons, thus driving the coherent network oscillations.
Expected results:

1. Bath perfusion of GABA_A antagonist should block the preictal discharges and prolong and/or enhance the ictal phase.

2. Individual interneurons should fire intensively in correlation with extracellular preictal events.

3. Individual CA3 pyramidal neurons should have strong inhibitory components during the interictal and early preictal phases.

ii) During the period of ictal initiation (i.e the preictal phase), there is increased spontaneously synchronized excitatory afferent glutamatergic activity and a marked decrease of the synchronous inhibitory GABAergic activity.

This hypothesis is based upon previous observations that the highly synchronized discharge of CA3 pyramidal cells has been shown to initiate fast ripples (Traub et al. 2001; Buzsaki et al. 1992). According to the in vivo studies in both chronic animal models and human temporal lobe epilepsy, the underlying mechanism of the HFOs generation at the ictal onset has been proposed to be the synchronous activation of clusters of highly interconnected principal neurons that overpowers feedback inhibition (Bragin et al. 2004, 2005; Le Van Quyen et al. 2008).

Expected results:

1. Bath perfusion of glutamate antagonists should affect on the interictal, preictal and ictal phases, since the activation of both pyramidal cells and interneurons require excitatory drive.
2. Compound EPSCs from individual neurons correlated with the recurrent field events should be enhanced during the late preictal period.

3. Compound inhibitory postsynaptic currents (IPSCs) correlated with the field events should be reduced during the late preictal period.

4. The reversal potentials of the spontaneous postsynaptic potentials correlated with the field population events should become more depolarized during the ictal initiation period.

iii) Alternatively, the transition to ictal onset could also be mediated by an increased synchronous GABAergic activity due to a depolarization of the GABA\textsubscript{A}-Cl reversal potential.

This hypothesis is based upon previous seizure transition studies which suggested that the loss of interneuron-mediated inhibition underlying the seizure generation is the result of chloride loading and over-activation of GABA\textsubscript{A} receptors (Ben-Ari 2002). After intense stimulation, there is an enhanced influx of Cl\textsuperscript{-} into the cell, subsequently reverse the Cl\textsuperscript{-} gradient in pyramidal cells, causing a switch from inhibition to excitation (Fujiwara-Tsukamoto et al. 2006; Kaila et al. 1997; Staley et al. 1995; Lasztoczi et al. 2009). The neurons of immature mice have weaker Cl\textsuperscript{-} exclusion ability than mature mice due to their low expression of KCC2. Thus the positive shift in the GABA\textsubscript{A}-Cl reversal potential is favoured in immature mice (Ben-Ari 2002).

Expected result:

1. Reversal potential of GABA\textsubscript{A} of the recorded pyramidal cell should be more depolarized in comparison with the neuron’s resting membrane potential, suggesting a positive driving force for Cl\textsuperscript{-} efflux.
3. MATERIALS AND METHODS

Hippocampal preparation

C57 male black mice (Charles River Laboratory, Montreal, QC, Canada; aged 6 to 12 postnatal days) were used to isolate whole hippocampus (Derchasky et al., 2004). The animals were anaesthetized with halothane and decapitated in accordance with the Canadian Animal Care Guidelines. The brain was quickly removed, hemisectioned, and immersed for 4-5 minutes in an ice-old, oxygenated (95% O2/5% CO2) solution of artificial cerebrospinal fluid (ACSF) containing (mM): 123 NaCl, 2.5 KCl, 1.5 CaCl₂, 2 MgSO₄, 25 NaHCO₃, 1.2 NaH₂PO₄, and 15 glucose. The whole intact hippocampus was then dissected out from each hemisphere, and immersed in oxygenated ACSF at room temperature for at least 1.5 h before recording.

Experimental environment

The isolated intact hippocampus was placed in a submerged recording chamber and maintained at 32±0.5°C. Epileptiform activity was induced by perfusing the tissue with low-Mg²⁺ ACSF: 123 NaCl, 5 KCl, 1.5 CaCl₂, 0.25 MgSO₄, 25 NaHCO₃, 1.2 NaH₂PO₄, and 15 glucose). The tissue was perfused with oxygenated ACSF and low-Mg²⁺ ACSF at a flow rate of 8 ml/min.

Electrophysiology

Simultaneous extracellular field potential, and whole-cell patch-clamp recordings in current and voltage clamp modes were obtained from stratum oriens interneurons and pyramidal
cells of the hippocampal CA3 region. Neurons were visualized by infrared differential interference contrast (IR-DIC) videomicroscopy (Olympus BX51 microscope, OLY-150IR camera-video monitor unit). The extracellular electrode was placed into the stratum pyramidale near the patched cells (≤150µm). The whole-cell patch pipettes (3-5 Mohm) were filled with solution containing (mM): 135 potassium gluconate, 10 NaCl, 0.0001 CaCl₂, 10 NaHEPES, 1 MgCl₂, 0.3 NaGTP, 2 NaATP (pH7.4). Whole cell recordings were done using an Axopatch 200B amplifier (Axon Instrument) in either the current- or voltage-clamp configurations. Data were collected using a sampling rate of 10 kHz and were subjected to a low-pass filter of 5 kHz.

The intrinsic properties of cells were measured in whole-cell current-clamp mode. The resting membrane potential was recorded in the absence of current injection. The input resistance was determined from the slope of the I-V plot recorded from voltage responses to current pulses (900 ms; -100 pA, at 25 pA increments). The analysis of the peak spiking frequency was calculated from the numbers of action potential evoked by a step current injection of 75 pA over 900 ms. The duration of the action potentials was measured at half-amplitude as was the duration of the fast spike afterhyperpolarization (fAHP).

Extracellular recordings were used to define the boundary between these states. The interictal state was characterized as the period between the end of the previous ictal state to the next preictal state. Preictal state was defined as the onset of rhythmic field discharges at a frequency >0.2 Hz (average frequency of 0.5 ± 0.15 Hz). The start of ictal state was identified by the high frequency discharges of >15 Hz (average frequency of 20 ± 3 Hz).

The reversal potentials of the spontaneous intracellular bursting activity correlating with the rhythmic recurring inter- and preictal epileptiform field potentials were determined by
voltage-clamping the cell at different voltages over several seconds. The reversal potential of GABA_A was determined by pressure-injection of muscimol onto the soma of a pyramidal cell using a Picospritzer (General Valve, TX, USA). Recorded neurons were also voltage-clamped at the reversal potential of glutamatergic and GABAergic currents; 0 mV and -60 mV respectively. After isolating the excitatory or inhibitory synaptic inputs, the charge transfers underlying the postsynaptic currents associated with the spontaneous rhythmic field potentials were estimated for both CA3 pyramidal cells and non-FS interneurons during the preictal period by quantifying the area under the curve.

**Pharmacology**

Bicuculline methiodide (BMI), dl-2-amino-5-phosphonopentanoic acid (APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were dissolved into low-Mg²⁺ ACSF for the desired final concentrations. Bath application was performed with these antagonists. Muscimol (10 μM) in saline (pH 7.4) was pressure injected locally near the soma of the recorded neuron via a Picospritzer.

**Data analysis**

The statistical test used was one-way ANOVA followed by Bonferroni’s multiple comparison test with P < 0.05 considered significant. Data are reported as means ± S.E.M. Data processing, analysis, and graphical representations were executed with pClamp9.2 (Molecular devices, Forest City, CA, USA) and CorelDRAW X4 (Corel, Ottawa, ON) software.
4. RESULTS

4.1 Recorded neurons were classified based on their morphological features and electrophysiology properties.

Recorded CA3 neurons were classified based on their morphological features and electrophysiology properties. An electrophysiological profile of recorded pyramidal cells, FS interneurons and non-FS interneurons is detailed in Table 1. Pyramidal cells were identified by their characteristic spike-frequency adaptation, their morphological features as well as by visual inspection based on the location of the electrode in the pyramidal cell layer. When recording from the intact mouse hippocampus with the stratum oriens layer facing up, the cell bodies of CA3 pyramidal neurons were found in horizontal sections of the stratum pyramidale layer below the stratum oriens layer. Pyramidal cells were more densely packed than the stratum oriens interneurons (Figure 2A, 4Ai,Aii). Recorded pyramidal cells had a more negative average resting membrane potential (-68.5 ± 3.4 mV) and lower input resistance (178.6 ± 12.5 M\(\Omega\)) than the interneurons (Figure 2B, Table 1). This is consistent with previous studies (Derchansky et al., 2008; Wu et al., 2002). The CA3 pyramidal cells barely fired action potentials from the resting membrane potentials, but discharged regularly with evident spiking adaptation when stimulated by intracellular injection of depolarizing current pulses greater than 75 pA (Figure 2C). As the injected current increased, the number of induced action potentials also increased.

To distinguish between FS and non-FS interneurons, FS interneurons were identified as those interneurons which exceeded an action potential frequency of 50 Hz with maximal current
injection, whereas non-FS interneurons had less. The same discriminator was used by Fujiwara-Tsukamoto et al. (2003) and Derchansky et al. (2008).

Stratum oriens interneurons are known to be morphologically and electrophysiologically diverse. The CA3 interneurons had more positive resting membrane potentials and higher input resistance than PCs (Table 1, Figure 3Bi,Bii). Interneurons often fire from resting membrane potentials. When stimulated by intracellular injection of depolarizing current pulses, interneurons discharged in high frequencies with little firing adaptation (Figure 3Ci,Cii). Without morphological reconstruction of the axonal and dendritic arborization, their electrophysiology properties were used to differentiate these recorded interneurons. There were no significant differences in the resting membrane potentials between the recorded non-FS interneuron and FS interneuron (Table 1). Non-FS interneuron but not the FS interneuron showed the clear “sag” at a strong hyperpolarizing current injection (Figure 3Bi,Bii). This “sag” is an indication of the hyperpolarization-activated cation current (Ih) (Gloveli et al, 2005). The firing frequencies of both types of interneurons increased as the injected depolarizing current increased (Figure 3Ci,Cii). The recorded non-FS interneurons discharged at a lower frequency than FS interneurons when the same amount of current was injected. When examining the first action potential elicited after 75 pA depolarizing current injection, the action potentials in non-FS interneurons had a longer duration and followed by a longer fAHPs in comparison with those of FS interneurons (Figure 3Di,Dii).

According to these electrophysiological features and comparing with previous studies, interneurons with FS properties are basket cells, bistratified cells and trilaminar cells (Freund and Buzsaki, 1996; Gloveli et al, 2005; Derchansky et al, 2008). Basket cells have dendrites localized
to strata oriens, radiatum and LM layers, while the axon arbor extended heavily into the stratum pyramidale. Trilaminar interneurons have dendrites localized in the stratum oriens, and axonal arbors pervade in the strata oriens, pyramidal and radiatum. Another class of FS interneurons, bistratified cells, is located around the border between strata oriens and pyramidale, with axon broadly spread into strata radiatum, oriens, but not into stratum pyramidale. O-LM interneurons are known to have non-FS properties. Previous morphology reconstruction showed that O-LM cells extend its axon directly towards the stratum lacunosum-moleculare, where the axonal arborization developed horizontally and constrained to the stratum oriens (Gloveli et al., 2005; Derchansky et al., 2008). Comparing with previous studies involving both morphology reconstruction and electrophysiological studies, the recorded non-FS interneurons were identified as the O-LM interneurons.
Figure 2. Intrinsic membrane properties and firing patterns of pyramidal cells
A, Infrared image showing the recorded pyramidal cell. Note that pyramidal cells were more densely packed than the stratum oriens interneurons. B, Hyperpolarizing responses to negative current injections (900 ms, -100 pA to -25 pA in 25 pA increments) (left) and the I-V relation (right) in a CA3 pyramidal cell. Ci, Firing patterns of the recorded neuron (upper traces, Membrane potential) in response to the depolarizing current injection indicated at the bottom of the trace (900 ms, 25 pA to 125 pA in 25 pA increments). Cii, Number of action potentials fired at various positive current injections were plotted. Note the recorded cells fired action potentials with depolarizing current injections of greater than 75 pA. There was also a gradual build up of the firing frequencies.
Figure 3. Intrinsic membrane properties and firing patterns of non-FS and FS interneurons

A, Infrared image showing the recorded non-FS interneuron (i) and FS interneuron (ii). B, Hyperpolarizing responses to negative current injections (900 ms, -100 pA to -25 pA in 25 pA increments) (left) and I-V relation (right) in the non-FS interneuron (i) and FS interneuron (ii). Voltage-current relationships for non-FS interneurons were plotted separately at the peak responses and steady state voltages. Note that non-FS interneuron but not the FS interneuron showed the clear “sag” at a strong hyperpolarizing current injection. C, Firing patterns (upper traces, Membrane potential) in response to the depolarizing current injection indicated at the bottom of the trace (900 ms, 25 pA to 125 pA in 25 pA increments) in the non-FS interneuron (i) and FS interneuron (ii). The plot of the number of action potential measured at various positive current injections for both neuronal types (bottom left and right). Note that in response to depolarizing current injection, FS interneurons showed significantly higher number of action potential discharges than non-FS interneurons. D, A representative recording of the first action potential elicited by a 75 pA depolarizing current injection in non-FS interneuron (i) and FS interneuron (ii). Note that the action potentials in non-FS interneurons are characterized by their longer duration and are followed by longer fAHPs in comparison with FS interneurons.
<table>
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<th>Fast-spiking Interneuron</th>
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<td>12</td>
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<tr>
<td>Resting membrane potential (mV)</td>
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<td>-62.4 ± 4.2</td>
<td>-59.5 ± 4.7</td>
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<tr>
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<td>394.0 ± 28.3</td>
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</tr>
<tr>
<td>Peak frequency (Hz)</td>
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</tr>
<tr>
<td>AP width (ms)</td>
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<td>0.95 ± 0.10</td>
<td>0.56 ± 0.04*</td>
</tr>
<tr>
<td>fAHP half-duration (ms)</td>
<td>8.5 ± 2.3</td>
<td>4.6 ± 1.8</td>
<td>1.1 ± 0.5*</td>
</tr>
</tbody>
</table>

**Table 1: Summary of the intrinsic properties of the recorded neurons**

N, the number of recorded CA3 pyramidal cell and non-FS interneurons with soma located at stratum oriens; resting membrane potential; input resistance calculated using linear regression of peak voltage changes evoked by negative current injections (-100 pA, increments of 25pA). Peak frequency was calculated from the numbers of action potential evoked by a step current injection of 75 pA. Action potential (AP) width is the duration of AP measured at half-amplitude. Half-duration of the fast afterhyperpolarization (fAHP) was also measured. Each value is expressed as the mean ± S.E.M.
4.2 The interictal and preictal states of CA3 pyramidal cells and non-FS
interneurons are characterized by dominant recurrent depolarizing potentials

By perfusing the intact isolated mouse hippocampus with low Mg\(^{2+}\) ACSF, spontaneous
recurrent SLEs were recorded in the isolated neonatal mouse hippocampus (Figure 4), consistent
with previous studies by Derchansky et al. (2004, 2008) in CA1 neurons. Intracellular and
extracellular recordings were obtained from the CA3 region simultaneously (Figure 4). The
recurrent SLEs or ictal events began after 243 ± 65s of low Mg\(^{2+}\) ACSF perfusion, with an
average duration of 97 ± 13s. Two characteristic preictal states were observed: interictal and
preictal.

The spiking activities during the interictal and preictal states were variable among
pyramidal cells and interneurons. Non-FS interneurons have a higher frequency of intracellularly
recorded spiking than the pyramidal cells (Figure 4). In the interical state, pyramidal cells had
recurrent depolarizing events corresponding to extracellular field potentials at a frequency of
0.07 ± 0.01 Hz, and the frequency of the discharges was 0.45 ± 0.05 Hz during the preictal state.
Similar depolarizing events corresponding to synchronized extracellular field events occurred in
interneurons at a frequency of 0.25 ± 0.08 Hz during the interictal state and 0.85 ± 0.5 Hz during
the preictal state. These intracellularly recorded electrical responses during the preictal state were
synchronized with field potentials and characterized as the large depolarizing potentials of
probable synaptic origin consisting of a sudden, large (20-40 mV), and long-lasting (50-250 ms)
depolarization, which triggered a train of action potentials at its peak. The intrinsic firing patterns
on top of these depolarizing potentials were different between the pyramidal cells and
interneurons during the preictal state (Figure 4). The frequencies of the preictal burst firing
discharges were much higher in interneurons (up to 50 Hz) than pyramidal cell. During the transition to the ictal state, the intracellular burst firings of both neuronal types became more frequent and the firing patterns were also more complex. Despite their different intrinsic firing frequencies and patterns, the interictal and preictal state of both pyramidal cells and non-FS interneurons are characterized by dominant phasic depolarizing activities, which are synchronized with the recorded field population excitation.
Figure 4. The interictal and preictal states of non-FS interneurons and CA3 pyramidal cells are characterized by dominant depolarizing activities.

Recurrent seizure-like events are recorded from the non-FS interneurons (A), and pyramidal cells (B). Seizure-like activities were recorded intracellularly under current-clamp with the correlated field activity. The field recording electrode was placed <150 µm away from the recorded neuron. Three characteristic states were observed: interictal, preictal and ictal. Extracellular recordings were used to define the boundary between these states. The bursting activities during the preictal state of both neuronal types are shown in a faster time base (red box). Note that dominant phasic depolarizing activities are observed during the interictal and preictal states of both neuronal types. During the preictal state, non-FS interneurons have a higher frequency of intracellularly recorded bursting activities than the pyramidal cells.
4.3 Glutamate receptor activation is necessary for the ictal state

To examine the balance between the inhibitory and excitatory synaptic inputs at the population level, the effects of glutamate receptor antagonists on SLEs were examined from the field potentials recorded in the CA3 pyramidal layer. When a mixture of glutamatergic receptor antagonists D-APV (60 µM) and CNQX (10 µM) were added to the chamber at the same time that Mg$^{2+}$ ions were washed out, SLEs were markedly diminished (Figure 5). After 10 minutes of perfusion, interictal epileptiform activities were observed with the absence of the high frequency ictal bursts. Washout of the receptor antagonists was associated with a return of SLEs. The fact that SLEs were markedly diminished by the glutamatergic receptor antagonists suggests that glutamatergic inputs were required for the ictus to occur.

![Figure 5. Effects of glutamate receptor antagonists on the epileptiform activity.](image)

Field recordings of SLEs were obtained from the pyramidal cell layer under the perfusion of low Mg$^{2+}$ ACSF, D-APV (60 µM) plus CNQX (10 µM), and washout. D-APV (60 µM) and CNQX (10 µM) markedly diminished the low Mg$^{2+}$ ACSF induced SLEs. After washout for 10 minutes, the SLEs reappeared. (n=5)
4.4 GABA<sub>A</sub> receptor activation is necessary for the expression of the preictal state and delays the transition to ictal state

The role of signalling by GABA<sub>A</sub> receptors in generation of the SLEs was also examined by using antagonist of the inhibitory receptors. Field potentials were recorded in the CA3 pyramidal layer. Blocking GABAergic inputs by the antagonist, BMI (10 µM), greatly diminished the spontaneous bursting activities of the interictal and preictal state. The interictal state was characterized by absent interictal epileptiform activity and the duration of the preictal state was significantly reduced (Figure 6A, B). Because of the decreased inter-seizure intervals, the frequency of ictal events increased when compared with SLEs in control. Interestingly, abolishing the GABAergic inputs did not significantly alter the ictus duration. After washout for 10 min, the effect of BMI was not completely reversible. The preictal state duration recovered but the interictal duration was still significantly less than that of the low Mg<sup>2+</sup> ACSF. In addition, ictus duration was also significantly reduced after the washout in comparison with the control.
Figure 6. Effects of GABAergic receptor antagonists on the epileptiform activity.

A, SLEs are recorded extracellularly from the CA3 pyramidal layer with perfusion of low Mg\(^2\+) ACSF, 10 µM BMI, and then washout (top trace). Boxed SLEs under each treatment were shown at a faster time base (bottom traces). The last SLE of each treatment was chosen to ensure a longer perfusion time for the antagonists. B, Quantitative analysis of the preictal, ictal, and inter-seizure durations were calculated in each condition. Note that BMI greatly reduced the inter-seizure interval and the duration of interical and preictal period, with little effect on the ictal duration. After washout, the interictal duration did not recover completely. Surprisingly, the ictal duration was reduced significantly after washout. *Significant differences in the values between low Mg\(^2\+) ACSF and BMI treatments. **Significant differences in the values between low Mg\(^2\+) ACSF and washout. (N=5, One-way ANOVA, P<0.05)
4.5 GABAergic transmission is required for the much of the slow membrane depolarization during the transition to seizure

When perfusing the tissue with low Mg$^{2+}$ while recording from CA3 pyramidal cells and the field simultaneously, a slow depolarization of the membrane potential during the preictal state was also observed (Figure 7). The changes in the resting membrane potentials were summarized in Table 2A. There were significant increases in the resting membrane potentials of both pyramidal cells and non-FS interneurons during the seizure transition period. The resting membrane potentials observed at the late preictal period were significantly more depolarized than at the interictal and early preictal states. With the application of 10 µM BMI, the presence of the slow depolarization was attenuated and the neurons’ intrinsic firing was also diminished at the interictal and preictal period (N=5). There were still some bursting activities recorded prior to the ictal onset and the ictus remained unaffected. This suggests that GABAergic transmission may play an important role for generating the depolarizing interictal and early preictal potentials and the intrinsic slow membrane depolarization during the transition to seizure.
Figure 7. The slow membrane depolarization during the transition to seizure is mainly GABA mediated.
Correlated intracellular and extracellular recordings of the SLEs from a CA3 pyramidal cell under the perfusion of low Mg\(^{2+}\) (A) and 10 µM BMI (B). The period of seizure transition (red bar) is shown at a faster time scale (left). Note that bursting activities prior to ictal onset are greatly diminished after BMI application. There is also a slow depolarization in the membrane potential during the seizure transition, which is also markedly diminished by BMI. (N=5)
4.6 Muscimol-induced postsynaptic inhibitory receptor responses remain intact throughout the SLEs

The postsynaptic inhibitory responses induced by muscimol were also examined throughout the transition to seizure and seizure epoch. Recordings voltage-clamped at different voltages demonstrated that postsynaptic muscimol-induced $\text{GABA}_A$ receptor responses were preserved throughout the interictal, preictal and ictal states. When voltage-clamping the recorded interneuron at 0 mV, the reversal potential of the glutamatergic inputs, large outward currents were evoked by injected muscimol (Figure 8). These large outward currents are the result of the activated postsynaptic GABAergic drive. Note that in the buildup to seizure, there were spontaneous rhythmic outward currents which then ceased just prior to the ictal state.

![Figure 8. Muscimol-induced postsynaptic inhibitory receptor responses remain intact during the ictus.](image)

Muscimol (* 500mM, 10ms, 10 p.s.i.) was pressure-injected near the soma of the recorded CA3 non-FS interneuron, V-clamped at different potentials. Muscimol, a $\text{GABA}_A$ receptor agonist, evoked postsynaptic inhibitory responses throughout the transition to seizure and during the ictus. ($N = 6$)
4.7 During the interictal period, GABAergic inputs may mediate a small depolarizing driving force.

The reversal potentials of the inhibitory postsynaptic potentials were also measured for examining specifically the GABAergic inputs. IPSCs of the pyramidal cells (N=3) were electrically evoked by somatic pressure-injection of the GABA_A agonist muscimol in normal ACSF and during the interictal and preictal state (Figure 9A, B). The reversal potential of GABA_A mediated current (E_{GABA_A}) was calculated from the amplitude of the evoked IPSCs at various holding voltages. The evoked E_{GABA_A} was -70 ± 3 mV in normal ACSF, but depolarized to -58 ± 4 mV in interictal state and -60 ± 2 mV in preictal state (Figure 9C, Table 2B). The average value of E_{GABA_A} recorded during the preictal state was further used as the holding potential for reflecting the glutamatergic currents.

E_{GABA_A} was more depolarized during the seizure transition period than in ACSF. At the same time, the resting membrane potential of the neurons also became more depolarized. During the interictal state, the E_{GABA_A} (-58 ± 4) and resting membrane potential (-67 ± 2) of pyramidal cells are closely associated, suggesting a small depolarizing driving force for Cl⁻ of only a few millivolts (Table 2). During the preictal state, it was hard to determine whether GABAergic activity is inhibitory or excitatory. This is because the resting membrane potential of the neuron depolarized gradually, and it is difficult to measure the E_{GABA_A} in correlation with the resting membrane potential slow depolarization. In addition, I performed whole-cell patch instead of perforated patch. As a result, the intracellular Cl⁻ concentration was altered leading to an underestimation of the reversal potentials.
Figure 9. Measurement of the reversal potentials of the muscimol-induced $E_{\text{GABA}}$ in a CA3 pyramidal cell during the period of seizure transition. The muscimol-induced $E_{\text{GABA}}$ is $-70 \pm 3$ mV in ACSF (A), $-58 \pm 4$ mV for the interictal period (B), and $-60 \pm 2$ mV for the preictal period (B). Current vs. voltage plot was used to determine the reversal potentials (C). Muscimol application occurred at the star (* 500mM, 10ms, 10 p.s.i.) (p<0.05).
4.8 The reversal potential of the recurrent spontaneous rhythmic postsynaptic currents depolarizes as the network transitions into the ictal state

Simultaneous extracellular and V-clamped intracellular recordings were utilized to determine the reversal potentials of the recurrent rhythmic whole-cell postsynaptic currents (PSCs) in CA3 pyramidal cells and non-FS interneurons, which correlated with the spontaneous rhythmic field potentials during the seizure transition states (Figure 10). Cells were voltage-clamped at various potentials and the spontaneous reversal potentials were calculated from the plotted IV curve. The observed reversal potentials of the spontaneous postsynaptic currents were summarized in Table 2B. When comparing different seizure periods, the postsynaptic reversal potential was significantly more depolarized during the preictal state than the interictal state. This suggests that both types of neurons became more excited after low Mg\(^{2+}\) perfusion. For the comparison between non-FS interneurons and pyramidal cells, the reversal potential of the PSCs was significantly more depolarized in non-FS interneurons than in the pyramidal cells during the preictal state, not the interictal state. Thus during the seizure transition preictal state, the excitatory drive onto non-FS interneurons is greater than those of the pyramidal cells. A comparison between the reversal potentials of PSCs with muscimol induced E\textsubscript{GABA\textalpha} revealed that pyramidal cells received significant greater amount of excitatory glutamatergic inputs during the preictal state, as indicated by the more positive PSCs reversal potentials.
Figure 10. Measurement of the reversal potentials of the rhythmic whole-cell postsynaptic currents in a CA3 pyramidal cell during the preictal period.

A, Simultaneous extracellular and V-clamped intracellular traces were recorded at various voltages during the preictal period in a CA3 pyramidal neuron. B, Intracellular and extracellular (corresponding to the -55 mV trace) recordings are shown in a faster time base. C, The currents during the preictal state were plotted versus the holding potentials. Data (mean) were measured from 7-9 consecutive events corresponding to the synchronous field potential at each voltage and were fitted with linear regression lines. In this example, the observed reversal potential of the PSCs during the interictal state was -44.2 mV.
### A

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<th>Preictal Early</th>
<th>Preictal Late</th>
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### B

<table>
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<tr>
<td>Non-FS Interneurons (n=17)</td>
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<td>-30±4*</td>
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<tr>
<td>Muscimol-induced $E_{\text{GABA}_A}$ in pyramidal cells (n=3)</td>
<td>-70±3</td>
<td>-58±4*</td>
<td>-60±2*</td>
</tr>
</tbody>
</table>

Table 2. Summary of the resting membrane potentials and the reversal potentials of the recurrent rhythmic whole-cell postsynaptic currents and the muscimol-induced $E_{\text{GABA}_A}$ in CA3 pyramidal cells and non-FS interneurons.

Resting membrane potentials of both neuronal types were measured in ACSF and several low-Mg$^{2+}$ induced seizure stages (A). Note that the resting membrane potentials at the late preictal state were significantly more depolarized than the early preictal state. Reversal potentials of the whole-cell postsynaptic currents and muscimol-induced $GABA_A$ were recorded in both types of neurons (B). These recorded currents occurred in correlation with the spontaneous rhythmic field potentials during the seizure transition states. Note that the reversal potentials of both neuronal types were significantly more depolarized during the preictal state than interictal state. Their reversal potentials were not recorded in the ACSF, because no spontaneous rhythmic potentials were seen in ACSF. Muscimol-induced $E_{\text{GABA}_A}$ was more depolarized in the interictal and preictal state than in ACSF. The chloride concentration in the whole-cell intracellular recording electrode was 13 mM. (* P<0.05)
4.9 Dynamics of the synchronized glutamatergic and GABAergic inputs during the preictal period

During the preictal state of low Mg$^{2+}$ induced SLEs, the CA3 network was characterized by accelerated synchronous bursting activities. To determine the relative contributions of the synchronized glutamatergic and GABAergic inputs during the perictal period, voltage-clamp recordings were performed on both non-FS interneuron and pyramidal cells. Recording currents at the EPSC reversal potential (0 mV) and IPSC reversal potential (-60 mV) permitted the isolation of GABAergic (outward) and glutamatergic (inward) currents respectively, as shown for both neuronal types (Figure 11A,B, Figure 12A,B). When the non-FS interneurons was voltage clamped at -60 mV, the inward glutamatergic currents increased as the network proceeded to ictal onset, and large inward glutamatergic currents remained throughout the ictal state (Figure 11A). When voltage clamping the non-FS interneuron at 0 mV, the outward GABAergic currents predominated in the early preictal phase, but disappeared just prior to the ictus (Figure 11B). The inward glutamatergic currents grew in the later preictal phase and predominated during the ictus. To quantify these observations, we measured the changes in the charge transfers of glutamatergic and GABAergic postsynaptic currents associated with the spontaneous rhythmic field potentials of CA3 interneurons during the preictal period. The charge transfers were measured for eight preictal bursts synchronized with the population spikes (p-8 to p-1), and were normalized against the burst at p-8 (Figure 11C,D). The inhibitory postsynaptic currents were enhanced during the early preictal phase, but gradually decreased during the late preictal period (Figure 11D). At the last burst prior to seizure onset, the polarity of the GABAergic current changed from outward to inward. On the other hand, the charge transfers of
excitatory glutamatergic postsynaptic currents were not significantly different between p-8, p-7, and p-6, whereas the postsynaptic current associated with late preictal discharges (p-5 to p-1) markedly increased to almost ten times control (Figure 11C).

Similar to the non-FS interneurons, relative contributions of the synchronized glutamatergic and GABAergic inputs of pyramidal cells were also measured during the preictal period. There was a gradual increase in the glutamatergic postsynaptic currents during the preictal state (Figure 12A). The outward (GABAergic) currents predominated the early preictal phase, but were greatly reduced during the ictal phase (Figure 12B). Unlike non-FS interneurons, inward currents were not present in pyramidal cells voltage-clamped at 0 mV just prior to seizure onset. Similarly, the charge transfers under the eight preictal bursts were also measured for the recorded pyramidal cells. Their inhibitory postsynaptic currents gradually decreased during the preictal period, at a faster rate than non-FS interneurons (Figure 12D). The charge transfer of the outward current observed at p-1 was significantly reduced, but it was not completely eliminated as seen in the non-FS interneurons. The increase in the charge transfer of the EPSCs was not significant in the early preictal period (p-8 to p-4) (Figure 12C), but in the late preictal period (p-3 to p-1), markedly increased to almost five times control, about half of that seen in the non-FS interneurons.
Figure 11. Changes in the glutamatergic and GABAergic PSCs associated with the spontaneous rhythmic field potentials of CA3 non-FS interneurons during the preictal period. Low Mg induced SLEs are recorded simultaneously from a CA3 non-FS interneuron (top traces) and the CA3 statum oriens layer (field, bottom traces). The electrodes are <100 µm apart. The glutamatergic and GABAergic currents were recorded by clamping the neuron at -60 mV (A) and 0 mV (B), respectively. The preictal activities seen in Ai and Bi are shown at a faster time base and the preictal bursts were numbered for quantitative analysis (Aii, Bii). The ictal onsets are indicated by the open arrows. The charge transfers were measured for eight preictal bursts synchronized with the population spikes (p-8 to p-1), and were normalized against p-8. Normalized charge transfer associated with different preictal discharges were plotted for both glutamatergic postsynaptic currents (C) and GABAergic postsynaptic currents (D). Note that the inhibitory postsynaptic currents were enhanced at the early preictal state, but gradually decreased during the late preictal period. Inward current was observed at p-1. On the other hand, EPSCs gradually but markedly increased (to almost 10X control) and became the dominant currents during the ictus. (N=15)
Figure 12. Changes in the glutamatergic and GABAergic PSCs associated with the spontaneous rhythmic field potentials of CA3 pyramidal cells during the preictal period. Low Mg induced SLEs were recorded simultaneously from the CA3 pyramidal layer (field, bottom traces) and a CA3 pyramidal cell (top traces) which were <100 µm apart. The glutamatergic and GABAergic driven currents were recorded by clamping the neuron at -60 mV (A) and 0 mV (B), respectively. The preictal activities seen in Ai and Bi were shown at a fast time base and the preictal bursts were numbered for quantitative analysis (Aii, Bii). The ictal onsets were indicated by the arrows. The charge transfers were measured for eight preictal bursts synchronized with the population spikes (p-8 to p-1), and were normalized against the burst prior to p-8. Normalized charge transfer associated with different preictal discharges were plotted for both glutamatergic postsynaptic currents (B) and GABAergic postsynaptic currents (A). Note that the inhibitory postsynaptic currents were gradually decreased during the preictal period. The charge transfer of the outward current observed at p-1 was significantly reduced, but it was not completely eliminated as in non-FS interneurons. EPSCs gradually but markedly increased (to almost 5X control) and became the dominant currents during the ictus. (N=10)
5. SUMMARY

1. Recorded CA3 neurons were classified into non-FS interneurons, FS interneurons, and pyramidal cells based on their intrinsic electrophysiological properties (Figures 2 and 3; Table 1).

2. Recurrent seizures were generated in the low Mg$^{2+}$ and high K$^{+}$ seizure model. The recorded activity was characterized into three states: interictal, preictal and ictal. Dominant recurrent rhythmic depolarizing potentials were recorded intracellularly during the interictal and preictal phases of all CA3 neuronal types (Figure 4).

3. Hypothesis #1: The rhythmic discharges at the interictal and preictal state are mediated by the increased synchronous GABAergic activity. This hypothesis was supported by the following findings:

   i. Abolishing GABAergic inputs diminished the gradual build-up of spontaneous activities during the early seizure transition and decreased the interictal and early preictal durations. These suggest that GABAergic inputs are important in generating the interictal and early preictal discharges. Interestingly, BMI did not prolong or enhance the ictal phase, suggesting that the generation of ictal phase is independent of GABA$_A$ receptor activity. (Figures 6 and 7).

   ii. Both pyramidal cells and interneurons fire intensively in correlation with the extracellular preictal events (Figure 4).

   iii. The more negative postsynaptic reversal potentials of the interical and preictal recurrent postsynaptic potentials in CA3 pyramidal neurons (-58±2 at interictal state and -45±2 at preictal state) suggests a strong GABA$_A$ mediated component during the interical and preictal phases (Table 2).
4. **Hypothesis #2:** During the period of ictal initiation, there is increased spontaneously synchronized excitatory afferent glutamatergic activity and a gradual decrease in the synchronous inhibitory GABAergic activity.

This hypothesis was supported by the following findings:

i. In field potential recordings from the CA3 pyramidal layer, blockade of glutamatergic receptors markedly diminished the preictal and ictal states, indicating that the generation of the ictal state in this model is dependent on excitatory synaptic transmission (Figure 5). Interestingly, glutamatergic receptor antagonists did not eliminate the low frequency interictal field discharges. This suggests that GABAergic activity may be capable of generating the interictal events with small amplitudes.

ii. The reversal potential of the spontaneous PSCs which correlated with the recurrent field potentials became more depolarized during the preictal state from that seen in the interictal state for both pyramidal cells and non-FS interneurons. This suggests that the balance moves towards excitation as the CA3 network proceeds to ictal onset (Table 2).

iii. Voltage-clamped intracellular recordings demonstrated that for both non-FS interneurons and pyramidal cells, the GABAergic currents were enhanced during the early preictal period and markedly diminished during the late preictal period, whereas the glutamatergic currents were gradually and markedly increased during the preictal period. When the GABAergic inputs were completely or almost completely abolished, the higher frequency ictal phase began (Figure 11, 12).

5. This loss of GABAergic input was not likely presynaptic since postsynaptic mucimol responses remained robust during the ictus (Figure 8).
6. Hypothesis #3: Alternatively, the transition to ictal onset could also be mediated by an increased synchronous GABAergic activity due to a depolarization of the GABA\textsubscript{A}-Cl reversal potential.

This hypothesis was not supported by the following findings:

i. During the interictal period, the recorded slightly more positive E\textsubscript{GABA\textsubscript{A}} than resting membrane potential may suggest a weak depolarizing action of GABAergic inputs onto pyramidal cells (Table 2). However, field recordings have suggested that synchronized GABAergic inputs are capable of generating interictal discharges in the absence of glutamatergic inputs.

ii. At the late preictal state, because the resting membrane potential of the neuron depolarized gradually, and it was difficult to measure the E\textsubscript{GABA\textsubscript{A}} in correlation with the resting membrane potential slow depolarization, thus it was hard to determine whether GABAergic activity is inhibitory or excitatory (Table 2).

iii. I would speculate that the strong excitatory drive observed at the seizure onset is not mediated by the depolarized GABA\textsubscript{A}-Cl reversal potential, because the E\textsubscript{PSCs} of both neurons are more depolarized during the preictal state.

However perforated patching technique and/or direct measurement of the intracellular Cl\textsuperscript{-} concentration are needed to determine the functional role of GABAergic inputs during the transition to seizure. Whole-cell patch-clamp could result an alteration in the intracellular Cl\textsuperscript{-} concentration, leading to an underestimation of the reversal potentials.

7. Comparing the PSCs reversal potentials between both neuronal types revealed that in the preictal phase, the reversal potential of the phasic rhythmic PSCs was more depolarized in the
non-FS interneurons than in the pyramidal cells, suggesting that the excitatory drive to non-FS
interneurons is greater than to pyramidal cells. The charge transfers underlying the synchronous
postsynaptic GABAergic and glutamatergic currents were also significantly larger in the CA3
non-FS interneurons than in the pyramidal cells (Table 2, Figures 11 and 12).
6. DISCUSSION

The strength and weakness of the low Mg$^{2+}$/high K$^+$ seizure model

As previously discussed (section 1.2.3), bath perfusing the hippocampal tissues with low Mg$^{2+}$ and high K$^+$ induce the generation of spontaneous and recurrent SLEs both in neonatal and adult rodents (Quilichini et al. 2002; Derchansky et al. 2004). Interictal, preictal, and ictal states can be easily defined according to the frequency of the discharges recorded extracellularly. Thus, this model allows us to study the changes occurred in the hippocampal network during the transition to seizure. In addition, the advantage of using isolated intact hippocampus is that this preparation preserves much of the hippocampal circuitry, thus it is useful to study both the cellular and network properties during seizure generation. However, in vitro studies of seizure transition are limited. Ultimately, this model must be translated into an in vivo model so that it has more clinical relevance.

Effects of glutamatergic and GABAergic blockade on epileptiform activity

In the epileptic brain, there exists an imbalance between the excitatory and inhibitory drives. Both glutamatergic and GABAergic synaptic transmission play a crucial role in the epileptogenesis. To study the effect of these synaptic transmissions on the epileptiform activity, glutamatergic and GABAergic antagonists were applied while prefusing the hippocampal tissue with the recurrent seizure producing perfusate, low Mg$^{2+}$/5mM K$^+$ SLEs. Low Mg$^{2+}$ and low K$^+$ perfusate does indeed produce robust seizure activity, as is well characterized from various studies (Anderson et al. 1986; Quilichini et al. 2002; Derchansky et al. 2004, 2006, 2008; Isaev et al. 2005). When the intact hippocampus was exposed to the AMPA receptor antagonist, CNQX,
and the NMDA receptor antagonist, APV, epileptiform activity was greatly diminished. The activities left had a low frequency and resembled the interictal firings observed in low Mg\(^{2+}\) induced epileptiform activities. BMI completely abolished the interictal state, diminished the preictal state, and decreased the duration of the inter-seizure interval, causing the system to become more sensitive to the depolarizing inputs. Together, these findings suggest that GABAergic inputs play a major role in generating the interical and early preictal bursts, and delay the transition to the ictal state. Glutamatergic inputs are required for the late preictal bursts and ictal onset.

The observed dominant glutamatergic inputs during the ictal state is consistent with previous studies wherein the low Mg\(^{2+}\) induced epileptiform activities in hippocampal slices were effectively attenuated with the application of APV and CNQX (Derchansky et al. 2004). AMPA receptor-channels mediate excitatory synaptic action by conduct Na\(^+\) and K\(^+\). NMDA receptor-channels are permeable to Ca\(^{2+}\) as well as to Na\(^+\) and K\(^+\), and the opening of the channel is voltage-dependent. Depolarization of the membrane is needed to remove the Mg\(^{2+}\) block at the pore of the channel allowing Ca\(^{2+}\) to move into the cell and lead to further depolarization. These excitatory bursts combined with action potential firings are synchronized among different neurons to produce the SLEs.

In addition to the excitatory glutamatergic synaptic inputs, the interneuronal GABAergic network is also involved in the generation of spontaneous rhythms in the intact isolated hippocampus (Wu et al., 2002), and in the entrainment of hyperexcitable states in the slice (Velazquez and Carlen, 1999; Kohling et al., 2000; Derchansky et al., 2004). Previous studies have shown that the functional alterations in GABA\(_A\) receptor-mediated effects occur for the
generation of SLEs. Picrotoxin and BMI have been used to shorten the inter-SLE interval and reduce the number of preictal discharges in CA1 and CA3 pyramidal cells (Marchionni et al. 2007; Derchansky et al. 2008; Lasztoczi et al. 2009). Surprisingly, we have also shown that the ictal phase was not prolonged by GABAergic blockade, suggesting that the generation of ictal phase is independent of GABA_A receptor activity. In addition, during the ictal phase, focal application of muscimol can induce outward currents and GABAergic interneurons still fire synchronously with the field. This suggests that the postsynaptic GABAergic receptor is intact. Thus we hypothesize that the lack of GABAergic activity during the ictal phase may be the result of impaired presynaptic GABA release or a depletion of GABA at the synaptic terminal. Future experiments testing this hypothesis are suggested in the next section.

Potential mechanisms responsible for the transition to seizure in CA3 region

The synergy of GABAergic and glutamatergic receptors plays a key role in enhancing neuronal activities in the hippocampal network and facilitating the generation of synchronized patterns of activities. By examining the functional dynamics of the GABAergic and glutamatergic synaptic inputs, we tried to elucidate the network mechanisms underlying the synchronous and hyperactive hippocampal network induced by low Mg^{2+} and high K^+. The observed neuronal hyperexcitability in epileptic seizures is associated with the altered oscillatory activity patterns ( Beenhakker and Huguenard 2009). The existing circuitry in the normal brain is the template upon which pathological changes are taken place. Thus alterations in the intrinsic and/or synaptic excitability would account for the pathological changes that promote network hyperexcitability ( BEENHAKKER AND HUGUENARD 2009). This study focuses on the CA3 region,
which is a critical region for the initiation of hippocampal SLEs in several different seizure models (Dzhala and Staley 2003; Derchansky et al. 2006, 2008). Neurons in CA3 region have enhanced cellular excitability and synaptic connectivity because of the recurrent circuitry. Especially, the CA3a subregion is considered to be the pacemaker region capable of initiating the synchronous activity of neuronal populations (Wittner and Miles et al. 2007).

In this study, the GABAergic inputs have shown to play a vital role in the generating the spontaneous and synchronized discharges during the interictal and early preictal periods. This is consistent with previous studies where they showed that the reinforcement and synchronization of inhibitory networks underlie the generation of ripples (Ylinen et al. 1995, Bragin et al. 1995) and the oscillations associated with preictal spikes (Khalilov et al. 2005, Lasztoczi et al. 2009). However, the exact mechanism for the generation of interictal and preictal spikes in the low Mg$^{2+}$/high K$^+$ seizure model remains unclear. Because GABA$_A$ plays a major role in the generation of interictal and early preictal discharges, the following mechanisms are proposed.

In the CA3 region, a population excitatory drive could be mediated by the recurrent EPSPs and spontaneous discharges of the pyramidal cells (Cohen and Miles, 2000). At the basal condition, the excitatory drive preferentially stimulates GABAergic inhibitory interneurons, because interneurons have a stronger EPSP-spike coupling than pyramidal cells (Fricker and Miles, 2000). After excitation, interneurons discharge cohesively and rhythmically because of their reciprocal inhibition and gap junctional intercommunication (Wu et al. 2002; Wu et al. 2009). These activated interneurons impose IPSPs onto a large number of pyramidal cells, thus generating spontaneous rhythmic field potentials (SRFPs) (Wu et al. 2002; Wong et al. 2005). With the perfusion of low Mg$^{2+}$/high K$^+$ solution, the CA3 recurrent circuitry is greatly
strengthened via NMDA receptor-dependent mechanisms, because of the removal of Mg$^{2+}$ block in the NMDA receptor. This strong CA3 excitatory drive would activate more interneurons and their reciprocal inhibitions as well as the gap junctional intercommunication. The repetitive IPSPs originating from a few interconnected presynaptic interneurons are effective in pacing and synchronize the spontaneous action potential generation in the postsynaptic pyramidal neurons (Cobb et al. 1997). As a result, spontaneous rhythmic interictal and preictal discharges are generated. These synchronous GABAergic activities play an important role in driving the coherent network oscillations. The induction of sharp waves has been suggested to be largely NMDA-dependent, which enhances the recurrent EPSPs among inter-connected CA3 pyramidal neurons (Wu et al. 2009).

In addition, strongly activated CA3 interneurons may fire rhythmically and synchronously to initiate the interictal events, since interictal discharges are shown to have a large inhibitory component. Field recordings have shown that synchronized GABAergic inputs are capable of generating interictal discharges in the absence of glutamatergic inputs. However because of the problems associated with the patch-clamp technique used, the interpretation of whether the close association of $E_{GABA_A}$ and resting membrane potential suggesting only a weak depolarizing action of GABAergic inputs onto pyramidal cells is in some question. Ben-Ari (2002) suggested that GABA$_A$ receptor-mediated activities are excitatory in neonatal (<postnatal week 2) hippocampal neurons. With low Mg$^{2+}$/high K$^+$ perfusion, the excited interneurons could fire rhythmically and synchronously through their reciprocal inhibitions and gap junctional coupling. The interictal discharges could be mediated by the excitatory actions of the postsynaptic GABAergic input onto pyramidal cells, through a positive shift in the GABA$_{A-Cl}$
reversal potential, which is evident comparing the reversal potential of the muscimol induced currents in ACSF and in low Mg/high K perfusate. The neurons of immature mice are known to have a weaker Cl\(^-\) exclusion ability than mature mice due to their lower expression of KCC2 (Ben-Ari 2002). This enhanced influx of Cl\(^-\) through NKCC1 would subsequently reverse the Cl\(^-\) gradient in pyramidal cells, causing a switch from inhibition to excitation (Fujiwara-Tsukamoto et al. 2006; Kaila et al. 1997; Staley et al. 1995; Lasztoczi et al. 2009). Thus, the recurrent connections among CA3 interneurons, pyramidal cells, as well as the gap junctional intercommunications, all synchronize into a rhythmic population event probably causing the interictal discharges. However, this depolarizing action of GABA was only observed during the interictal period. The transition from preictal to ictal was still dominantly by the enhanced excitatory glutamatergic drive. In the low Mg\(^{2+}/\)K\(^+\) seizure model, these two mechanisms of interictal discharge initiation could function together to generate the spontaneous and recurrent interictal events.

The mechanism for the transition from preictal to ictal state is likely due to a time-dependent dynamic change in the balance between GABAergic input and glutamatergic input. During the period of ictal initiation, there is increased spontaneously synchronized excitatory afferent glutamatergic activity and a gradual decrease in the synchronous inhibitory GABAergic activity (Figure 12). Previous studies have suggested similar mechanisms for the ictus initiation. The underlying mechanism for the ictogenesis has been proposed to be the synchronous activation of clusters of highly interconnected principal neurons that overpowers feedback inhibition (Bragin et al. 2004, 2005; Le Van Quyen et al. 2008). Derchansky et al. (2008) also suggested that mechanism in the CA1 region of the immature mouse hippocampus, both
inhibition persistence and enhanced excitation contributing to ictogenesis. In contrast, my study of the CA3 region of immature mouse hippocampus suggests a time-dependent decrease in the afferent inhibitory GABAergic activity together with an enhanced excitatory drive during the transition to ictal phase, possibly related to the idea that the afferent excitatory drive into the CA1 region from the “driving” CA3 region is continuously growing in the CA3 region during the transition to seizure.

**CA3 pyramidal cells vs. non-FS interneurons**

Similar patterns of postsynaptic current changes underlying the synchronized preictal discharges were observed in both CA3 non-FS interneurons and pyramidal cells, where activity-dependent weakening of GABAergic inhibition and enhanced glutamatergic excitation were recorded during the transition to seizure. However, the observed synaptic drive between pyramidal cells and non-FS interneurons were different. During the preictal phase, the reversal potential of the phasic rhythmic PSCs was more depolarized in the non-FS interneurons than in the pyramidal cells. The relative charge transfers underlying the synchronous postsynaptic GABAergic and glutamatergic currents were also significantly larger in the CA3 non-FS interneurons than in the pyramidal cells. These could be explained by the fact that excitatory drive preferentially stimulates GABAergic inhibitory interneurons than pyramidal cells (Fricker and Miles, 2000). Horizontal interneurons of the CA3 region have been suggested to be strongly active during epileptiform activity (Aradi and Maccaferri, 2004; McBain, 1994). These horizontal interneurons were also found to be more excitable than pyramidal cells because their latency to the first spike of the burst is shorter when compared to pyramidal cells (Maccaferri
2005). This suggests that the excitatory drive in non-FS interneurons is greater than in pyramidal cells, which explains the more depolarized $E_{\text{PSCs}}$ and larger excitatory drive in interneurons than pyramidal cells. In addition, the reciprocal inhibition among non-FS interneurons could result from the larger inhibitory drive received by interneurons than pyramidal cells.
7. LIMITATIONS AND FUTURE EXPERIMENTS

Potential mechanisms responsible for the transition to seizure

The functional role of GABA<sub>A</sub> receptors during the transition to seizure was studied only using GABA<sub>A</sub> receptor antagonist, BMI. It would also be interesting to apply GABA<sub>A</sub> receptor enhancers (ie, muscimol) to examine how the SLEs would be altered in this low Mg<sup>2+</sup>/high K<sup>+</sup> seizure model. Because GABA<sub>A</sub> receptors have been shown to play a crucial role in mediated the interictal and early preictal discharges using GABA<sub>A</sub> antagonist, I would expect that GABA<sub>A</sub> agonists should prolong or enhance the discharges during the preictal phase, with no effect on the ictal phase. This would further strengthen the hypothesis that the rhythmic discharges at the interictal and preictal state are mediated by the increased synchronous GABAergic activity. However, if the GABA<sub>A</sub> agonist application shows a reduction in the discharges during the ictal phase, this would suggest that during the ictal phase, the excitatory drive is significantly larger than the inhibitory drive. Thus blocking the GABA<sub>A</sub> receptors showed no effect on the duration of the ictal phase. When the GABAergic activities were enhanced with GABA<sub>A</sub> receptors agonist, the discharges during the ictal phase may be reduced due to the disrupted balance between excitation and inhibition.

Benzodiazepines and barbiturates have been clinically used for treating seizure (Shorvon 2009). They are positive allosteric modulators, which increase the activities of GABA<sub>A</sub> receptors indirectly via the activation of allosteric sites on the proteins. Benzodiazepines bind to the GABA<sub>A</sub> receptor complex and promote the binding of GABA, which in turn increase the conduction of chloride ions across the neuronal cell membrane (Riss et al. 2008). If there is an exhaustion of presynaptic GABA neurotransmitters release during the ictal phase, then the effect
of benzodiazepines on enhancing the activities of GABA<sub>A</sub> receptors will be limited. Thus, it would be interesting to examine how these clinically used anticonvulsants could alter the transition to transition.

In this study, the reversal potential of GABA<sub>A</sub>-mediated currents was determined by measuring the amplitude of the synchronous currents produced at various holding potentials using whole-cell patch clamp technique. Then current versus voltage curve was plotted to determine \( E_{GABA}\). There are several limitations of using this technique for measuring the reversal potential of Cl\(^-\). First, the high [Cl\(^-\)]<sub>i</sub> in neonatal hippocampal neurons is altered by the lower concentration of Cl\(^-\) in the recording pipette. Thus, the \( E_{GABA} \) is underestimated. Physiologically, \( E_{GABA} \) should be more depolarized. Second, changing in the membrane potentials can influence the activation of other voltage sensitive channels. The quality of the reversal potential measured is likely to be contaminated by the currents produced from other activated channels. Because of these limitations, the recorded \( E_{GABA} \) is not a good representation of the physiological \( E_{Cl} \). To overcome this issue, perforated patch technique, which prevents the alteration in [Cl\(^-\)]<sub>i</sub>, could be used to measure the GABA-evoked currents and also the \( E_{Cl} \). To determine the steady-state \( E_{Cl} \), Brumback and Staley (2008) used perforated patch technique to record the GABA-evoked currents during a series of voltage steps. They used bicarbonate-free ACSF to minimize bicarbonate flux through the GABA channel so that the \( E_{GABA} \) would approximate \( E_{Cl} \). To minimize the possibility of altering [Cl\(^-\)]<sub>i</sub> with large Cl\(^-\) influxes or effluxes associated with the different holding potentials, they limited the holding potential range to within ±15 mV of \( E_{Cl} \). In addition to the perforated patch technique, Nardou et al. (2009) used non-invasive single GABA channel recordings in chronic epileptic tissue to determine the \( E_{GABA} \).
GABAergic activities have been suggested to support the generation of interictal events, better techniques such as perforated patch-clamp or single channel recording should be used to determine the chloride reversal potential. This will allow a better understanding of how the Cl\textsuperscript{−} dynamic is actually shifting during the transition to seizure.

**Possible mechanisms underlying the weakening of GABAergic inputs**

The IPSCs correlated with field potentials were markedly decreased during the late preictal period and abolished at the ictal onset. The loss of inhibitory input onto pyramidal cells in the hippocampus may be mediated by the possible GABA depletion from over-active hippocampal interneuronal synaptic terminals. Failure of action potential mediated release of neurotransmitter may induce the weakening of GABA-mediated inhibition underlying the recurrent hyperactive neuronal activity in the epileptic states. Neurotransmitter depletion of the releasable glutamate pool was found at the recurrent synapses of the hippocampal CA3 pyramidal cells (Staley et al. 1998). During the epileptiform activity, CA3 interneurons are strongly activated (Aradi and Maccaferr 2004). The loss of inhibition in these hyperactive interneurons may be the result of a depletion of GABA or failure of GABA release.

To elucidate the underlying mechanism, I propose the following experiments:

One possible synaptic depression mechanism is the transient presynaptic ‘exhaustion’ of inhibitory neurotransmitter release. The enhanced CA3 interneuronal spiking activity would promote an increase of vesicle release of GABA. Several experiments can be performed to further explore the hypothesis that the abolished IPSCs at the ictal onset may be mediated by the
depletion of readily releasable GABA pool. First, we can focally apply a high concentration hyperosmotic fluid, 200-500 Osm sucrose, onto the soma of CA3 pyramidal cells to elicit the release of readily releasable pool of GABA. Picrospritzed sucrose should enhance presynaptic neurotransmitter release, thus allowing direct assessment of the supply of available releasable neurotransmitters (Staley et al., 1998). We predict that if there is nerve terminal exhaustion of GABA release during the late preictal phase, then focal sucrose application should not elicit any spontaneous IPSP/Cs, but after the SLE ends, this effect will recover. Another drug NO-711, which blocks the reuptake of GABA neurotransmitters, can also be used to determine if the depletion of releasable GABA pool is necessary for the seizure initiation. Marchionni et al. (2007) have shown that blocking the clearance of GABA with NO-711 enhanced the tonic conductance in pyramidal cells but not in stratum radiatum interneurons. If the diminished GABA-mediated current is mediated by the depletion of GABA release, NO-711 should enhance the IPSCs in neurons during the interictal and preictal state, delay the ictal onset, but have no effect on the activities of the ictal state.

Other possible explanations for the disappearance of large IPSCs are the internalization or desensitization of postsynaptic GABA<sub>A</sub> receptors (Goodkin et al. 2007; Thompason and Gahwiler, 1989). However, these mechanisms do not address the decreased GABAergic currents, because robust IPSCs were elicited with picospritzing muscimol during the ictal state where diminished spontaneous synchronous IPSCs were observed. Thus the function of postsynaptic GABA<sub>A</sub> receptors remains intact, and modulations of the GABAergic inputs are likely to occur presynaptically.
Interneuron diversity: Non-FS interneurons and FS interneurons

The location of the postsynaptic domain has a profound impact in determining the kinetics of the IPSC recorded on the soma of the target pyramidal cell (Maccaferri et al. 2000). Non-FS interneurons (likely to be O-bistartified and O-LM cells) synapse at more distal sites on the postsynaptic neurons, and thus the IPSCs originating from these cell types have small amplitude and slow kinetics (Ganter et al. 2004). Another group of interneurons found in the stratum oriens, the FS interneurons, which are mostly identified as the basket or axo-axonic cells with periosomatic-targeting horizontal axons (Maccaferri et al. 2000). FS interneurons are associated with higher rise-time kinetics and faster spiking phenotype than non-FS interneurons (Ganter et al. 2004). Persistent firing activity in perisomatic- versus dendritic-targeting interneurons would generate different responses in the postsynaptic cell. Thus it would be interesting to see if the dynamic change in the balance between inhibitory and excitatory inputs is similar between non-FS interneurons and FS interneurons.

Perisomatic vs. dendritic GABA<sub>A</sub> receptor-mediated inhibition

Our recordings were obtained somatically, but it is possible that significant dendritic GABA receptor-mediated inhibition and glutamate receptor-mediated excitation of pyramidal cells and interneurons were also present. There are evidences for functional difference between periosomatic inhibition and dendritic inhibition. Cossart et al. (2001) showed that loss of dendritic tonic inhibition was evident in brain tissue from patients with temporal lobe epilepsy, while the somatic inhibition from GABA<sub>A</sub> receptor was sustained. In our study, both EPSCs and IPSCs recorded were presumably from somatic and proximal dendrites, due to the space-clamp
issues. O-LM interneurons are known to have extensive dendritic regions innervating the pyramidal cells, because of their long axon arborisation and extensive branching at terminals (Sik et al. 1995). Thus GABAergic and glutamatergic inputs onto the distal dendrite of non-FS interneurons were overlooked. Future work is required to determine if the weakening of GABAergic inputs observed somatically in CA3 pyramidal cells and non-FS interneurons during seizure transition is also mediated by currents received in remote dendritic sites. The most direct experimental approach is duel voltage-clamp recording measuring the GABAergic input to the soma and distal dendrite (Marchionni and Maccaferri 2009).

**Technical issues:**

- Space-clamping issue in voltage-clamped recording from O-LM interneurons and pyramidal cells. This is common problem for voltage-clamped recordings from spatially distributed central neurons. Intracellular cesium could be used to block K currents and to improve the quality of the voltage clamp.

- When using the whole-cell patch clamp technique to measure the $E_{\text{GABA}_A}$, the intracellular $\text{Cl}^-$ concentration is altered by the lower concentration of $\text{Cl}^-$ in the pipette. Perforated patching or non-invasive single GABA channel recordings should be utilized for more accurate measurement of $E_{\text{GABA}_A}$ and $E_{\text{Cl}^-}$. 

8. REFERENCES


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