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HCN Channels and Regulation of Neocortical Network Activity

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ABSTRACT

Hyperpolarization activated non-specifc cation (HCN) channels are unique channels that activate following membrane hyperpolarization. Expressed primarily along the apical dendrites of pyramidal neurons, they pass a non-inactivating, inward current (I_h). HCN channel activation at resting membrane potentials profoundly impacts the synaptic and intrinsic properties of pyramidal neurons. Activated channels decrease intrinsic membrane excitability, hyperpolarize the resting membrane potential, and increase excitatory post-synaptic potentials (EPSP) summation. Loss of HCN channels is commonly observed in models of epilepsy. This dissertation tests the hypothesis that the influence of HCN channels on individual neuron excitability translates to an influence on network excitability. Furthermore, we hypothesize that loss of HCN channels in a model of malformation epilepsy contributes to observed hyperexcitability. We found that rats with freeze induced cortical lesions, a well-established model of malformation epilepsy, have reduced I_h, significantly increased EPSP summation, and increased membrane excitability. Using voltage sensitive dye imaging, we found that freeze lesioned rats exhibit significantly increased network excitability. Inhibiting HCN channels with ZD 7288 similarly increases network activation. Enhancing HCN channels with the anticonvulsant lamotrigine reduces network excitability. The ability of lamotrigine to reduce network excitability is significantly reduced in freeze lesioned rats. We next examined whether HCN channels influence epileptiform network events. Epileptiform

events were evoked *in situ* using strong stimulation in disinhibited acute cortical slices. We found that HCN channel inhibition significantly increases the area of epileptiform events in pyramidal neurons from layers 5 and 2/3 and well as interneurons from layers 5 and 1. Interesting, ZD 7288 also increases the number of action potentials overlying epileptiform events, but only in layer five pyramidal neurons. This increase in area is mimicked when neurons are voltage clamped at -60 mV indicating that the increase in area is a network effect. We also found reduced I_h in layer 5 interneurons in freeze lesioned rats. ZD 7288 increases summation in layer 5 interneurons from control, but not lesioned rats. HCN channel inhibition decreases interneuron membrane excitability, and rats with freeze lesions have reduced baseline membrane excitability.

DEDICATION

This work is dedicated to my mom.

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TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

LIST OF FIGURES vii

INTRODUCTION 1

Epilepsy 3
HCN Channels 13
HCN Channels in Epilepsy 21

DECREASED HYPERPOLARIZATION-ACTIVATED CURRENTS IN LAYER 5 PYRAMIDAL NEURONS ENHANCES EXCITABILITY IN FOCAL CORTICAL DYSPLASIA 28

REGULATION OF NEOCORTICAL EPILEPTIFORM DISCHARGES BY HCN CHANNELS 67

RATS WITH CORTICAL MALFORMATIONS HAVE REDUCED IH IN LAYER V INTERNEURONS 101

CONCLUSIONS AND SUMMARY 132

LIST OF GENERAL REFERENCES 142

APPENDIX: IACUC APPROVAL FORM 156

LIST OF FIGURES

Figure *Page*

INTRODUCTION

1 Summary of Changes in HCN Channel Gating Observed in the Pilocarpine Model of Epilepsy 26

2 Summary of the Excitability Changes Observed in Following Loss of I_h.....27

DECREASED HYPERPOLARIZATION-ACTIVATED CURRENTS IN LAYER 5 PYRAMIDAL NEURONS ENHANCES EXCITABILITY IN FOCAL CORTICAL DYSPLASIA

1 L5 pyramidal neurons from freeze lesioned rats have depolarized membrane potentials and increased input resistances.....57

2 Reduction in I_h-dependent voltage changes in L5 pyramidal neurons from lesioned animals.....58

3 L5 pyramidal neurons from freeze lesioned rats have increased intrinsic excitability.....59

4 Voltage clamp recordings of I_h in neurons from sham-operated and lesioned animals 60

5 Effects of ZD7288 on EPSP summation in sham-operated and lesioned animals .61

6 Voltage sensitive dye imaging of evoked activity. *A:* photograph showing the typical position of the brain slice over the diode array.....62

7 Comparison of voltage-sensitive dye signals in control and lesioned animals.....63

8 HCN channel inhibition increases the duration of evoked network activity 64

LIST OF FIGURES CONTINUED

Figure *Page*

9 Effects of the anticonvulsant lamotrigine on evoked network activity.....65

10 Sensitivity of network activity to lamotrigine 66

REGULATION OF NEOCORTICAL EPILEPTIFORM DISCHARGES BY HCN CHANNELS

1 Ih Inhibition Increases the Severity of Epileptiform Events in Layer V and Layer 2/3 Pyramidal Neurons 94

2 Ih Inhibition Increases the Severity of Epileptiform Events in Layer V and Layer 2/3 Pyramidal Neurons 95

3 Ih Inhibition Increases the Severity of Epileptiform Events in Layer V Interneurons 96

4 Ih Inhibition Increases the Severity of Epileptiform Events in Layer I Interneurons. 97

5 Ih Inhibition Increases Epileptiform Events Severity by Enhancing Network Activation.....98

6 Increases Epileptiform Event Severity Masks a Late GABAB Mediated Hyperpolarization 99

7 Ih Enhancement Reduced the Severity of Epileptiform Events in Layer One Interneurons 100

RATS WITH CORTICAL MALFORMATIONS HAVE REDUCED IH IN LAYER V INTERNEURONS

1 I_h Associated “Sag” and “Rebound” Voltages are Reduced in Interneurons from Animals with Transcortical Freeze Lesions.....126

LIST OF FIGURES CONTINUED

Figure *Page*

2 HCN Channel Inhibition Does not Significantly Influence Single EPSP in Layer V Fast Spiking Interneurons 127

3 HCN Channel Inhibition Significantly Increases EPSP Summation in Layer V Fast Spiking Interneurons 128

4 HCN Channel Inhibition does not Increases EPSC Summation in Layer V Fast Spiking Interneurons.....129

5 HCN Channels do not Signficantly Influence Release at Excitatory Synapses on to Layer Five Interneurons.....130

6 HCN Channel Inhibition Significantly Decreases the Firing Frequency of Fast Spiking Layer V Interneurons.....131

INTRODUCTION

As our understanding of the central nervous system grows, the diversity and complexity of the individual neurons within it becomes more apparent. With this complexity comes the challenge of understanding how these units interact as a network. The brain consists of distinct but interacting networks of neurons. These include the hippocampus, cerebellum, and neocortex (Bock et al. 2011; Apps and Hawkes 2009; Varga et al. 2009). Neuronal networks are composed of two components, the excitatory which drives cells toward action potentials and the inhibitory which drives them away. The inhibitory component is mediated primarily by interneurons (Markram et al. 2004) while the excitatory component primarily by principle cells like pyramidal neurons (Yuste 2011). The interaction between these two network components on post-synaptic elements largely determines the likelihood of neurons reaching action potential threshold and eliciting information carrying spikes (Yuste 2011).

The behavior of inhibitory and excitatory neurons and their interaction regulates the network in which they subsist (Sun et al. 2006). Imbalance of inhibitory and excitatory neurons causes aberrant network activity (Yizhar et al. 2011). Strong excitatory signaling is often followed by a slower inhibitory signal which may serve to dampen network activity (Silberberg and Markram 2007). Blocking *gamma*-Aminobutyric acid (GABA) type A mediated inhibition permits the occurrence of evoked epileptiform discharges in brain slices (Gutnick et al. 1982) while anesthesia can be achieved by enhancing inhibition (Franks 2008).

The behavior of neurons is regulated in large part by their intrinsic conductances. These conductances regulate their ability to elicit spikes (Gutnick et al. 1982), their ability to detect inputs from surrounding cells (Williams and Stuart 2000), and their resting membrane potential (Sutor and Hablitz 1993; Huang and Trussell 2011). Different classes of neurons, including excitatory and inhibitory neurons, express unique intrinsic conductances (Markram et al. 2004; Connors et al. 1982). Since these conductances regulate the behavior of individual neurons within the network, they also regulate the behavior of the network as a whole. Interestingly, intrinsic conductances are subject to activity dependent plasticity (Desai et al. 1999).

As a tribute to the crucial nature of intrinsic neuronal conductances in determining network behavior, disruptions of these conductances often cause epileptic events in the network (Catterall et al. 2008). The work in this dissertation focuses primarily on one of these conductances: the hyperpolarization activated non-specific cation (HCN) channel and its role in regulating the properties of both excitatory and inhibitory elements of the neocortical network. The work has several broad goals. The first is to better understand how HCN channels modulate the way in which normal activity spreads through cortical networks, and how that modulation might be altered in cases of cortical dysplasia. The second is to understand how HCN channels contribute to the shape and nature of epileptiform discharges in disinhibited cortex. The third is to understand how HCN channels modulate individual excitatory and inhibitory elements of the neocortical network and how differential modulation of these neuron types may shape network behavior.

Epilepsy

Epilepsy is a disorder characterized by spontaneous, recurring seizures. Seizures are the result of pathologic, synchronous, electrical activity within the brain often associated with hyperexcitable neurons (Scharfman 2007). Seizures have the ominous history of once being associated with demonic possession. (Scharfman 2007). It is now understood that epileptic seizures are caused by many factors. Genetic mutations may cause epilepsy such as the especially severe Dravet Syndrome (Claes et al. 2001). Post-traumatic brain injuries commonly precedes the development of epilepsy via multiple mechanisms (Hernandez and Naritoku 1997; Prince et al. 2009). Severe fevers may cause seizures, and in some cases lead to epilepsy (Dube et al. 2010). Hyper and hypoglycemia both cause seizures (Moien-Afshari and Tellez-Zenteno 2009). Seizures themselves are also hypothesized to contribute to future seizure occurrence (Ben-Ari et al. 2008). Interestingly, a ketogenic diet is hypothesized to be anticonvulsant (Bough and Rho 2007). While the majority of epilepsy patients gain seizure control either through anticonvulsant therapy or surgery, a small number are resistant to all treatment (Cascino 2008). Developmental cortical malformations are associated with severe, pharmaco-resistant epilepsies (Leventer et al. 2008). Parts of this dissertation will focus on understanding the pathology of malformation epilepsy.

Epilepsy Mechanisms

The causative mechanisms underlying epilepsy are poorly understood. Given the diversity of epilepsy syndromes, the mechanisms are likely also diverse (Kandel et al. 2000). Disruptions of the balance between excitation and inhibition are often cited as a

possible cause of epilepsy. For example, excessive glutamate release from glioblastomas may contribute to tumor associated seizures (Buckingham et al. 2011). Another example is seen in the unique expression pattern of the chloride transporters, NKCC1 and KCC2 in the developing brain. Early in development, the pattern of NKCC1/KCC2 expression depolarizes the reversal potential for chloride. This causes $GABA_A$ mediated currents to be excitatory (Ben-Ari 2002) and may disrupt excitation and inhibition. Epileptiform activity in young brains is resistant to barbiturate treatment which acts through $GABA_A$ receptors, but can be ameliorated by pharmacologically shifting internal Cl^- concentration until GABA is inhibitory (Dzhala et al. 2005). Additionally, intracellular accumulation of chloride during seizures may also shift intracellular Cl^- concentration in the depolarizing direction, potentially contributing to future seizure occurrence (Dzhala et al. 2010).

Changes in ion channels occur with a variety of epilepsy syndromes. Many such ion channel pathologies are due to genetic mutations (Catterall et al. 2008). Mutations in low-voltage activated T-type calcium channels are associated with absence epilepsy in rats (Powell et al. 2009) as well as humans (Chen et al. 2003). Dravet syndrome, a severe, pharmacoresistant, childhood epilepsy characterized by tonic-clonic seizures, is caused by mutations in voltage gated sodium channels (Claes et al. 2001). Mutations in the voltage gated potassium channel KCNQ2 are associated with benign familial neonatal convulsions (Biervert et al. 1998). Polymorphisms in voltage gated chloride channels are hypothesized to contribute to epilepsy (D'Agostino et al. 2004). Finally mutations in GABA and aceylcholine receptor ion channels may also contribute to epilepsy (Scheffer and Berkovic 2003).

Seizures and epilepsy are also associated with non-genetic changes in ion channels which may contribute to hyperexcitability. Whether these changes are secondary to, or causative of, epileptic phenomena is debatable. Persistent sodium currents are unique currents which do not rapidly inactivate like traditional sodium currents. Small increases in the current may significantly increase neuronal firing, and persistent sodium currents are reduced by several anticonvulsant drugs (Stafstrom 2007). Increased persistent sodium currents have been indicated in resected subiculum tissue from patients with temporal lobe epilepsy (Vreugdenhil et al. 2004). Additionally, CA1 pyramidal neurons from rats exposed to high doses of pilocarpine, a commonly used model of epilepsy, exhibit increased persistent sodium currents (Vreugdenhil et al. 2004). Hippocampal T-type calcium channels are also upregulated in this model of epilepsy and may also contribute to epileptogenesis (Becker et al. 2008). This dissertation focuses on non-genetic changes in another ion-channel, the HCN channel which may contribute to epileptic hyperexcitability.

Models of Epilepsy

The relative rarity of human epilepsy tissue paired with the absence of proper human control tissue has driven the development of animal models of epilepsy and seizures. The diversity of epilepsy syndromes has also promoted the development of many different types of animal models. While knock-out and transgenic models have furthered understanding of genetic channelopathies and associated epilepsies, many induced animal models of epilepsy are used to aid understanding of more idiopathic syndromes such as generalized temporal lobe epilepsy or malformation epilepsy.

Exposure to a convulsant agent is one of the most commonly used models of temporal lobe epilepsy. High doses of the muscarinic agent pilocarpine or the glutamatergic agent kainic acid cause a severe seizure followed by a seizure-free latent period. Interestingly, following the latent period, which lasts a variable number of days, spontaneous seizures begin to occur (Curia et al. 2008; Le et al. 2008; Williams et al. 2009). The latent stage is characterized by greatly increased hippocampal excitatory drive (Smith and Dudek 2001; Shao and Dudek 2004). Animals reaching the latent phase also exhibit histological signs of temporal lobe epilepsy including mossy fiber sprouting and cell loss (Le et al. 2008; Curia et al. 2008). A limitation of the convulsant model is its dependence on an initial high grade seizure. This confounds determination of which observed brain changes are epileptogenic and which are non-epileptogenic, but still secondary to the high levels of brain activity seen during the convulsant exposure.

The kindling model of epilepsy uses gradual and repetitive electrical stimulation of the amygdala or hippocampus to eventually elicit chronic spontaneous seizures. As with chemical induction, spontaneous seizures typically begin after a latent period of variable length (Loscher 2002). The occurrences during the latent period which lead to spontaneous seizures are an enticing area of study. Similar to convulsant models, cell loss and mossy fiber sprouting (Li et al. 2003; Loscher 2002) occurs. Also, as with convulsant models, determining which brain changes following kindling contribute to recurring seizures is problematic.

Febrile seizures are a relatively common and usually benign occurrence in children. They are however correlated with increased risk of temporal lobe epilepsy (Cendes et al. 1993; Huang and Chang 2009). Modeling febrile seizures simply involves

exposing rat pups to hyperthermia until seizures are observed (Dube et al. 2010). Rat pups which experience prolonged febrile seizures may developed temporal lobe epilepsy (Dube et al. 2006) and repeated febrile seizures may impair long term potentiation in the hippocampus (Huang and Chang 2009). Similar to kindling and convulsant models of epilepsy, febrile seizures induce mossy fiber sprouting, but in contrast to both kindling and convulsant models, they do not induce cell loss (Bender et al. 2003). The differences between the long term consequences of febrile seizures and the kindling/convulsant induced seizures raises interesting questions regarding the pathogenesis of long term epilepsy. Both models are dependent on initial episodes of seizures, but the outcome of an early life febrile seizure is clearly less severe. The exact role of single epileptiform events in inducing future seizures and the nature of initial epileptogenesis requires continued investigation.

As discussed, head trauma is often associated with the development of epilepsy (Hernandez and Naritoku 1997). A commonly used model of post-traumatic epilepsy is the "undercut model." Generation of this model involves isolating part of the cortex by cutting through cortical layers with a bent needle in an anesthetized animal. Acute cortical slices containing the undercut cortex exhibit spontaneous and evoked epileptiform events (Prince and Tseng 1993; Li et al. 2005). Neurons also have significant changes in synaptic physiology. It is interesting to consider the differences in the genesis of hyperexcitability in this model, which does not rely on an initiating electrical event like kindling or convulsant exposure. It further begs the question of whether epileptogensis is reliant on common mechanisms or if it is a symptom of multiple different causative factors.

Seizure-like activity is also modeled in acute brain slices. This is accomplished by disrupting the balance of excitation and inhibition. Reducing inhibitory transmission with the $GABA_A$ antagonist bicuculine is a well-established model of in-situ epileptiform events (Gutnick et al. 1982). Stimulating the neocortex under these conditions causes run-away excitation characterized by large, long-lasting, depolarizations with overlying action potentials (Gutnick et al. 1982; Lee and Hablitz 1991). Removing extracellular magnesium and increasing extracellular potassium from the solution surrounding acute hippocampal slice from young rats causes spontaneous epileptiform discharges (Derchansky et al. 2004). Such *in vitro* models allow more direct study of hyper-excitability mechanisms than induced models of epilepsy due to precise pharmacologic control of the epileptiform inducing changes. An especially interesting model of *in vitro* epilepsy is the 4 amino-pyridine model (4AP). Stimulating the cortex in the presence of 4AP, a potassium channel blocker, as well as glutamatergic antagonists results in long-lasting, synchronous inhibitory discharges (DeFazio and Hablitz 2005; Benardo 1997). This model is useful for determining the role of synchronized inhibition in epileptiform events as well as studying inhibitory network connections.

Cortical Dysplasia

Malformations of cortical development such as lissencephaly, polymicrogyria, or schizencephaly are commonly associated with epilepsy (Guerreiro 2009). Much of the work in this dissertation focuses on changes in the intrinsic properties of neurons in a particular type of developmental malformation, focal cortical dysplasia. It is estimated that up to 40% of intractable childhood epilepsies occur with cortical dysplasia (Leventer

et al. 2008). Cortical dysplasia is usually characterized by disruptions of normal cortical lamination with nearby gliosis. This malformation may occur with graded severity ranging from a near microscopic disruption to much larger abnormalities (Guerreiro 2009). Regions of dysplasia are not associated with macroscopic abnormalities, a characteristic which impedes identification and surgical removal of lesions (Taylor et al. 1971). Neurons in dysplastic tissue may have abnormal morphologies such as cytomegally, disrupted branching, and an immature profile (Cepeda et al. 2003). These neurons also exhibit abnormal conductances and may receive excitatory GABA signals (Cepeda et al. 2003; Cepeda et al. 2007). Dysplastic tissue is characterized by intrinsic epileptogenicity (Guerreiro 2009). Current antiepileptic drugs are often ineffective in patients with cortical dysplasia (Mathern et al. 1999) leaving surgical excision of the dysplastic cortex as the preferred treatment (Sisodiya 2000). Even surgery however may be ineffective at giving patients seizure freedom (Krsek et al. 2009; Sisodiya 2000).

Epilepsy associated with cortical dysplasia motivates many intriguing questions. What is the nature of the intrinsic epileptogenesis associated with the abnormal cortical structure? What is unique about mal-developed cortex which causes hyperexcitability? The resistance of epilepsies associated with cortical malformations to anticonvulsant treatment suggests unique mechanisms of epileptogenesis. Given the unsatisfactory success rate in treating epilepsy associated with cortical dysplasia, understanding the nature of its drug resistance is especially crucial. Finally, as with any disease model system, changes in the normal physiology of the system allow for conclusions to be drawn about that system's normal function. Study of cortical dysplasia associated epilepsy provides a unique opportunity for better understanding the normal activity of the

neocortical network. The findings described above were observed in resected human tissue; however, several animal models of cortical malformations also exist.

Models of Cortical Dysplasia

The aptly named *mind bomb* strain of zebrafish has a large, developmental, total-brain malformation due to errors in the developmental signaling protein, notch. Given the association of epilepsy with a variety of gene mutations, zebrafish, *Danio rerio*, are an interesting and useful model for study of epilepsy. Their fast reproduction rate, aquatic environment, and low cost of captivity make them an ideal tool for mutagenic screening (Hortopan et al. 2010b). *Mind bomb* zebrafish have severe behavioral seizures as well as spontaneous spike-burst discharges apparent in forebrain field recordings (Hortopan et al. 2010a).

In utero radiation exposure also results in wide spread brain malformations. Radiation produces wide-spread cortical injury primarily effecting younger neurons early in development. Radiation exposed rats develop microcephally, diffuse cortical dysplasia, and the presence of ectopic neuron clusters (Roper 1998). Radiation exposed rats also exhibit ictal events visible with EEG as well as tissue hyperexcitability (Roper 1998). Neurons in this dysplastic cortex have reduced GABAergic signaling (Zhu and Roper 2000) and decreased staining for excitatory terminal markers (Zhou and Roper 2010). The severity of the cortical dysplasia in this model limits its usefulness as a tool for understanding malformation epilepsy in humans.

In utero exposure to the DNA methylating agent methylazoxymethanal (MAM) also results in severe cortical malformations. MAM exposed rats have homogenous

cortical dislamination, microencephally, as well as large heterotopic cell groups in the hippocampus and cortex (Garbossa and Vercelli 2003; Garbossa and Vercelli 2003). Hippocampal and cortical neurons from MAM exposed rats have increased excitability (Baraban and Schwartzkroin 1995; Colacitti et al. 1999). Rats also exhibit subclinical seizures visible with EEG (Harrington et al. 2007) and increased susceptibility to kainic acid induced seizures (Germano and Sperber 1997). As with pre-natal radiation exposure, the severity and extent of the malformation limits its relevance to study of human disease. The *Mind bomb*, radiation, and MAM models all fail to replicate the more focal nature of many human cortical dysplasias. The severity of the defects may preclude elucidation of the specific changes underlying the epileptogenic nature of small groups of dysplastic neurons.

Exposing the skull of post-natal day 1 (P1) rats to a cooled copper rod produces a small focal lesion in the cortex by killing young, migrating cells in deep cortical layers (Dvorak and Feit 1977). This is followed by abnormal migration of neurons into more superficial layers. The lesion is characterized by having only four cortical layers, gliosis, and a small visible microgyrus against the normally smooth surface of the rat brain (Dvorak and Feit 1977; Dvorak et al. 1978; Hablitz and Defazio 1998). The microgyrus does not develop if the lesion is made following the end of cortical neuronal migration at P4 (Dvorak et al. 1978). The focal nature of the cortical malformation in the freeze lesion model and its developmental dependence make it an ideal system to model human developmental cortical dysplasia. The lesion is characterized by a surrounding region of hyperexcitability. Stimulation within this region elicits epileptiform events in acute slices (Jacobs et al. 1996; Hablitz and Defazio 1998) and rare spontaneous epileptiform events

in this region have been reported (Jacobs et al. 1999). Freeze lesioned rats are also more susceptible to hyperthermic seizures (Scantlebury et al. 2004).

Many of the causative factors behind the hyperexcitability and epileptogenic nature of the dysplastic tissue in the freeze lesion model and human cortical dysplasia still need to be elucidated; however several factors have been implicated. NMDA receptor subunit type 2B (NR2B) expression is enhanced in freeze lesioned cortex and contributes significantly to epileptiform activity evoked there (DeFazio and Hablitz 2000). Radioligand binding to $GABA_A$ is significantly reduced in lesioned cortex while binding to kainate and AMPA receptors is significantly increased (Zilles et al. 1998). The hyperexcitable cortex near the lesion expresses decreased glial glutamate transporter activity (Campbell and Hablitz 2008) as well as decreased astrocytic inwardly rectifying K^+ currents (Bordey et al. 2001). Both interneurons and pyramidal neurons within the cortex near the freeze lesion receive increased excitatory drive (Jacobs and Prince 2005; Brill and Huguenard 2010). This drive is hypothesized to stem from developmentally mistargeted axons (Jacobs and Prince 2005). Another possible contributor to hyperexcitability are alterations in intrinsic conductances. Such changes have been implicated in the freeze lesion model (Luhmann et al. 1998). The work in this dissertation focuses on how HCN channel mediated conductances may change network behavior and how alterations in this channel may contribute to the hyperexcitability observed in the freeze lesion model of epilepsy.

HCN Channels

HCN channels are a unique set of ion channels which progressively activate following by hyperpolarization negative to -60 mV. These channels conduct a slowly-activating, inward, cationic current (I_h) that counteracts hyperpolarization and depolarizes the cell (Robinson and Siegelbaum 2003). I_h was first identified in cardiac sinatrial node cells in which it is often referred to as I_f for funny current (Brown et al. 1979). Here it activates following the large afterhyperpolarization of sinatrial spikes and drives the cell toward depolarization (DiFrancesco 1993) facilitating cardiac pacemaking. HCN channels in concert with Ca^{2+} channels serve a similar role in the pacemaking of some neuronal cell types (Pape 1996). The channels flux both Na^+ and K^+ with a greater conductance for K^+; however activation at hyperpolarized potentials means that the majority of I_h is mediated by Na^+ (Robinson and Siegelbaum 2003). The channel can be blocked with either extracellular cesium (Cs^+) or the bradycardic agent ZD 7288 (Harris and Constanti 1995).

HCN channels are encoded by 4 genes (HCN1-4) and are members of the voltage gated K^+ ion channel super-family. They express six hydrophobic segments and form tetrameric channels (Much et al. 2003; Ludwig et al. 1998; Robinson and Siegelbaum 2003; Santoro and Tibbs 1999). Exclusive expression of only one subunit of HCN channel (1-4) reveals unique activation kinetics of each channel with HCN1 having the fastest rate of activation and HCN4 having the slowest (Moosmang et al. 2001). The distinct properties of each subunit raise the possibility of unique expression patterns of I_h in individual neurons. This could either occur via expression of a unique pattern of homomeric tetramers or through the expression of heteromeric tetramers. Co-expression

of different subunits in oocytes results in currents which are intermediate to the currents produced by exclusive expression (Chen et al. 2001b). HCN channel subunits are capable of co-assembly in cultured expression systems (Much et al. 2003).

Another unique property of HCN channels is their ability to be modulated by cyclic AMP. All four subunits express a C-terminal cyclic nucleotide binding domain (Santoro and Tibbs 1999). Direct binding of cAMP to the cyclic nucleotide binding domain of the subunits greatly speeds the time constant of channel activation. cAMP also shifts the voltage dependent activation of I_h to more depolarized potentials (Wainger et al. 2001). Furthermore, cAMP has different levels of influence over different HCN channel subunits (Chen et al. 2001b; Wainger et al. 2001). The cAMP sensitivity of the channel allows neuromodulatory compounds to influence channel properties through activation of cAMP. β-adrenergic modulation of cAMP and consequently cardiac HCN channels modulates heart rate (Santoro and Tibbs 1999). Dopamine in the central nervous system also has a large influence on I_h through a cAMP dependent mechanism (Wu and Hablitz 2005; Rosenkranz and Johnston 2006)

In addition to their distribution within cardiac tissue, HCN channels are also widely expressed within the central nervous system. HCN1 and HCN2 are by far the most prominently expressed subunits within the brain with HCN3 and HCN4 showing only minimal expression (Notomi and Shigemoto 2004). Immunoreactivity for HCN channels is most prominent in the dendrites of hippocampal and cortical pyramidal neurons (Notomi and Shigemoto 2004; Lorincz et al. 2002). Spines and shafts of pyramidal neuron dendrites express HCN channels. Additionally, some HCN channels have been observed within inhibitory neurons of the hippocampus (Lorincz et al. 2002).

HCN channel expression increases greatly with age, though developmental channel regulation differs amongst brain sub-regions (Brewster et al. 2007). Perhaps the most interesting aspect of nervous system HCN channel distribution is its polarized expression along pyramidal dendrites. Distal dendrites express significantly more HCN channels than proximal dendrites (Lorincz et al. 2002). This distribution places HCN channels in a unique position to filter dendritic inputs.

The role of I_h within non-pacemaking, hippocampal or cortical pyramidal neurons of the central nervous system has been extensively investigated. Most of its role within these cells is due to the channel's moderate activation level at rest and non-uniform dendritic distribution. The polarized distribution of HCN channels along the membrane of the apical dendrites of pyramidal neurons is mirrored by gradual increases in the amplitude of I_h in dendritic recordings made progressively further from the soma (Berger et al. 2001). This contributes to dendritic membranes having a much greater conductance than the soma (Magee 1998). Dendritic I_h increases the electrotonic length of the dendrite meaning that current changes in the distal dendrite are subject to more filtering prior to reaching the integration point at the soma (Fernandez et al. 2002). HCN channels are therefore positioned to modulate the properties of synaptic inputs and influence the integration of information. EPSPs from distal and proximal inputs have a similar time course of decay upon reaching the soma, despite distal inputs having propagated through a significantly greater length of membrane. This normalization of EPSP time constant is lost when HCN channels are inhibited (Williams and Stuart 2000; Berger et al. 2001). The shortening of the time course of distal EPSPs in the presence of higher HCN channel density is hypothesized to be caused by HCN channel closing following EPSP

depolarization. The loss of the inward I_h current is a net outward current that may hasten EPSP decay (Williams and Stuart 2000).

The attenuating influence of HCN channels on synaptic inputs has strong functional consequences for integration. Blockade of HCN channels greatly increases the peak voltage reached by temporal summation of EPSPs (Magee 1999). Enhancing I_h with the anticonvulsant drug lamotrigine decreases the peak voltage reached by summation of EPSPs (Poolos et al. 2002). HCN channels normalize EPSP summation from both distal and temporal inputs (Magee 1998; Magee 1999). In agreement with the polarized distribution of HCN channels along dendrites, HCN channel inhibition fails to influence the summation of EPSPs originating near the soma (Varga et al. 2009). The increased time constant of EPSPs following HCN channel inhibition allows subsequent EPSPs to start at more depolarized voltages. Interestingly, I_h similarly reduces EPSP summation in the elaborate dendrites of cerebellar purkinje cells (Angelo et al. 2007).

HCN channels active at rest also significantly influence the intrinsic properties of neurons. The inward current mediated by I_h depolarizes the resting membrane potential by approximately 4 to 5 mV (Magee 1998). Inhibition of I_h and consequent loss of the depolarizing conductance significantly increases the input resistance of cellular membranes. Despite the hyperpolarizing effect of I_h inhibition, this is associated with an increase in membrane excitability (Magee 1998). Enhancement of the HCN channels with the anticonvulsant lamotrigine decreases dendritic excitability (Poolos et al. 2002). HCN channels also decrease the peak voltage reached by EPSPs by activating the slow K^+ current called the M current (George et al. 2009). Furthermore, synaptically evoked dendritic Ca^{2+} spikes are constrained by active I_h (Tsay et al. 2007). Taken together loss

of HCN channels, while associated with membrane hyperpolarization significantly increases cellular excitability, both by increasing the intrinsic excitability of membranes and increasing effectiveness of EPSP summation at driving the membrane toward threshold.

Another potential site of action for HCN channels is the presynaptic terminal. HCN channels have been reported in several types of presynaptic terminals. At these locations activation of the channel at rest could significantly influence release. Within the entorhinal cortex, HCN channel inhibition significantly increases release probability from layer 3 excitatory synapses onto pyramidal neurons. I_h reduces release probability from these sites via a slight depolarizing effect of its current which increases resting level inactivation of T-type Ca^{2+} channels (Shah et al. 2004). HCN channels do not influence release from pyramidal neuron synapses in other layers suggesting cell specific use of this mechanism to regulate release. HCN channel inhibition also increases release probability at immature, but not mature perforant pathway synapses (Bender et al. 2007). I_h enhancement and consequent membrane depolarization via serotonin mediated upregulation of cAMP, increases release probability at crayfish neuromuscular junction (Beaumont and Zucker 2000). Greater I_h increasing release probability at this synapse in contrast to less I_h increasing release probability in entorhinal cortex and the immature hippocampus indicates heterogeneity of possible mechanisms by which axonal I_h may influence release.

HCN channels are expressed in some interneurons (Lorincz et al. 2002), however their function is not as well understood. Interneurons express varying levels of I_h (Maccaferri and McBain 1996; Wu and Hablitz 2005; Aponte et al. 2006). However,

interneurons lack the long apical dendrites along which HCN channels are usually expressed (Markram et al. 2004) which may preclude HCN channels from playing a similar role in synaptic integration. HCN channels have been highly implicated in the control of presynaptic GABA release from some interneurons. Immunogold labeling reveals HCN channels in the presynaptic compartments of cerebellar basket cell terminals (Lujan et al. 2005a). Inhibiting HCN channels decreases the frequency of mIPSCs in granule cells within the dentate gyrus by presumably decreasing the release probability of fast spiking basket cell terminals (Aponte et al. 2006). HCN channels also affect the action potential dependent properties of some interneurons. The I_h enhancing drug lamotrigine increases the frequency of spontaneous IPSCs in CA1 pyramidal neurons and I_h inhibition decreases their frequency (Peng et al. 2010). Blockade of HCN channels alters the firing properties of statum oriens interneurons (Maccaferri and McBain 1996; Matt et al. 2011). Inhibiting HCN channels decreases spontaneous but not miniature IPSCs in principle neurons from the baso-lateral amygdala. Together, these data suggest that HCN channels have a unique role in inhibitory interneurons, separate from that in excitatory pyramidal neurons. It is interesting to note that in pyramidal neurons, I_h inhibition is associated with increased glutamate release (Huang et al. 2011), in addition to increasing summation and excitability. This in contrast to Aponte et al. 2006, suggesting that HCN channel inhibition results in less GABA release. It is important to consider how HCN channels may differentially regulate these two network elements and how changes in channel expression may alter excitatory verses inhibitory network functionality.

HCN Channel Function

Functionally, HCN channels in the central nervous system have been implicated in several roles. Given their role in information processing, it is not surprising that HCN channels are hypothesized to be important parts of learning and memory pathways. HCN channel plasticity is a potential mechanism for regulation of synaptic strength. To this end, different forms of LTP induction both up and down-regulate HCN channels in CA1 pyramidal neurons (Campanac et al. 2008). Furthermore, HCN inhibition blocks long term potentiation (LTP) at mossy fiber synapses, likely by decreasing pre-synaptic excitability. HCN channels in granule cells are hypothesized to depolarize the presynaptic compartment, with LTP enhancing this depolarization via a Ca^{2+} mediated increase in the HCN activator cAMP (Mellor et al. 2002). In contrast to this, HCN channel inhibition enhances LTP at perforant path to CA1 synapses which contact the distal dendrites of CA1 neurons. HCN channels do not similarly oppose LTP at schaffer collateral synapses that contact at more proximal dendritic locations resulting in CA1 inputs that are not exposed to as much HCN channel filtering (Nolan et al. 2004). This suggests that the effect of HCN channels on LTP at this synapse is through its effect on dendritic integration. LTP is also enhanced in perforant path to CA1 synapses in animals lacking HCN2 channels in GABAergic interneurons (Matt et al. 2011)

HCN channels also influence behavior. Forebrain specific HCN1 knockout mice have enhanced short and long-term memory (Nolan et al. 2003). HCN channels are highly expressed in the cerebellum, both in perkinje cells and in the terminals of basket cells (Notomi and Shigemoto 2004; Lujan et al. 2005b; Angelo et al. 2007). HCN1 knockout animals have deficits in motor learning via a hypothesized HCN channel

dependent alteration of excitatory integration onto perkinje neurons (Nolan et al. 2003). Finally, HCN channel knockdown in the prefrontal cortex improves working memory performance and HCN channel inhibition increases firing of pre-frontal cortex pyramidal neurons in memory related paradigms (Wang et al. 2007).

HCN channel involvement in learning pathways suggests the possibility for plasticity. While channel properties are subject to regulation by cAMP, HCN channels themselves are subject to dynamic trafficking (Noam et al. 2010). Given the inhibitory role HCN channels have on cellular excitability and synaptic integration, regulation of HCN channel membrane expression could drastically modify cellular properties. As discussed, LTP induction is associated with changes in I_h amplitude (Campanac et al. 2008). Action potential blockade for 24 hours downregulates HCN channel expression (Arimitsu et al. 2009). Glutamate exposure inhibits HCN channel trafficking (Noam et al. 2010). Trafficking of HCN channels to their membranous expression points is regulated by association with the proteins TPR-containing RAB8b interacting protein (TRIP8b) and Pex5p-related protein (PEX5Rp) (Santoro et al. 2004). Knockdown of TRIP8b significantly reduces I_h in neurons and hyperpolarizes membrane potentials and increases input resistance. Additionally, knockdown of TRIP8b disrupts the normal dendritic expression of HCN channels (Piskorowski et al. 2011). TRIP8b knockout mice have increased EPSP summation, are relatively insensitive to HCN inhibition with ZD-7288, and lack the normal polarized expression of dendritic HCN channels. These knockout mice also exhibit reductions in motor learning and increased anti-depressant behavior (Lewis et al. 2011).

While the role of HCN channels in individual neuron excitability seems clear, how these changes influence network properties is less well understood. Blocking or enhancing HCN channels disrupts synchronous network bursts in organotypic, hippocampal cultures bathed in 0 Mg^{2+} (Adams et al. 2009). HCN inhibition alters the structure of thalamic network oscillations (Yue and Huguenard 2001) as well as subthreshold oscillations in hippocampal and cortical neurons (Dickson et al. 2000; Marcelin et al. 2009). These data suggest that I_h may contribute to normal brain rhythms and oscillations. Many questions about the role of I_h in network activity remain. Does the inhibitory influence of HCN channels on cellular excitability translate to a dampening effect on network activity? Does regulation of HCN channel expression influence network activity? Do changes in HCN channels influence excitatory elements of the network differently than inhibitory elements, and could this contribute to aberrations in network activity?

HCN Channels in Epilepsy

Given the inhibitory nature of HCN channels on neuronal excitability, taken in context with their plastic nature, it is perhaps not surprising that HCN channels are almost universally downregulated in models of epilepsy. Down regulated channels and consequently increased cellular excitability may contribute significantly to the pathophysiology of seizures and epilepsy. In support of this, the commonly used anticonvulsants lamotrigine (Poolos et al. 2002) and gabapentin (Surges et al. 2003) both increase I_h.

HCN channels and I_h are reduced in the kainate model of epilepsy 28-30 days following the initial seizure. This is mirrored by a concomitant increase in input resistance and temporal summation (Shin et al. 2008). The pilocarpine model of epilepsy also has decreased HCN channel expression acutely following a seizure as well as after the latent period and the onset of epilepsy (Jung et al. 2007). These changes are associated with increased input resistance and dendritic excitability (Jung et al. 2007). In agreement with these data, decreased HCN channel mRNA is observed within the hippocampus of animals following amygdala kindling (Powell et al. 2008). I_h is reduced following hypoxia induced seizures in perinatal rat pups (Zhang et al. 2006). Resected human cortical tissue from epilepsy patients also demonstrates evidence of reduced I_h (Wierschke et al. 2010). The spontaneously epileptic Wag/Rij strain of rats has reduced I_h (Strauss et al. 2004). Somewhat paradoxically, febrile seizures are associated with increased I_h (Chen et al. 2001a).

The ability of seizures to be epileptogenic has been hypothesized (Ben-Ari et al. 2008). In convulsant models of epilepsy, it is clear that the observed HCN channelopathy is caused by the initial seizure activity, though the mechanism by which this occurs is unclear. It seems likely that the observed loss of HCN channels contributes to the development of later epilepsy in these models given the correlation between decreased HCN channels and increased intrinsic excitability and effectiveness of EPSP summation. The pilocarpine model of epilepsy is also associated with a hyperpolarizing shift in the activation curve for HCN channels such that less I_h is activated with the same level of hyperpolarization (Jung et al. 2007). This shift can be blocked by using anticonvulsants to suppress electrographic seizure activity in the acute period following the initial seizure

without suppressing the downregulation of total HCN channels (Jung et al. 2007). Epileptiform activity in vitro can mimic this change in voltage dependent activation (Jung et al. 2010). The change in the voltage dependent activation is dependent on changes in phosphorylation mediated by calcineurin or p38 mitogen-activated kinase. Furthermore, downregulated HCN channel activation may be sufficient to increase dendritic excitability (Jung et al. 2010). These findings are summarized in Figure 1. These data suggest HCN channel phosphorylation as a third mechanism for HCN channel regulation beyond cAMP signalling and membrane trafficking. These findings raise further questions regarding the mechanism behind the seizure induced epileptogenesis in convulsant models of epilepsy.

Genetic alterations in HCN channels are also associated with increased excitability. The *apatheic* line of mice has spontaneously mutated HCN2 subunits and exhibits an absence epilepsy phenotype (Chung et al. 2009). Mice lacking the HCN2 channel altogether also have an absence epilepsy phenotype, an abnormal cardiac rhythm, and increased hippocampal pyramidal neurons excitability (Ludwig et al. 2003). Mice lacking the HCN1 subunit have increased seizure susceptibility as well as increased excitability and temporal summation of EPSPs in entorhinal cortex pyramidal neurons.

Overall, these data suggest that reductions in HCN channels contribute significantly to the excitability observed in cases of epilepsy. This is summarized in Figure 2. However, the relative contribution of HCN channel pathology verses other pathologies is not fully understood. Also whether loss of HCN channels is causative of or results from epileptogenesis is not fully understood, although it seems clear that loss of

HCN channels contributes to future hyperexcitability. Furthermore, whether similar changes in HCN channels are observed in interneurons in cases of epilepsy is unknown.

Remaining Questions

The overall goal of this dissertation is to better understand how HCN channels contribute to network behavior. This goal can be subdivided into many smaller questions. For example: Does the inhibitory influence of HCN channels on neurons translate to a role for the channel in the regulation of network behavior? Does loss of HCN channels only contribute to excitability following seizure dependent down regulation? Do HCN channels change epileptiform events? Do HCN channels influence the excitability of cortical interneurons? If HCN channels influence primary cells and interneurons separately, what is the consequence for network behavior?

The work in this dissertation addresses these questions and others using three sets of experiments. The first asks the question of whether or not HCN channels influence network activity. This was examined by viewing the spread of activity throughout the neocortical network in the presence or absence of HCN channels using voltage sensitive dyes. This work also examines the influence of HCN channels in the freeze lesion model of malformation epilepsy that is not dependent on an initial seizure event. The second set of experiments examines the role of HCN channels in epileptiform discharges. These experiments ask whether inhibition of HCN channels increases epileptiform network events *in vitro* and if such HCN channel inhibition differentially influences the behavior of pyramidal neurons and interneurons in the neocortical network. The final set of experiments examines whether I_h influences the intrinsic and integrative properties of

neocortical interneurons in the fashion as excitatory pyramidal neurons. It also asks if loss of HCN channels could differentially contribute to the spike output of excitatory or inhibitory neurons.

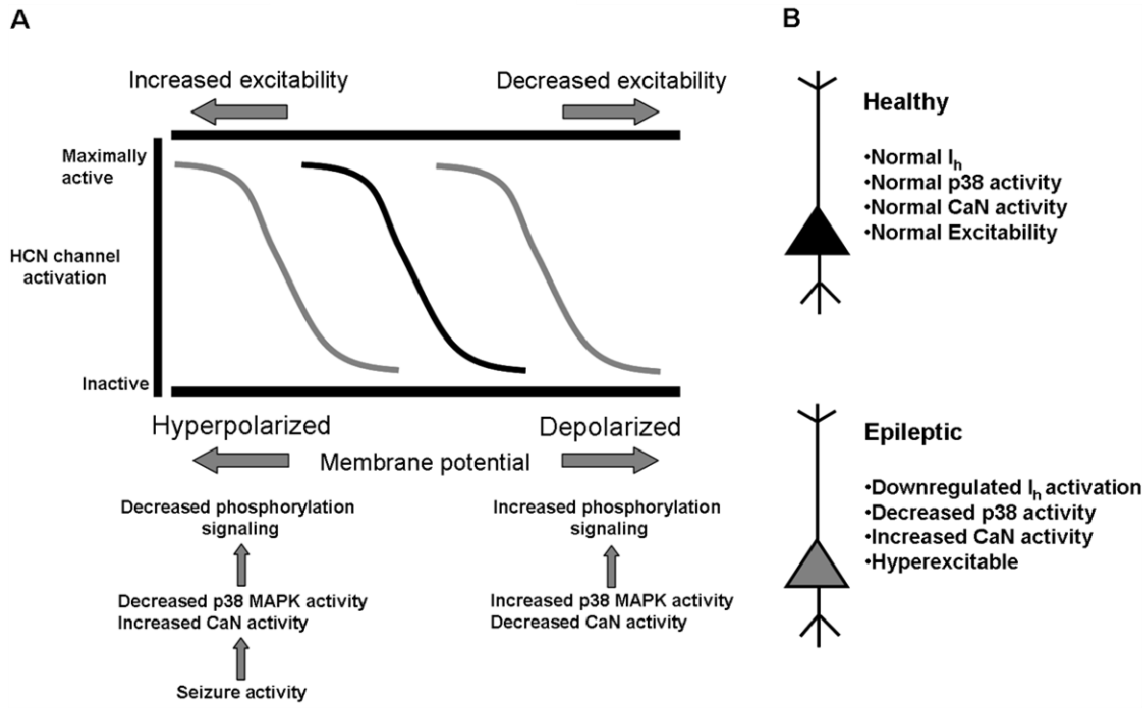

Figure 1. Summary of Changes in HCN Channel Gating Observed in the Pilocarpine Model of Epilepsy. A. Schematic of the shift in HCN channel gating observed. B. Summary of the accompanying molecular changes

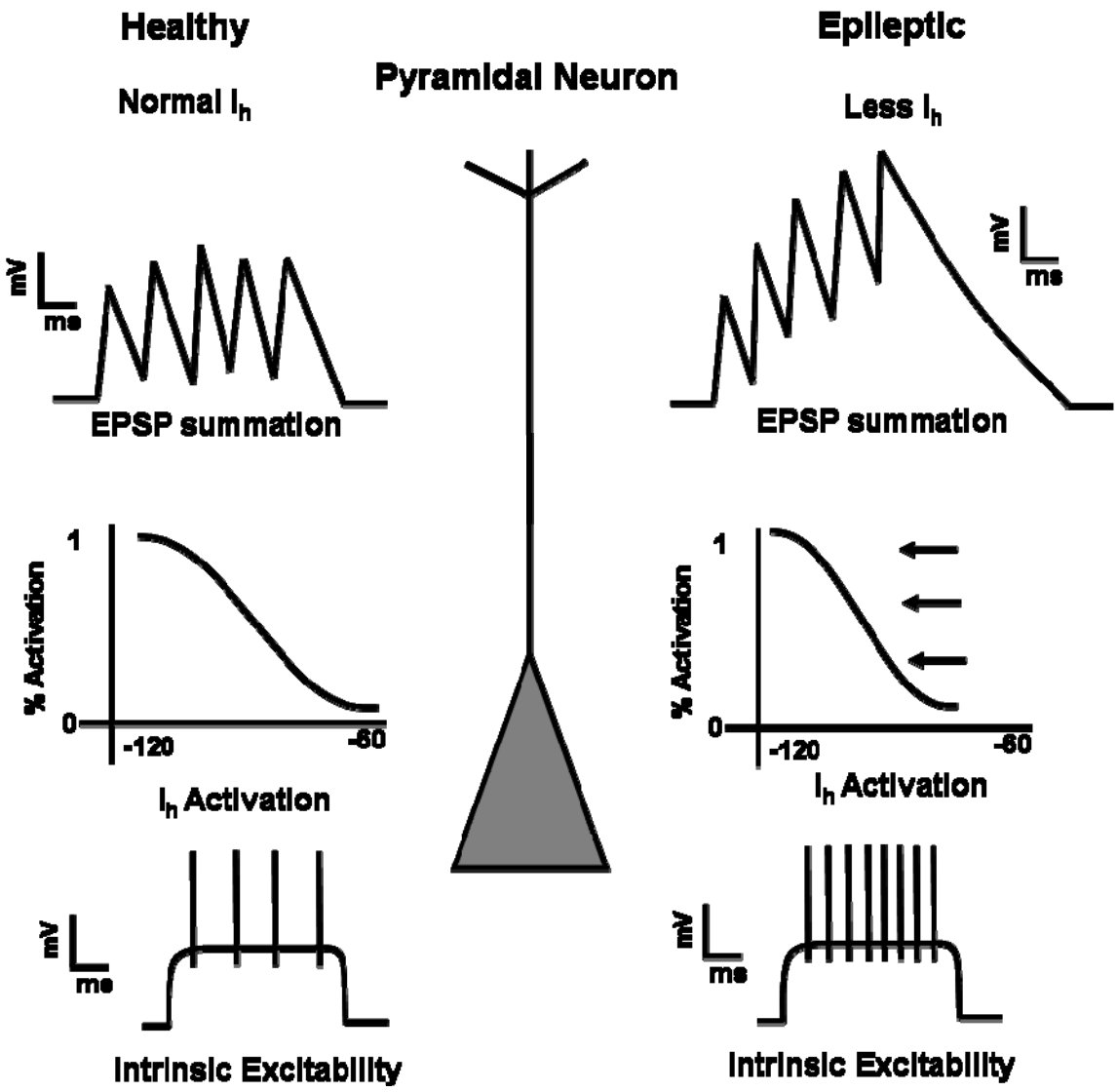

Figure 2. Summary of the Excitability Changes Observed in Following Loss of I_h

DECREASED HYPERPOLARIZATION-ACTIVATED CURRENTS IN LAYER 5 PYRAMIDAL NEURONS ENHANCES EXCITABILITY IN FOCAL CORTICAL DYSPLASIA

by

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ABSTRACT

Focal cortical dysplasia is associated with the development of seizures in children and is present in up to 40% of intractable childhood epilepsies. Transcortical freeze-lesions in newborn rats reproduce many of the anatomical and physiological characteristics of human cortical dysplasia. Rats with freeze lesions have increased seizure susceptibility and a region of hyperexcitable cortex adjacent to the lesion. Since alterations in hyperpolarization activated non-specific cation (HCN) channels are often associated with epilepsy, we used whole-cell patch-clamp recording, and voltage sensitive dye imaging to examine alterations in HCN channels and I_h currents in cortical dysplasia. L5 pyramidal neurons in lesioned animals had hyperpolarized resting membrane potentials, increased input resistances and reduced voltage "sag" associated with I_h activation. These differences became non-significant following application of the I_h blocker ZD7288. Temporal EPSP summation and intrinsic excitability was increased in neurons near the freeze lesion. Using voltage sensitive dye imaging of neocortical slices, we found that inhibiting I_h with ZD7288 increased the half-width of dye signals. The anticonvulsant lamotrigine produced a significant decrease in spread of activity. The ability of lamotrigine to decrease network activity was reduced in the hyperexcitable cortex near the freeze lesion. These results suggest that I_h serves to constrain network activity in addition to its role in regulating cellular excitability. Reduced I_h may contribute to increased network excitability in cortical dysplasia.

INTRODUCTION

Focal cortical dysplasia is associated with the development of seizures in children (Krsek et al. 2009) and is present in up to 40% of intractable childhood epilepsies (Leventer et al. 2008). Current anti-epileptic drugs are often ineffective in these patients (Mathern et al. 1999) leading to surgical treatment (Sisodiya 2000). Brain slices prepared from human dysplastic cortex display abnormal synaptic connections and increased excitability (Cepeda et al. 2006). Transcortical freeze-lesions in the newborn rat (Dvorak and Feit 1977; Dvorak et al. 1978) reproduce many of the anatomical and electrophysiological characteristics of human focal cortical dysplasias (Jacobs et al. 1996, 1999; Luhmann and Raabe 1996; DeFazio and Hablitz 1998). Such lesions also increase susceptibility to complex hyperthermic seizures (Scantlebury et al. 2004). Reduced inhibition (Zhu and Roper 2000), alterations in glutamate receptors (DeFazio and Hablitz 2000) and transporters (Campbell and Hablitz 2008) have been shown to contribute to hyperexcitability in cortical dysplasia, possibly interacting with local changes in connectivity (Jacobs and Prince 2005) to further increase excitability. Although abnormalities in several voltage-dependent currents have been implicated in epilepsy (Avanzini et al. 2007; Becker et al. 2008; Catterall et al. 2008), changes in intrinsic excitability in cortical dysplasia have not been extensively investigated.

Hyperpolarization-activated, non-selective cation (HCN) channels are encoded by four mammalian genes, termed HCN1-4. Distinct patterns of activation and inactivation and varying sensitivities to cyclic nucleotides are displayed by each subunit (Santoro et al. 2000; Wainger et al. 2001). Depending on the cell type and brain region, the inwardly rectifying hyperpolarization-activated current I_h contributes to generation of rhythmic

activity (McCormick and Pape 1990), determination of the resting membrane potential (Robinson and Siegelbaum 2003) and synaptic integration (Magee 2000; Berger et al. 2001). Alterations in I_h and HCN expression occur in a variety of seizure models including kainic acid and pilocarpine-induced epilepsy (Jung et al. 2007; Shin et al. 2008), early-life hyperthermia (Chen et al. 2001), temporal lobe kindling (Powell et al. 2008) and absence seizures (Strauss et al. 2004; Schridde et al. 2006; Kole et al. 2007). HCN1 subunit specific knockout mice have a reduced seizure threshold (Huang et al. 2009) whereas HCN2 knockout mice exhibit an absence epilepsy phenotype (Ludwig et al. 2003). Paradoxically, hyperexcitability has been associated with both up- and downregulation of HCN channels (reviewed by Dyhrfjeld-Johnsen et al. 2009). Modifications in I_h have not been examined in cortical dysplasia.

Despite the relatively well-characterized role of I_h in cellular excitability, its contribution to network activity is not well understood. Maturation of rhythmic slow-wave sleep activity patterns is dependent on the density and the properties of I_h during development (Kanyshkova et al. 2009). Working memory networks are strengthened by inhibition of HCN channel signaling in prefrontal cortex (Wang et al. 2007). Theta activity in hippocampus (Hu et al. 2002; Xu et al. 2004; Marcelin et al. 2009) and subthreshold oscillations in entorhinal cortex (Dickson et al. 2000) are disrupted by I_h blockers. The timing of interictal bursts in the neonatal rat hippocampus is positively modulated by I_h (Agmon and Wells 2003). The contribution of I_h to network hyperexcitability in cortical dysplasia has not been established. In the present study, we have used whole-cell patch-clamp recordings and voltage-sensitive dye imaging to determine the effect of HCN channel alterations on intrinsic excitability of individual

cells and activity in local circuits. Results suggest that I_h contributes significantly to the normal pattern of spread of activity across the cortical mantle. Decreases in I_h in cortical dysplasia augment network excitability, possibly contributing to the hyperexcitability seen in malformed cortex.

METHODS

Animals

All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals using protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Every effort was made to minimize pain and discomfort. Focal freeze-lesions were induced in postnatal (PN) day 1 Sprague-Dawley rats. In brief, newborn rat pups were anesthetized by hypothermia and a small incision was made to expose the skull. A 2 mm copper rod cooled to approximately -50º C was placed on the surface of the skull for 3 s. Sham-operated animals received similar treatment without cooling the probe. After suturing the scalp, the animals were warmed and returned to their home cage. Rats were allowed to recover for 21-27 days before recordings were made.

Preparation of in vitro brain slices

Rats were anesthetized and decapitated. The brain was quickly removed and placed in ice-cold cutting solution consisting of (in mM): 135 N-Methyl-D-glucamine, 1.5 KCl, 1.5 KH_2PO_4, 23 choline HCO_3, 0.4 ascorbic acid, 0.5 $CaCl_2$, 3.5 $MgCl_2$ and 25 D-glucose (Tanaka et al. 2008). The solution was bubbled with 95% O_2/5% CO_2 to maintain a pH around 7.4. Coronal brain slices (300 μm thick) were cut using a vibratome (Microm, Walldorf, Germany). Slices were obtained from an area of somatosensory neocortex

containing the microgyrus in freeze-lesioned animals and a corresponding location in sham-operated controls. The slices were stored for 40-60 minutes at 37º C in oxygenated recording solution containing (in mM) 124 NaCl, 2.5 KCl, 10 D-glucose, 26 $NaHCO_3$, 2.0 Ca^{2+}, 2.0 Mg^{2+}, then kept at room temperature. For recording, individual slices were transferred to a recording chamber and continuously perfused (4 mL/min) with oxygenated recording solution.

Whole-cell recording

A Zeiss Axioskop FS (Carl Zeiss, Thornwood, NY) microscope, equipped with Nomarski optics, a 40X-water immersion lens and infrared illumination, was used to view neurons in the slices. L5 pyramidal neurons were identified by their pyramidal shape and size, presence of a prominent apical dendrite, distance from the pial surface and their spiking properties. In addition, cells were intracellularly labeled with biocytin to confirm identification. Labeled cells were processed as described previously (Zhou and Hablitz 1996).

Whole-cell recordings were obtained from visually identified L5 pyramidal neurons. Signals were acquired using a MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA) controlled by Clampex 8.0 software via a Digidata 1322A interface (Molecular Devices). Responses were filtered at 5 kHz, digitized at 10-20 kHz and analyzed offline using Clampfit 8.0 software. Tight seals (>2 GΩ before breaking into whole-cell mode) were obtained using patch electrodes that had an open tip resistance of around 3 MΩ. Series resistance during recording varied from 9 to 20 MΩ. Under voltage-clamp conditions, series resistance was compensated 50–70% and continually monitored throughout the experiment. Recordings were terminated whenever significant increases

(>20%) in series resistance occurred. In current clamp recordings, the Bridge Balance control of the MultiClamp amplifier was used to compensate for the voltage drop across the electrode. All current clamp records were visually checked for proper compensation during analysis. The intracellular solution for recording contained (in mM): 125 K-gluconate, 10 KCl, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP, and 0.5 EGTA. pH and osmolarity were adjusted to 7.3 and 290 mOsms, respectively. Bicuculline methiodide (BIC) (10 μM) (Sigma-Aldrich, St. Louis, MO) was present during all whole-cell recording experiments in order to block $GABA_A$ receptors. Synaptic responses were evoked with a bipolar stimulating electrode (twisted pair of 25 μm Formvar insulated nichrome wires) positioned 150-200 μm above the recording pipette. Stimuli were current pulses 50-200 μA in amplitude and 50 μs in duration. A stimulation frequency of 0.05 Hz was used. All traces of synaptic currents shown are the average of 10 consecutive responses. Recordings were done at 32±1°C.

Data are expressed as mean ± SEM. Statistical analysis of response amplitudes from control and freeze-lesioned animals was carried out using two-tailed Student's t-test or one-way ANOVA. $P<0.05$ was considered significant.

Voltage-sensitive dye imaging

Imaging experiments were conducted using the voltage-sensitive fluorescent dye *N*-(3-(triethylammonium)propyl)-4-(4-(*p*-diethylaminophenyl)butadienyl)pyridinium dibromide (RH 414). Individual slices were stained with 30 μM RH 414 for at least 60 min at room temperature and then placed in the recording chamber on the stage of the microscope (Axiovert 135TV, Zeiss) used for optical recording. Slices were continuously perfused with recording saline at a rate of 4 ml/min for at least 30 min prior

to recording in order to wash out excess dye. A bipolar stimulating electrode was positioned intracortically in middle cortical layers. Activity was evoked using single shocks 40-100 μA in amplitude with duration of 190 μs. A hexagonal photodiode array containing 464 diodes (Neuroplex, Red Shirt Imaging, Fairfield, CT) was used to detect activity dependent changes in fluorescence. Excitation of the dye was achieved using a stabilized power supply (Hewlett-Packard, Palo Alto, CA), 100W halogen lamp and a 535 ± 40 nm filter. The emitted light passed through a 590 nm long pass filter. Optical signals were amplified and stored on a computer for later analysis. The resting light intensity measured for each diode was used to normalize fluorescent measurements. Correction for dye bleaching was done using measurements taken in the absence of stimulation. All optical signals are represented as changes in fluorescence with stimulation divided by resting fluorescence ($\Delta F/F$ where F is the fluorescence measured in the absence of stimulation and ΔF is the change in fluorescence following stimulation). Responses to three stimulations were averaged. RH 414 responds to membrane depolarization with a decrease in fluorescence. This is plotted as an upward deflection in all figures. Using fixed scaling for individual figures, pseudocolor images were generated to visualize spatiotemporal patterns of activity in the slice. A digital image of the slice in the recording chamber was taken with a CCD camera attached to a dissecting microscope in order to document the position of the photodiode array with respect to cortical layers.

Data Analysis

For analysis of changes in amplitude and duration of dye signals, a region of interest containing 18 diodes showing significant dye signals before drug application was

selected. The peak amplitudes and half widths of these responses were compared before and during drug administration. To examine changes in spread of activity, the number of diodes showing peak signal amplitudes three times the baseline noise levels was determined. The baseline noise level was determined from ten diodes which exhibited no obvious activity. A two-way ANOVA was used for statistical comparison with differences being considered significant if $P < 0.05$. Data are expressed as mean ± SEM.

Drugs

Drugs were stored in frozen stock solution and dissolved in the recording solution prior to each experiment. After recording control responses, drugs were bath applied for twenty minutes. Lamotrigine and ZD7288 were obtained from Tocris Bioscience (Ellisville, MO).

RESULTS

Membrane properties of L5 pyramidal neurons

To observe the impact of I_h changes on L5 pyramidal neurons, we obtained somatic whole-cell patch-clamp recordings from 22- to 28-day-old sham-operated and lesioned animals. Recordings in lesioned animals were obtained 1-2 mm lateral to the lesion. Consistent with a reduction in the expression level of I_h, the somatic resting membrane potential (V_m) in L5 pyramidal neurons was more hyperpolarized in slices from lesioned (−76.5 ± 1.0 mV, $n = 24$) than sham-operated animals (−73.8 ± 0.9 mV, $n = 26$, $P<0.05$) (Fig. 1A left). Furthermore, L5 pyramidal neurons in slices from lesioned animals had a significantly larger somatic input resistance (R_{in}) (lesioned: 77.4 ± 4.2 MΩ, n=24; sham-operated: 64.9 ± 3.7 MΩ, n=26, $P<0.05$) (Fig. 1B left). These differences in membrane properties were no longer significant after bath application of the I_h channel blocker

ZD7288 (10 μM) (V_m: lesioned: -79.1 ± 1.4 mV; sham-operated: -78.5 ± 0.81 mV; R_{in}: lesioned: 92.3±6.5 MΩ; sham-operated: 85.6 ± 5.3 MΩ), suggesting that the initial differences arose from alterations in I_h expression (Fig. 1 A and B, right).

When neurons exhibit a prominent I_h current, hyperpolarizing current pulses evoke a voltage response that reaches a peak and then "sags" back toward rest (Maccaferri et al. 1993; Sutor and Hablitz 1993; Berger et al. 2001). Fig. 2A shows responses to a series of hyperpolarizing current pulses in an L5 neuron from a sham-operated animal. Sag responses were prominent. When the same currents were applied to a neuron from a lesioned animal, sag responses were reduced (Fig. 2B), indicating a decreased I_h. For example, when employing a current pulse of -400 pA, the sag response was 2.26 ± 0.3 (n=9) in controls and 1.91 ± 0.2 (n=9) in lesioned animals. These differences were statistically significant ($P > 0.05$, one-way ANOVA). At the end of the current pulse, the smaller de-activating I_h in cells from lesioned animals led to a smaller rebound depolarization. Using a -400 pA current pulse, rebound amplitudes were 1.99 ± 0.2 and 1.26 ± 0.2 pA in control and lesioned animals, respectively. The differences between control and lesioned animals were significant ($P > 0.05$, one-way ANOVA). A summary plot of the changes in responses to hyperpolarizing current pulses is shown in Fig. 2C. It can be seen that that sag responses (left) and rebound depolarization's (right) were significantly reduced in neurons from lesioned animals.

Intrinsic excitability changes in L5 pyramidal neurons

The changes described above, a more hyperpolarized V_m and an increased $R_{in,}$ make it difficult to predict the net effect on intrinsic excitability of L5 neurons (Dyhrfjeld-Johnsen et al. 2009). We therefore examined the relationship between somatic current

injection and AP firing in neurons from sham-operated and lesioned animals. At least 5 min after obtaining a whole-cell recording, cells were stimulated, at their resting potential, with 500 pA depolarizing current pulses. Fig. 3A (upper left) shows a typical response in a neuron from a sham-operated animal. The depolarizing current pulse evoked a train of action potentials. In L5 pyramidal neurons from lesioned animals (Fig. 3B, upper right), the number of action potentials was significantly higher than in sham-operated animals (lesioned: 9.8 ± 0.4 spikes/pulse, n=9; sham-operated: 5.6 ± 0.4 spikes/pulse, n=9, $P < 0.05$) despite the fact that the membrane potential was more hyperpolarized. When 10 μM ZD7288 was bath applied, the number of action potentials in the sham operated neuron was markedly increased whereas the cell from the lesioned animal showed a smaller increase. A summary plot of the results from a group of cells is shown in Fig. 3C. It can be seen that neurons from lesioned animals are more excitable under baseline conditions. This difference was no longer significant in the presence of ZD7288 (lesioned: 12.4 ± 0.9 spikes/pulse; sham-operated: 11.3 ± 0.8 spikes/pulse, n=9, $P > 0.05$). These results suggest that decreased I_h in neurons from lesioned animals results in increased intrinsic excitability of L5 pyramidal cells.

Voltage-clamp analysis of I_h

Somatic voltage-clamp recordings were performed to examine I_h currents. Cells were held at –50 mV in the presence of TTX (1 μM). Voltage steps 500 ms in duration were given from –50 to –130 mV in 10 mV increments to activate I_h currents. ZD7288 (10 μM) was then bath applied to block HCN channels. Currents evoked following a 10 min perfusion with ZD were subtracted from control to obtain the ZD-sensitive current. Specimen records of ZD-sensitive currents from a sham-operated animal are shown in

Fig. 4A. The ZD-sensitive currents recorded in a neuron from a lesioned animal were significantly smaller in amplitude (Fig. 4B). Currents began to activate around –60 mV. When the membrane potential was held at -120 and -130 mV, I_h currents showed a significant decrease in lesioned compared with sham operated animals. (V_h = -120 mV: sham-operated: -298.1 ± 23 pA, n=9, lesioned: -230.6 ± 22 pA, n=9, $P < 0.05$; V_h = -130 mV: sham-operated: -342.6 ± 25 pA, lesioned: -262.1 ± 27.2 pA, $P < 0.05$). However, due to space-clamp errors, which result in the incomplete control of dendritic membrane potential, it is likely that these somatic voltage clamp data underestimated the HCN channel conductance, in particular, at more hyperpolarized potentials. The higher input resistance in lesioned animals is expected to reduce this potential confound. Currents evoked at -130 mV were fitted to single exponential functions to determine activation time constants. There were no significant difference between sham-operated and lesioned groups (sham-operated: 24.7 ± 4 msec, n=9; lesioned: 29.6 ± 4 msec, n=9; p>0.05). This value is in the range for I_h in thalamic neurons (Santoro et al. 2000), hippocampal interneurons (Santoro et al. 2000), and neocortical pyramidal cells (Williams and Stuart, 2000b) and is consistent with mediation by HCN1-HCN2 subunits.

Alterations in EPSP temporal summation

During a train of evoked EPSPs in L5 pyramidal neurons, summation is reduced or prevented by the presence of I_h (Berger et al. 2001). To determine if the observed I_h decreases in lesioned animals altered synaptic integration, distal EPSPs were evoked by a bipolar stimulating electrode positioned 150-200 μm above the recording pipette. A train of five stimuli at 20 Hz was used to evoke EPSPs in L5 neurons. As shown in Fig. 5, sublinear temporal summation was observed in neurons from both sham-operated and

lesioned animals under control conditions. When the ratio of the amplitude of the fifth to the first EPSP in the train ($EPSP_5/EPSP_1$) was calculated, a significantly increased ratio was observed in the lesioned group (sham-operated: 1.7 ± 0.2 (n = 26); lesioned: 2.3 ± 0.2 (n = 24) $P < 0.05$), indicative of an increased summation in the latter group due to a decreased I_h.

The effect of I_h blockade on synaptic activation was further examined using ZD7288 (10 μM). In the presence of the I_h channel blocker, temporal summation during the EPSP train was significantly increased in both sham-operated and lesioned groups. However, in the presence of ZD7288, the groups were no longer statistically different from each other (sham-operated: 3.28 ± 0.4; lesioned: 3.35 ± 0.4 mV, P >0.05). These results suggest that dendritic I_h is reduced but not abolished in the lesioned animals.

Spatial-temporal spread of activity in dysplastic cortex

Multi-electrode field potential recordings of paroxysmal discharges in freeze-lesioned cortex have demonstrated propagation over long distances in the horizontal direction (Luhmann and Raabe 1996; Jacobs et al. 1996). Voltage-sensitive dye studies of evoked activity in normal neocortex have shown that the time course of dye signals are similar to those of locally recorded field potentials. Dye signal responses peak rapidly (Yuste et al. 1997) and spread horizontally over relatively short distances (Langenstroth et al. 1996). Using voltage-sensitive dye imaging, we have shown that spread of activity in lesioned animals was greater in upper cortical layers in the paramicrogyral area relative to sham-operated controls (Bandyopadhyay and Hablitz 2006). More persistent activation of local cortical circuits was also seen in dysplastic cortex. Experiments

described below examine the role of I_h in regulating spread of activity in dysplastic neocortex.

The voltage-sensitive dye RH 414 and optical imaging were used to quantify how alterations in I_h modify spatiotemporal patterns of activity. The hexagonal photodiode array used for this purpose covered an area of approximately 1.8 x 1.8 mm of the slice at the magnification (x10) used. Figure 6A shows the typical positioning of the photodiode array over the neocortex. The arrow indicates the location of the stimulating electrode. Figure 6B shows the typical position of the array over slices from lesioned animals. The small arrow shows the location of the microgyrus. Four stimulus intensities were tested in each slice (40, 60, 80 and 100 uA). A typical pseudocolored voltage sensitive dye response is shown overlaid an image of the cortex in Fig. 6C. Examples of individual diode responses from a slice from a sham operated animal are shown in Fig. 6D. Fluorescence changes had a rapid rising phase and a slower decay (Fig. 6D).

Stimulation in slices from sham operated controls evoked responses near the site of stimulation with subsequent vertical and horizontal spread. Fig. 7A is a montage of 20 pseudocolor maps showing the spatial distribution of dye signals ($\Delta F/F$) at given points in time. The first panel displays activity 2-5 ms following stimulation and additional panels are shown at 3 ms intervals. Warm colors represent larger-amplitude dye signals, i.e., high levels of activity. The pial surface is up in each panel. Activity first spread to more superficial layers and then laterally. When the same stimulation intensity was used in a slice from a lesioned animal, activity rapidly spread across large portions of the superficial layers and was more persistent (Fig. 7B), as described previously (Bandyopadhyay and Hablitz 2006). To quantify these results, the average peak

amplitude from selected diodes (see methods) and the number of diodes activated (indicative of activity spread) were determined. Peak amplitudes and the number of diodes activated were significantly increased at all stimulus intensities in slices from lesioned animals (Fig. 7) ($P < 0.05$, two-way ANOVA).

I_h and spread of activity

I_h has significant effects on dendritic excitability and attenuation of EPSPs in L5 pyramidal cells (Williams and Stuart 2000; Berger et al. 2001; Day et al. 2005). Blockade of I_h results in enhanced temporal summation (Berger et al. 2001) and increased dendritic calcium action potential generation (Berger et al. 2003). Given these changes, it was reasoned that I_h blockade should result in enhanced spatial-temporal spread of activity.

The spatio-temporal distribution of evoked activity from a sham-operated animal under control conditions is shown in Fig. 8A. The first panel shows activity approximately 2-5 ms after stimulation. Subsequent panels show responses at 3 ms intervals. The control images acquired following intracortical stimulation show an area of activity that appears first near the stimulating electrode and was generally constrained to a columnar shape. Responses following application of the HCN channel blocker ZD7288 (10 μM) are shown in Fig. 8*B*. Activity was seen to persist longer in the presence of ZD7288. Pseudocolor scaling was the same for all conditions. Superimposed dye signals from three different diodes under control conditions and in the presence of ZD7288 are shown in Fig. 8*C*. Response half-widths were significantly increased when the I_h blocker was present at all stimulation intensities (Fig. 8*D*). We did not observe a significant difference in the effect of ZD7288 on response half width between lesion and control animals ($P > 0.05$, two-way ANOVA). We also observed a slight decrease in response

amplitude following twenty minutes of ZD7288 that did not differ between lesion and control animals (data not shown). ZD7288 did not significantly change the number of diodes activated (indicating spread of activity) in either group.

Anticonvulsant drugs such as lamotrigine (Poolos et al. 2002; Peng et al. 2010) and gabapentin (Surges et al. 2003) have been shown to enhance I_h. The effect of enhancing I_h on network behavior has received little attention. We therefore tested the effect of bath application of lamotrigine (100 μM) on spatial-temporal spread of activity in neocortical slices. A montage of 20 pseudocolored maps under control conditions is shown in Fig. 9A. Activity 2-5 ms after stimulation is shown in the first panel. Panels are subsequently shown at 2.5 ms intervals. Fig. 9B shows responses to the same stimulation 20 min after bath application of lamotrigine (100 μM). Lamotrigine altered the spatial temporal pattern of evoked neocortical activity. When individual responses before and after lamotrigine were superimposed, a decrease in amplitude was observed (Fig. 9C & D). Lamotrigine also decreased the number of diodes activated (indicating spread of activity) (Fig. 9E). Additionally, we observed a small, but significant, decrease in diode half-width after lamotrigine (data not shown). This is in contrast to the increase observed following ZD7288.

Lamotrigine is known to have effects on ion channels other than I_h (Thompson et al. 2011). We therefore tested the effect of lamotrigine when applied in the presence of ZD7288. Figs. 9C and D show that lamotrigine had a significantly reduced effect on the amplitude of voltage sensitive dye signals and number of diodes activated, respectively, in the presence of ZD7288 ($P < 0.05$, two-way ANOVA). This suggests that a significant portion of lamotrigine's effect on network activity is mediated via an action on I_h.

The effects of lamotrigine on response amplitude in slices from sham-operated and lesioned animals are summarized in Fig. 10. In both groups, bath application of lamotrigine produced a significant reduction in amplitude at all intensities ($P < 0.05$, two-way ANOVA) (Fig. 10A). The effect of lamotrigine on response amplitude was significantly decreased in lesion compared to control animals ($P < 0.05$, two-way ANOVA). The effect of lamotrigine on the number of diodes activated is shown in Fig. 10B. A significant decrease in the number of diodes reaching threshold levels of activation was also observed in both groups. Again, this effect was significantly reduced in the lesion group ($P < 0.05$, two-way ANOVA) A decrease was observed in the ability of lamotrigine to reduce half-width in lesioned animals compared to control animals.

We also examined the ability of lamotrigine to alter the I_h dependent voltage sag and rebound (as seen in Fig. 2) in control and lesioned animals. Lamotrigine significantly ($P < 0.05$, 2-way ANOVA) increased both the voltage sag (-250 pA injection, 1.48 mV increase ± 0.82 mV, $P < 0.05$) and rebound depolarization (-250 pA injection, 1.31 mV increase ± 0.86 mV, $n = 7$, $P < 0.05$) in neurons from controls. Lamotrigine did not have a significant effect on I_h dependent voltage sag or rebound in neurons in dysplastic cortex(Sag -250 pA current injection, -0.29 ± 0.6 mV change, $P< 0.05$; rebound -0.27 ± 0.8 mV change $n = 7$, $P < 0.05$). The lack of a significant effect of lamotrigine on sag and rebound in lesioned animals further suggests that animals with freeze lesions have reduced I_h. This reduction may contribute to the reduced effectiveness of lamotrigine in constraining network activity in lesioned animals.

DISCUSSION

In the current study, we used whole-cell patch-clamp recording and voltage sensitive dye imaging to examine alterations in HCN channels and I_h currents in the rat freeze-lesion model of cortical dysplasia. L5 pyramidal neurons in lesioned animals demonstrated hyperpolarized resting membrane potentials, increased input resistances and a reduction in the voltage "sag" associated with I_h activation. Temporal EPSP summation and intrinsic excitability was increased in neurons near the freeze lesion. These differences became non-significant following application of the I_h blocker ZD7288. Furthermore, we demonstrated a role for I_h in constraining network activity, finding that this effect was reduced in dysplastic cortex.

I_h changes in epilepsy

Alterations in I_h have been described in several animal models of epilepsy. A progressive, persistent downregulation of dendritic HCN channels is seen in the rat pilocarpine model of epilepsy (Jung et al. 2007). Rats with pilocarpine-induced epilepsy exhibit increases in input resistance and dendritic excitability. A reduction in I_h and increased dendritic EPSP summation also has been observed following status epilepticus induced by kainic acid (Shin et al. 2008). Similarly, the spontaneously epileptic WAG/Rij rat exhibits reduced I_h associated with increased input resistance and enhanced synaptic summation (Strauss et al. 2004; Kole et al. 2007). Perinatal seizures induced by hypoxia are also accompanied by a downregulation of I_h (Zhang et al. 2006). The present study indicates that reductions in I_h associated with increases in cellular excitability and enhanced EPSP summation are found in a non-chemically induced, malformation

epilepsy model. This suggests that persistent I_h downregulation associated with increased excitability may be a pervasive finding in many types of epilepsy.

Genetic reduction in HCN channels is strongly associated with epilepsy. HCN2 knockout animals exhibit spontaneous absence type seizures (Ludwig et al. 2003) whereas HCN1 knockouts have enhanced seizure susceptibility (Huang et al. 2009). Additionally, *Apathetic* mice, which possess spontaneously truncated HCN2 channels, display an absence epilepsy phenotype (Chung et al. 2009). Whereas our findings suggest a decrease in I_h as one potential mechanism for hyperexcitability in cortical dysplasia, increases in I_h have been reported to produce increased excitability in a of febrile seizure model (Chen et al. 2001). Although differential effects on I_h may occur depending on the initial insult, it appears that proper network function can be perturbed by up- or down-regulation of HCN channels.

I_h has a well-characterized role in regulating dendritic excitability. I_h activation increases resting membrane conductance, depolarizes the resting membrane potential, and decreases dendritic excitability (Magee, 1998;Poolos et al. 2002;Robinson and Siegelbaum, 2003). In the present study, L5 pyramidal neurons from lesioned animals have significantly reduced I_h currents, increased input resistances and hyperpolarized membrane potentials. Despite the membrane hyperpolarization, depolarizing current pulses of the same amplitude elicited more spikes from neurons near the lesion compared to sham-operated controls. This counterintuitive inhibitory effect of I_h on action potential firing in the sham-operated group has previously been attributed to HCN channels active at the resting membrane potential decreasing the input resistance (Robinson and Siegelbaum 2003; Poolos et al. 2002). In addition to changes in intrinsic excitability, I_h

blockade also enhances temporal summation of distal excitatory inputs (Magee 1999; Williams and Stuart 2000). Our observed increase in EPSP summation coupled with enhanced intrinsic excitability may be an underlying mechanism contributing to the hyperexcitability seen in dysplastic cortex. HCN channels are highly expressed in the apical dendrites of L5 pyramidal neurons (Lorincz et al. 2002) where they regulate excitability (Berger et al. 2001). Somatic recordings, like those employed here, do not faithfully reproduce dendritic responses (Williams and Mitchell 2008). Computational modeling studies have shown that somatic measurements underestimate dendritic I_h currents Day et al. 2005). Changes observed at the somatic level are nonetheless informative since they can potentially influence neuronal output. Spike initiation in L5 pyramidal neurons occurs in the distal portion of the axon initial segment (Palmer and Stuart 2006). Somatic membrane potential changes resulting from alterations in dendritic I_h could influence action potential generation. Our results indicate that, despite the presumptive dendritic localization of HCN channels in L5 pyramids, I_h modulates membrane potential, intrinsic excitability and synaptic responses at the somatic level.

Presynaptic HCN channels have been reported in hippocampus (Notomi and Shigemoto 2004; Aponte et al 2006; Bender et al 2007), brainstem (Cuttle et al. 2001) and enthorinal cortex (Huang et al 2011). Such presynaptic channels have been reported to reduce both GABA (Southan et al. 2000; Aponte et al 2006) and glutamate (Huang et al. 2011) release. If present in neocortex, presynaptic HCN channels would increase the repertoire of mechanisms whereby HCN channels could influence network excitability.

The factors responsible for I_h alterations in cortical dysplasia are unclear. A single seizure episode can decrease I_h (Shah et al. 2004) and long-term downregulation of total

I_h has previously been shown to occur independent of repeated seizure activity in the pilocarpine model of epilepsy (Jung et al. 2007). Although spontaneous seizures are not typically seen in the freeze-lesion model, increases in synaptic activity have been observed (Jacobs and Prince 2005) and high frequency stimulation is known to downregulate I_h in CA1 pyramidal neurons (Campanac et al. 2008). Increased extracellular glutamate levels have been shown to be present in dysplastic cortex (Campbell and Hablitz 2008). In cultured hippocampal neurons, activation of AMPA and NMDA receptors is capable of acutely augmenting HCN1 surface expression while diminishing channel trafficking (Noam et al. 2010). It is currently unclear if the observed decreases in I_h are activity dependent or result from the initial cortical injury.

Regulation of activity in local circuits by I_h

Although numerous studies have characterized the role of I_h in regulating the excitability of individual neurons (Magee 1998; Williams and Stuart 2000;George et al. 2009; Rosenkranz and Johnston 2006), the functional outcome on network activity has received less attention. The ability of I_h to constrain synaptic excitability suggests that I_h also could serve to restrict activity across networks of neurons. Using voltage sensitive dye imaging to quantify cortical circuit organization and dynamics, we have found significant changes in network activity in sham-operated animals following either I_h blockade or enhancement, effects which were altered in dysplastic cortex. As previously reported, intracortical stimulation elicited synchronized, horizontally restricted areas of activity extending from L1 to L5 (Kubota et al. 1999; Yuste et al. 1997; Bandyopadhyay and Hablitz 2006). In the presence of ZD7288, activity persisted significantly longer. A similar increase in half-width has previously been described for distally evoked EPSPs in

single cells (Williams and Stuart 2000). It is tempting to hypothesize that the network effect of ZD7288 is simply due to a net increase in the time constant of EPSPs. Enhancement of I_h with the anticonvulsant lamotrigine decreased response half width, dampened network excitability, and reduced the spatiotemporal spread of activity.

The ability of the anticonvulsant lamotrigine to constrain network activity was significantly reduced in lesioned animals. This suggests that the ability of I_h to constrain network activity in dysplastic cortex was reduced. We did not observe a similar decrease in the ability of ZD 7288 to enhance the duration of activity. The hyperexcitability in dysplastic cortex, low concentration of ZD7288 used, remaining I_h, and variability in epileptiform events may mask subtle alterations in the ability of ZD7288 to enhance the duration of network activity. Although HCN staining is prominent in L5 pyramidal neurons, I_h currents have been reported in L2/3 pyramidal cells (Sutor and Hablitz 1993; Strauss et al. 2004) and GABAergic neurons (Wu and Hablitz 2005). How I_h properties in these cells are altered in cortical dysplasia has not been established.

The decrease in HCN channel staining (Hablitz and Yang 2010), total I_h current, and accompanying voltage sag observed in the freeze lesion animals is associated with increases in synaptic integration and intrinsic excitability. These changes are mimicked in sham operated controls when I_h is blocked. This includes increased summation, increased spiking following current injection, as well as decreased membrane conductance. We also observed greatly increased network activation following electrical stimulation. Decreased I_h may contribute to the excitability changes observed in cortical dysplasia and malformation epilepsy. Blockade of I_h increased the duration of network activity whereas enhancement of I_h limited the spread of network activity. The ability of the

anticonvulsant lamotrigine to limit network activity was significantly reduced in freeze lesioned rats. These novel observations lead us to hypothesize that I_h serves to constrain network activity in addition to its role in constraining cellular excitability. Reduced I_h in rats with cortical malformations may contribute the increased network excitability.

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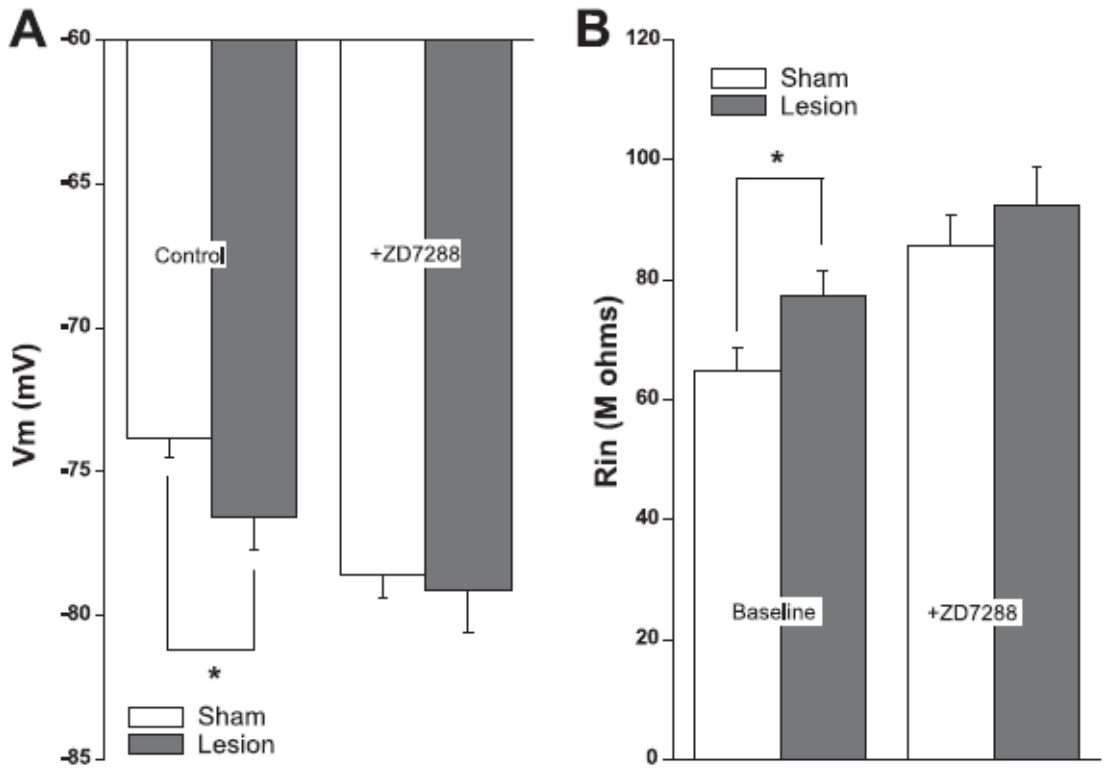

Figure 1. L5 pyramidal neurons from freeze lesioned rats have depolarized membrane potentials and increased input resistances. *A:* bar graph showing that the resting membrane potential of pyramidal neurons near the freeze lesion is significantly hyperpolarized compared to sham operated controls. This difference is not significant following HCN channel inhibition with ZD7288. *B:* bar graph illustrating that the input resistance of pyramidal neurons near the freeze lesion is significantly higher than that of sham operated controls. This difference is not significant following HCN channel inhibition.

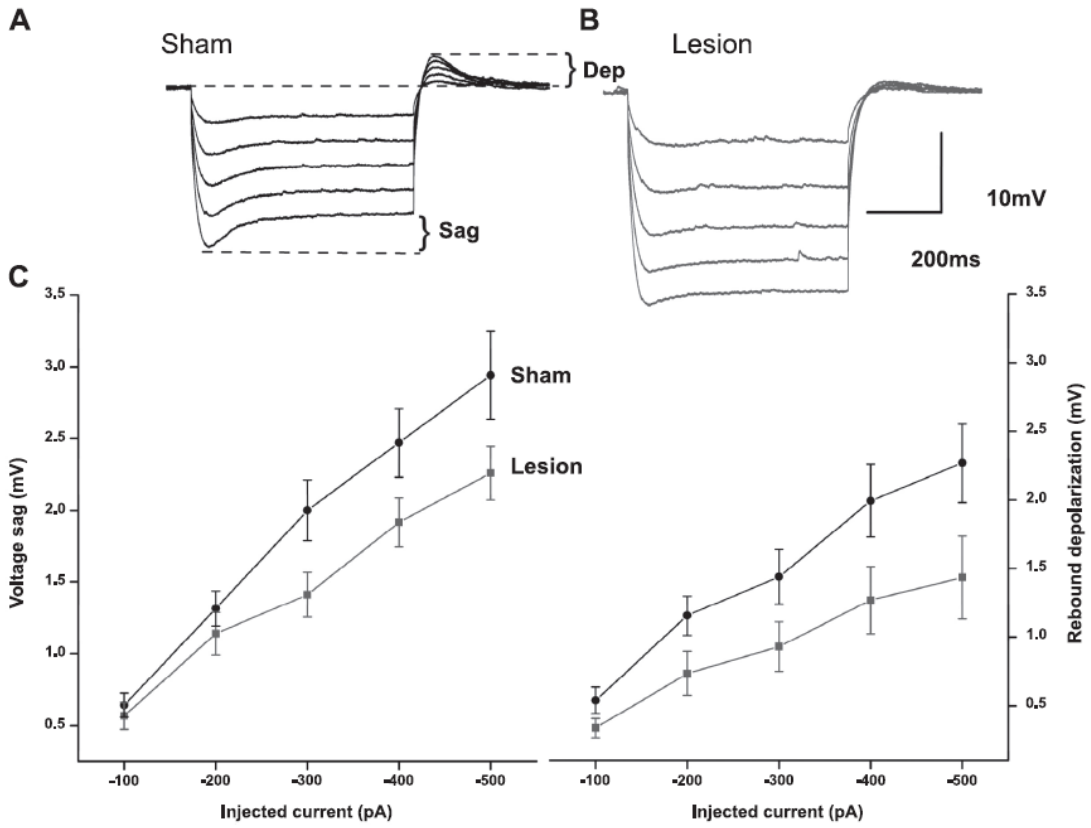

Figure 2. Reduction in I_h-dependent voltage changes in L5 pyramidal neurons from lesioned animals. *A:* specimen records showing that membrane hyperpolarization in sham operated animals is associated with a depolarizing "sag" in membrane voltage caused by I_h activation. Rebound depolarizations (Dep) are also seen. *B:* superimposed specimen records showing that sag responses are reduced in a pyramidal neuron near the freeze lesion. Rebound depolarizations upon current offset are also reduced. *C:* summary graphs showing a significant reduction in the amplitude in the voltage sag and rebound depolarization in pyramidal neurons from freeze lesioned animals.

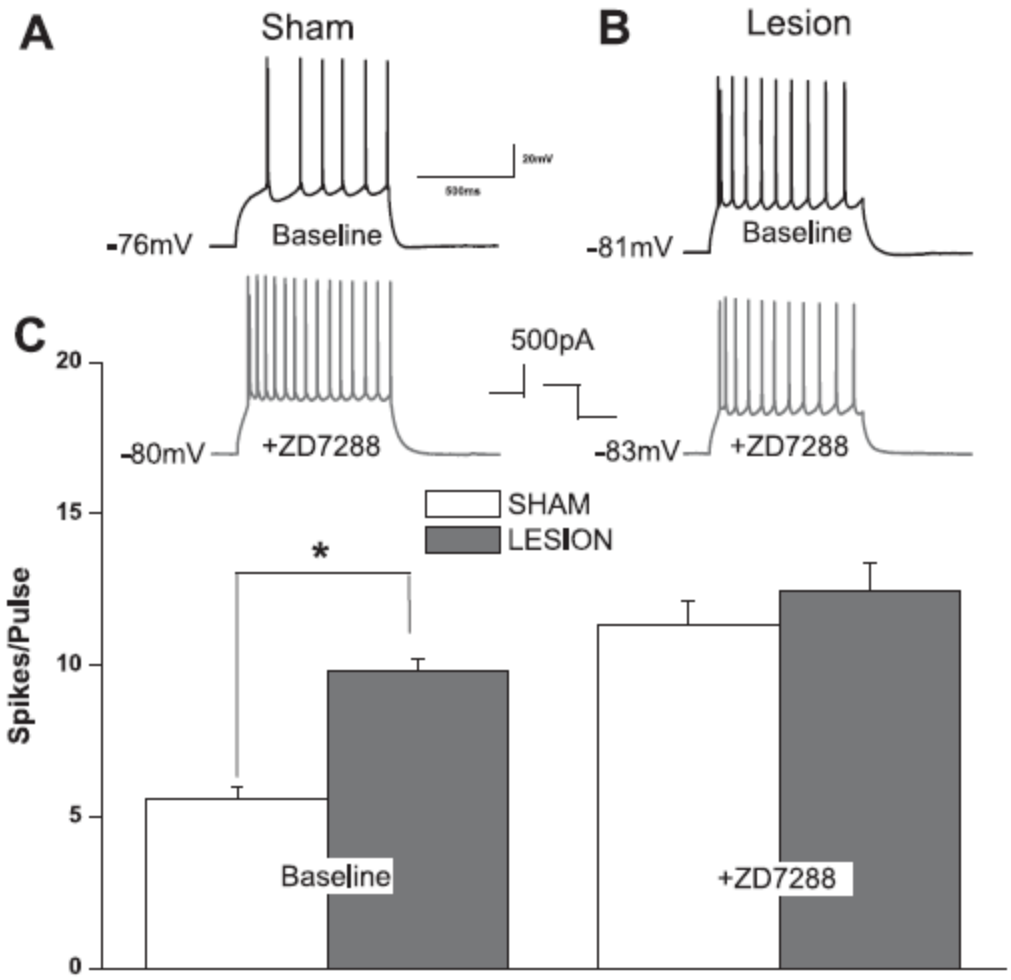

Figure 3. L5 pyramidal neurons from freeze lesioned rats have increased intrinsic excitability. *A:* recordings showing a somatically evoked train of action potentials in a neuron from a sham operated animal (upper). In the same cell during bath application of ZD7288, the membrane potential is hyperpolarized and the number of action potentials is increased (lower). *B:* records obtained from a pyramidal neuron near a lesion. The same current injection resulted in a greater number of spikes under control conditions (upper). After ZD7288, the membrane potential and number of evoked action potentials is virtually unchanged. *C:* summary graphs showing difference in number of action potentials (APs) between sham-operated and lesioned animals before (left) and during ZD7288 (right). The difference in AP number is not significant following I_h inhibition.

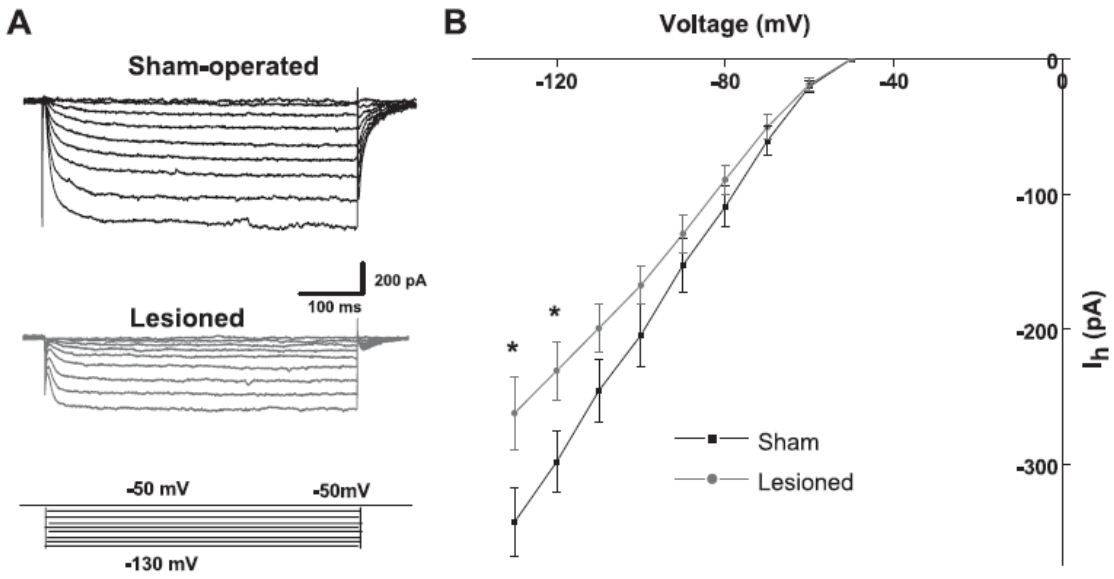

Figure 4. Voltage clamp recordings of I_h in neurons from sham-operated and lesioned animals. *A:* Upper, ZD-sensitive somatic I_h currents obtained by subtracting obtained before and after bath application of ZD7288) in a L5 pyramidal neurons from a sham-operated animal. Slowly activating I_h currents are observed. Lower, recordings from a neuron near the freeze lesion revealed a significant decrease in I_h amplitude following membrane hyperpolarization. *B:* summary diagram showing current-voltage plots for a group of neurons in sham-operated and lesioned animals.

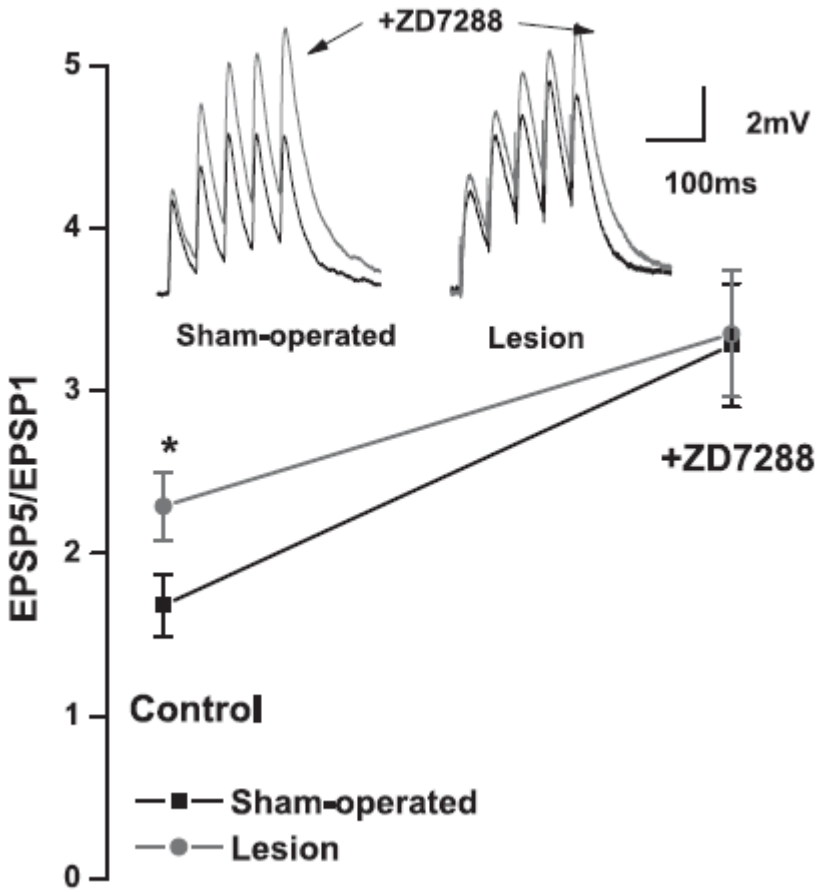

Figure 5. Effects of ZD7288 on EPSP summation in sham-operated and lesioned animals. *Upper left:* specimen records of EPSPs evoked by a train of stimuli at 20 Hz. In a slice from a sham-operated animal, under control conditions (Black trace) EPSPs show weak facilitation. After ZD7288 (red traces) amplitudes of EPSPs in this neuron were increased. *Upper right:* similar experiment in a neuron from a slice from a lesioned animal. EPSPs evoked at 20 Hz. summated to a significantly greater degree in pyramidal neurons from freeze lesioned rats. EPSPs showed increased facilitation in presence of ZD7288. Graph of EPSP5/EPSP1 ratios show that under control conditions ratios were significantly higher in sham-operated group. This difference was not significant after ZD7288.

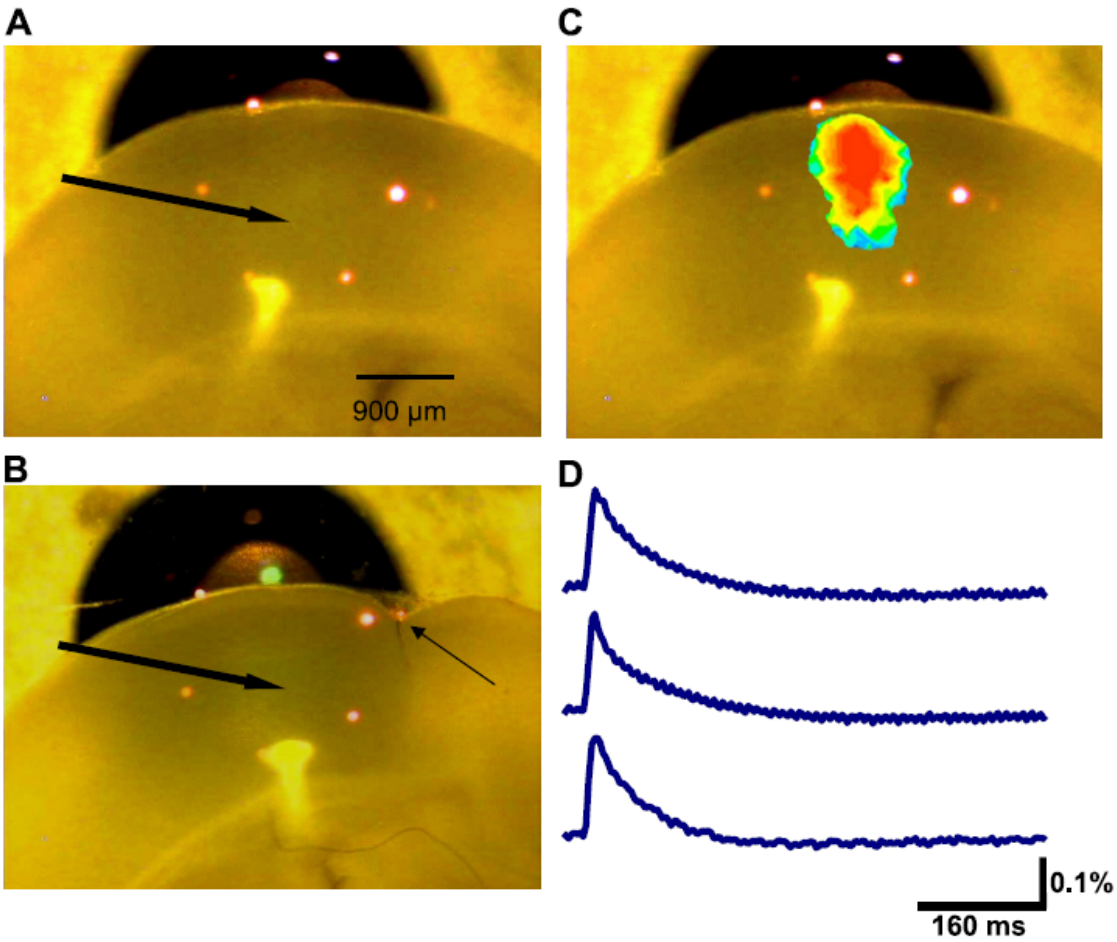

Figure 6. Voltage sensitive dye imaging of evoked activity. *A:* photograph showing the typical position of the brain slice over the diode array. The red dots indicate the borders of the hexagonal photodiode array. The array was positioned so that the upper limit was approximately in line with the pial surface. The arrow indicates the approximate position of the stimulating electrode. *B:* similar picture showing the typical position of the array relative to the freeze lesion. Small arrow indicates location of lesion. *C:* a pseudocolored image of peak activity is shown superimposed on the image of a slice. *D:* typical responses from selected individual diodes showing time course of fluorescence change.

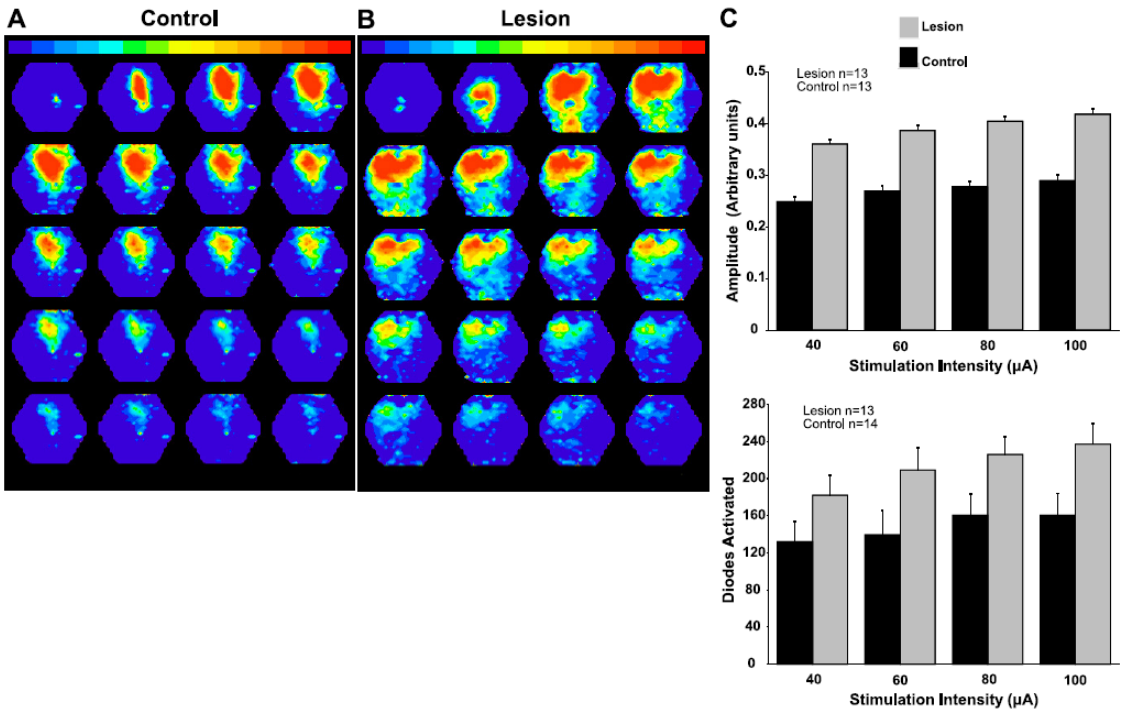

Figure 7. Comparison of voltage-sensitive dye signals in control and lesioned animals. *A:* specimen record of a typical network response evoked from control cortex. *B:* a typical network response evoked in the hyperexcitable region adjacent to the freeze lesion. Evoked activity near the malformation in freeze lesioned rats spreads further and is of higher amplitude. *C:* summary diagrams showing differences in response amplitude (upper) and number of diodes activated (lower) in lesion versus control animals.

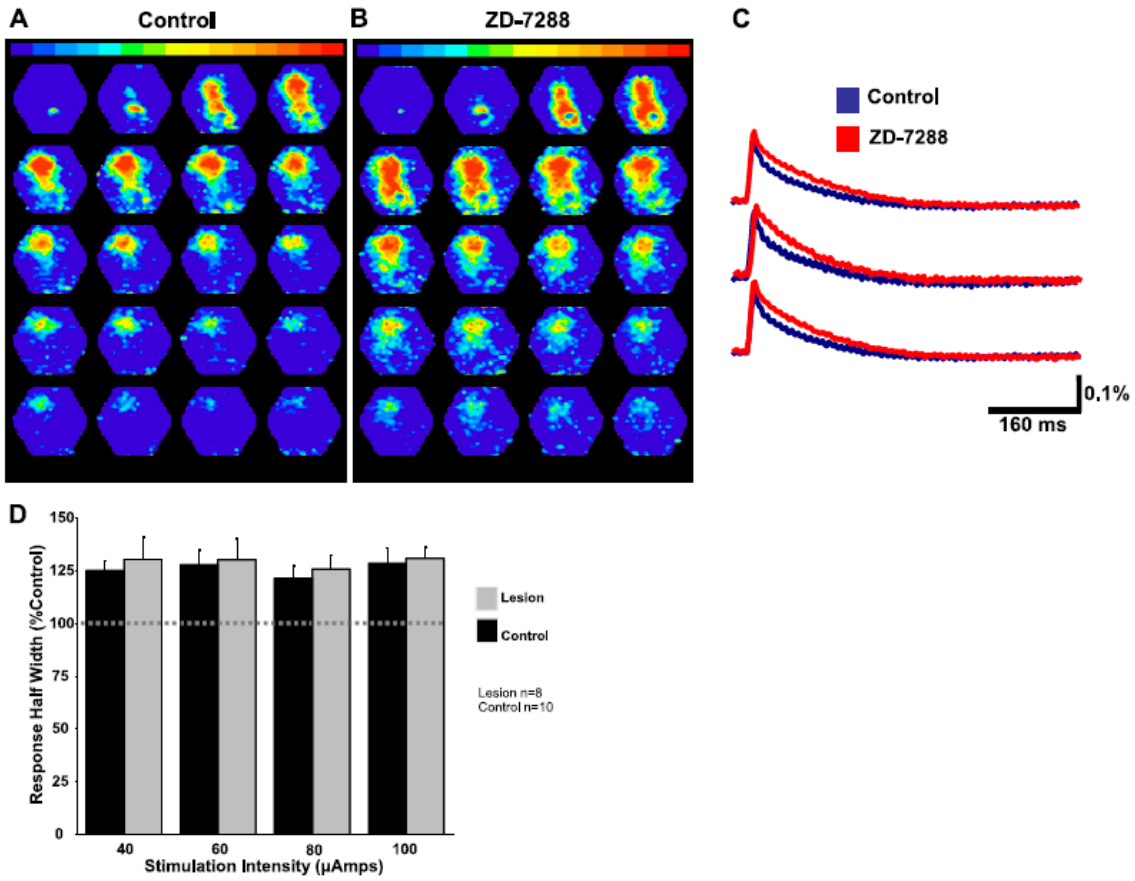

Figure 8. HCN channel inhibition increases the duration of evoked network activity. *A:* a typical network response evoked before HCN channel inhibition in a control animal. *B:* the same response following HCN channel inhibition with 10 µM ZD7288. *C:* responses from individual diodes before (blue) and after (red) HCN channel inhibition are shown superimposed. HCN channel inhibition increased the half width of these responses. *D:* bar graphs showing that I_h inhibition increases the duration of evoked activity in both control and lesioned animals.

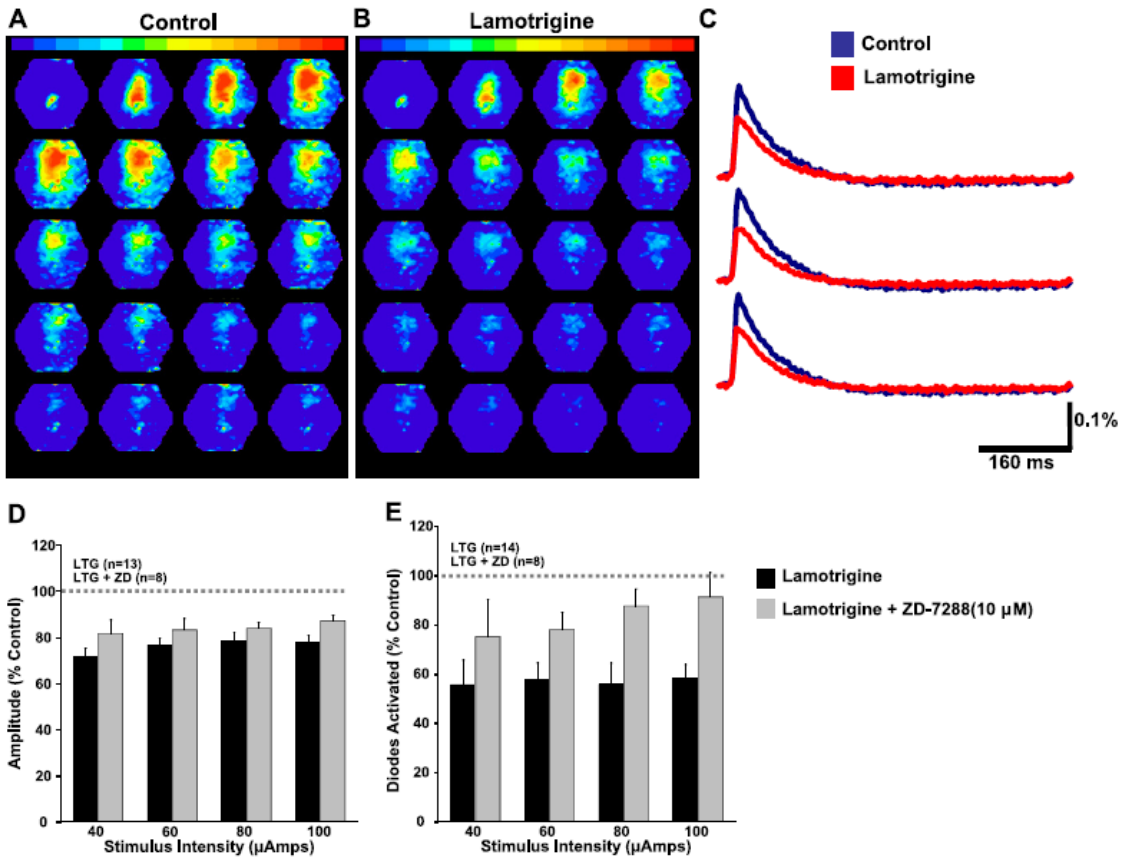

Figure 9. Effects of the anticonvulsant lamotrigine on evoked network activity. *A:* a typical network response evoked in a control animal before lamotrigine. *B:* response to the same stimulation 20 min after application of lamotrigine. *C:* responses from individual diodes before (blue) and after (red) lamotrigine. *D:* Lamotrigine reduced the amplitude of diode responses in control in control animals. This effect was significantly attenuated by co-application of ZD7288. *E:* Lamotrigine reduced the number of diodes activated (indicating spread of activity) in control animals. This effect was also significantly attenuated by co-application of ZD7288.

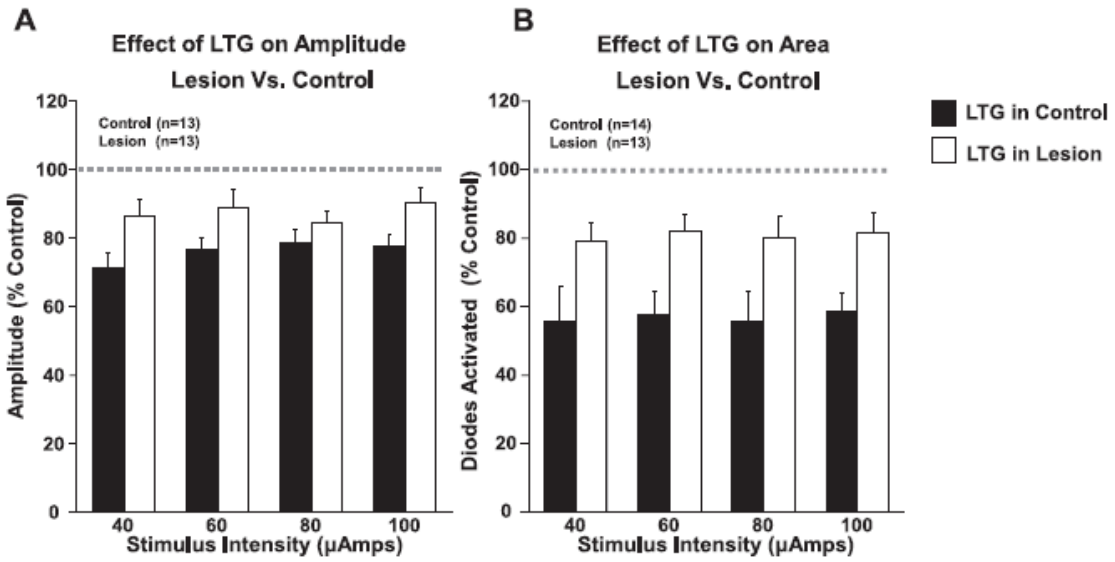

Figure 10. Sensitivity of network activity to lamotrigine. The ability of the anticonvulsant lamotrigine to reduce network activity is partially blocked by ZD7288. is reduced in animals with freeze lesions. *A:* The ability of lamotrigine to decrease voltage sensitive dye signal amplitude was significantly reduced in lesioned animals (white bars). *B:* The ability of lamotrigine to reduce the spread of the voltage sensitive dye signal was significantly reduced in lesioned animals (white bars).

REGULATION OF NEOCORTICAL EPILEPTIFORM DISCHARGES BY HCN CHANNELS

by

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ABSTRACT

Hyperpolarization activated non-specific cation (HCN) channels have a well-characterized role in the reduction of individual cellular excitability as well as a role in the constraint of normal network activity. The channel's role in the dynamics of epileptiform events is poorly understood however. This is especially pertinent given their almost universal reduction in models of epilepsy. We hypothesized that inhibition of HCN channels would increase the severity of epileptiform events evoked in disinhibited neocortex. When recording from L5 and L2/3 pyramidal neurons, we found that HCN channel inhibition significantly increased the magnitude of evoked epileptiform events. Furthermore, HCN channel inhibition increased the number of action potentials overlying events in layer 5 pyramidal neurons. We also tested the hypothesis that HCN channels regulated epileptiform events differently in interneurons and pyramidal neurons. We recorded events in both layer one and layer five interneurons. Interestingly, epileptiform events in both inhibitory cell types were not associated with large numbers of overlying action potentials. HCN, channel inhibition increased the magnitude of epileptiform events in both cell types, but we did not observe an increase in spiking. The increased magnitude of epileptiform events was due to an effect on increased network activity. Holding cells at depolarized potentials to preclude HCN channel opening did not eliminate the effect. The increased magnitude of epileptiform events following HCN channel inhibition often masked a late GABAB response in layer one interneurons. Finally, enhanced Ih with the anticonvulsant lamotrigine decreased the magnitude of evoked epileptiform events.

INTRODUCTION

In the neocortex, hyperpolarization-activated, non-specific cation (HCN) channels are highly expressed in the apical dendrites of layer 5 (L5) pyramidal neurons (Notomi and Shigemoto 2004; Lorincz et al. 2002). HCN channels are coded by four genes (HCN 1-4), resulting in channels with unique activation and expression characteristics (Santoro and Tibbs 1999; Wainger et al. 2001; Notomi and Shigemoto 2004). These channels have functional roles in long term potentiation (Nolan et al. 2004) and motor learning (Nolan et al. 2003). When active at resting membrane potentials, the HCN-mediated non-specific cation current, I_h, depolarizes neurons, increases membrane conductance and reduces dendritic excitability (Magee 1998; Berger et al. 2001; Robinson and Siegelbaum 2003). HCN channels act in a well-characterized fashion to filter distal synaptic inputs. I_h normalizes the decay time of distally evoked EPSPs (Williams and Stuart 2000) and decreases temporal summation (Berger et al. 2003). In hippocampal CA1 pyramidal neurons, I_h also inhibits the peak voltage reached by strongly evoked EPSPs via interaction with the M-current (George et al. 2009).

Given the inhibitory action of I_h on dendritic excitability, EPSPs, and temporal summation, considerable attention has been given to the role of HCN channels in epilepsy. Reductions I_h have been described in human epileptogenic cortex (Wierschke et al. 2010). Decreases in I_h or HCN channels have been reported in pilocarpine (Jung et al. 2007), kainate (Shin et al. 2008), and kindling models (Powell et al. 2008) of epilepsy. Reduced seizure thresholds are seen in HCN1 knockout mice (Huang et al. 2009). HCN2 knockout mice (Ludwig et al. 2003) and animals with spontaneous mutations in the HCN2 gene (Chung et al. 2009) exhibit absence epilepsy. Spontaneously epileptic

WAG/Rij rats (Strauss et al. 2004) and rats with cortical malformations (Albertson et al. 2011) also exhibit changes in I_h. The effect of Ih inhibition on acutely induced epileptiform discharges has not been widely examined.

Considering the role of I_h in regulating neuronal excitability and synaptic integration, as well as the correlation between loss of I_h and epilepsy, there has been limited investigation of the role of I_h in regulating network activity. HCN currents enhance subthreshold oscillations in entorhinal cortex, potentially facilitating the generation of rhythmic and synchronous hippocampal activity (Dickson et al. 2000). I_h inhibition disrupts theta frequency oscillations in hippocampal interneurons (Griguoli et al. 2010) and CA1 pyramidal neurons (Marcelin et al. 2009) and alters the strength and frequency of intrathalamic network oscillations (Yue and Huguenard 2001). Interictal burst frequency and regularity in mouse neonatal hippocampus is reduced by I_h blockers (Agmon and Wells 2003). The precise mechanisms underlying these network effects have still under investigation.

GABAergic interneurons are important and diverse elements of cortical networks (Markram et al. 2004). Various interneuron subtypes differential and specifically target localized regions of principal cells in both hippocampus (Danglot et al. 2006) and neocortex (Markram et al. 2004). These inhibitory cells modulate network activity in various ways, including regulating the firing probability of target cells (Miles et al. 1996), synchronizing neuronal activity (Cobb et al. 1995) and tuning synaptic integration (Pouille and Scanziani 2001). Electrically induced seizure-like oscillations can be driven by fast spiking hippocampal interneurons (Fujiwara-Tsukamoto et al. 2010). Immunoreactivity for HCN channels is not prominent in neocortical GABAergic

interneurons (Lorincz et al. 2002) but has been observed in hippocampal interneurons (Lorincz et al. 2002) as has HCN mRNA (Brewster et al. 2007). Physiological studies indicate that GABAergic interneurons have I_h currents which vary in amplitude and kinetics (Wu and Hablitz 2005; Aponte et al. 2006; Lujan et al. 2005). HCN channel modulation of GABAergic interneuron excitability in epilepsy has not been investigated.

The $GABA_A$ receptor disinhibition model has been extensively used to identify ionic and synaptic mechanisms underlying epileptiform activity both in vivo (Matsumoto et al. 1969) and in vitro (Gutnick et al. 1982). In the neocortex, $GABA_A$ receptor antagonists induce epileptiform events that are mediated by both NMDA and non-NMDA receptors (Hwa and Avoli 1991; Lee and Hablitz 1991). Paroxysmal activity is initiated by a population of intrinsically bursting neurons located in L4 and upper L5 (Connors 1984; Chagnac-Amitai and Connors 1989). Propagation of epileptiform activity occurs in a sterotyped and reliable fashion (Alefel et al. 1998). In the present study, we examined the influence of HCN channel inhibition on bicuculline-induced epileptiform discharges in L2/3 and 5 pyramidal cells and GABAergic interneurons in L1 and L5. We found that I_h inhibition enhances the duration of evoked epileptiform events and increased the number of superimposed action potentials (APs) in pyramidal neurons. The increase in the duration of epileptiform discharges was due, in part, to increased excitatory network activity. Finally, we found that I_h enhancement with the anticonvulsant lamotrigine decreased the amplitude of epileptiform discharges and reduced the number of overlying APs.

METHODS

Slice preparation

Neocortical slices were prepared from Sprague-Dawley rats (PND 20-26). Rats were anesthetized with isoflurane and decapitated. The brain was removed and immediately placed in ice-cold oxygenated (95% O_2/5% CO_2, pH 7.4) cutting solution consisting of (in mM): 135 N-Methyl-D-glucamine, 1.5 KCl, 1.5 KH_2PO_4, 23 choline HCO_3, 0.4 ascorbic acid, 0.5 $CaCl_2$, 3.5 $MgCl_2$ and 25 D-glucose (Tanaka et al. 2008). Coronal brain slices (300 µm thick) of somatosensory cortex were made using a Microm HM 650 vibratome (Microm, Walldorf, Germany). Slices were stored in saline containing (in mM) 124 NaCl, 2.5 KCl, 10 D-glucose, 26 $NaHCO_3$, 2.0 $Ca2^+$, 2.0 Mg^{2+} at 37°C for 40-60 minutes, then kept at room temperature until recording. Individual slices were transferred to a submerged recording chamber mounted on the stage of a Zeiss Axio Examiner D1 (Carl Zeiss Inc. Thornwood, NY) microscope. Dodt contrast optics, a 40X-water immersion lens and infrared illumination were used to view neurons in the slices. The recording chamber was continuously perfused with oxygenated saline (3 ml/min at 32°C). Pyramidal cells (L5 and L2/3) and interneurons (L1 and L5) were identified by their distance to the pial surface, morphology, and spiking properties. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals using protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Whole-cell recording

Whole-cell current-clamp recordings were obtained using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Patch electrodes, with an open tip

resistance of 3-5 MΩ, were filled with an intracellular solution containing (in mM): 125 K-gluconate, 10 KCl, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP, 0.5 EGTA, which had an adjusted pH and osmolarity of 7.3 and 290, respectively. In most experiments, biocytin (0.5%; Sigma-Aldrich, St. Louis, MO) was added to the intracellular solution for post-hoc morphological analysis. Tight seals of 1 GΩ or greater were obtained under visual guidance before breaking into whole-cell mode. Synaptic responses were evoked using a concentric bipolar electrode (FHC, Bowdoin, ME) positioned 100-200 μm lateral to the recording electrode, using 20-250 μA current pulses of 100 μs duration.

Data collection and analysis

Signals were acquired using Clampex 8.2 software and Digidata 1322A interface (Molecular Devices). Evoked responses were filtered at 2 kHz, digitized at 10 kHz, and analyzed using Clampfit 8.2 software (Molecular Devices). Data are expressed as mean ± SEM. Statistical analysis of responses before and during drug application was performed using a two-tailed Student's t-test, for which $p < 0.05$ was considered significant.

Drug application

ZD-7288, lamotrigine and SCH-50911 were all purchased from Tocris Bioscience (Ellisville, MO). Bicuculline methiodide (BIC) was obtained from Sigma-Aldrich. Drugs were prepared as stock solutions and frozen. Individual aliquots were added to the saline for each experiment. All drugs were bath applied and each neuron served as its own control.

RESULTS

I_h constraint of epileptiform discharges in neocortical pyramidal neurons

Synaptic integration in L5 pyramidal cells is strongly influenced by I_h which attenuates EPSPs (Berger et al. 2001). L2/3 pyramidal neurons also exhibit I_h (Sutor and Hablitz 1993) despite an absence of HCN1 immunoreactivity (Lorincz et al. 2002). BIC induced epileptiform discharges are prominent in both cell types (Gutnick et al. 1982; Lee and Hablitz 1991). The effect of I_h inhibition on paroxysmal activity in the GABA disinhibition epilepsy model has received little attention. We therefore examined the effect of I_h inhibition with ZD 7288 on evoked epileptiform activity in both L5 and L2/3 pyramidal neurons.

We obtained whole-cell patch clamp-recordings from visually identified L5 and L2/3 pyramidal neurons in rat sensorimotor cortex. Neurons were identified on the basis of distance below the pial surface, pyramid-like cell body, and presence of a prominent apical dendrite. In the first series of experiments, the bipolar stimulation electrode was placed 100-200 μm lateral to the recording electrode. The strength of stimulation was set to the lowest value that consistently evoked an epileptiform event. As reported previously (Gutnick et al 1982), stimulation in the presence of $GABA_A$ receptor antagonists evoked a characteristic, long-lasting (200-300 ms) epileptiform discharge. This response consisted of a large depolarization with superimposed APs that was similar both L5 (Fig. 1A) and L2/3 (Fig. 1C) pyramidal neurons. After bath application of the HCN channel inhibitor ZD-7288 (20 μM, 10 min), an enhancement of epileptiform discharges was observed in both cell types (Fig. 1A & C). This was quantified by measuring the depolarization area and the number of superimposed action potentials before and after ZD-7288 application. As shown in Fig. 2 A and B, areas were significantly increased in the presence of ZD 7288 in both L5 (Control: 10561.56 ±

2452.28 mV*ms; ZD 7288: 25896.74 ± 4663.85 mV*ms, t-test $p<0.05$, n=9) and L2/3 pyramidal neurons (Control: 86787.9 ± 25818.24 mV*ms; ZD 7288: 149176.4 ± 22453.6 mV*ms, t-test $p<0.05$,n=7). The number of APs observed before and after drug application was not significantly different in either L5 (Control: 10.52 ± 6.1 spikes; ZD 7288: 16.66 ± 10.5 spikes, t-test $p>0.05$, n=9) or L2/3 cells (Control: 3.0 ± 0.82 spikes; ZD 7288: 3.43 ± 1.31 spikes, t-test $p<0.05$ n=7) (Fig. 2A and B).

Stimulation either in the white matter or at pial surface of neocortical slices in vitro is effective in eliciting epileptiform discharges (Gutnick et al 1982). We used L1 stimulation to determine if paroxysmal events evoked at sites remote to the neuron being recorded were altered by reductions in I_h. We recorded from L5 pyramidal neurons as before, but positioned the stimulation electrode in L1. Epileptiform events were similar to those observed with proximal stimulation observed (Fig. 1B). I_h inhibition significantly increased the area (Control: 11126.13 ± 4067.71 mV*ms; ZD 7288: 31011.23 ± 7938.21 mV*ms, t-test $p<0.05$, n=9) of the epileptiform events (Fig. 2C) similar to the effect of ZD 7288 on proximally evoked events (Fig. 2A). In contrast to locally evoked events, the number of superimposed APs following distal stimulation was increased in the presence of ZD-7288 (Control: 6.68 ± 0.95 spikes; ZD-7288: 13.69 ± 3.1 spikes, t-test $p<0.05$, n=9) in L5 pyramidal neurons (Fig. 2C). This increased output could provide an increase in local recurrent excitatory drive thus leading to the observed area changes. We also recorded from L2/3 pyramidal neurons while stimulating in L5. ZD-7288 again significantly increased event area (Control: 68158.68 ± 21450.14 mV*ms; ZD 7288: 106684.12 ± 24352.09 mV*ms, t-test $p<0.05$, n=5) (Fig. 2D) but did

not increase the number of associated APs (Control: 3.6 ± 1.12 spikes ZD 7288 3.4 ± 0.81 spikes, t-test $p>0.05$, n=5) (Fig. 2D).

Excitation of GABAergic interneurons during epileptiform activity

Interneurons have critical roles in the regulation of network activity (Cobb et al. 1995; Manseau et al. 2010; Gastrein et al. 2011). Although HCN antibodies do not typically stain interneurons in the neocortex (Lorincz et al. 2002), electrophysiological studies have revealed functional I_h channels in several types of interneurons (Aponte et al. 2006; Yan et al. 2009; Peng et al. 2010; Zemankovics et al. 2010). Excitation of GABAergic interneurons during epileptiform discharges is poorly understood (Scharfman 2007) and the role of I_h in governing this activity has not been examined. We therefore studied the effect of I_h inhibition on evoked paroxysmal responses in cortical GABAergic interneurons.

Recordings were first obtained from visually identified non-pyramidal neurons in L5. Neurons were included only if their physiological properties were consistent with those of basket cells, namely, input resistances around 200 MΩ, narrow spike width and high frequency, non-accommodating AP firing in response to current injection (Kawaguchi and Kondo 2002; Kawaguchi and Kubota 1993). An example of such a neuron, labeled with biocytin, is shown in Fig. 3A. Consistent with basket cells (Okhotin and Kalinichenko, 2002), filled neurons were characterized by short, laterally oriented dendritic projections (Fig. 3A). A step depolarization elicited repetitive AP firing which did not show appreciable accommodation (Fig. 3A). To test for the presence of I_h in these cells, a voltage clamp protocol holding the cell at -50mV and stepping by -10 mV intervals to -210 mV was used. Under voltage clamp conditions, hyperpolarizing voltage

steps revealed that L5 interneurons had small I_h currents (Fig. 3 B&C). I_h activation following hyperpolarization causes the membrane potential to characteristically "sag" back toward rest (Berger et al. 2001). Consistent with our current measurements, L5 interneurons exhibited small voltage sags at hyperpolarized membrane potentials (Fig. 3C).

Intracortical proximal stimulation, at strengths which evoked epileptiform events in neighboring L5 pyramidal cells, elicited large EPSP-like responses of approximately 100 ms in duration and 20 mV amplitude in interneurons (Fig. 3D). Superimposed APs were not typically observed. Inhibition of I_h with ZD 7288 increased the amplitude and duration of evoked events in interneurons. As shown in Fig. 3E, ZD 7288 significantly increased response area (Control: 8278.56 ± 932.05 mV*ms; ZD 7288: 31085.08 ± 9563.08 mV*ms, t-test one tail, $p<0.05$, n=5). This was not associated with the appearance of APs, suggesting basket cell output was not enhanced. Increasing the stimulation intensity from 20-100 μA to 150-1000 μA, elicited paroxysmal bursts with superimposed APs in L5 interneurons (Fig. 3D). ZD 7288 significantly increased the area (Control: 11226.41 ± 1766.14 mV*ms; ZD 7288 21632.65 ± 1779.14, t-test $p<0.05$, n=4) of evoked epileptiform events evoked with strong stimultaion. We did not observe a consistent effect of ZD 7288 on spiking. Furthermore, ZD-7288 did not drive cells which were not bursting to elicit spikes. This raises questions about the effect of HCN channel loss on the balance of excitation and inhibition.

L1 consists primarily of interneurons (Winer and Laru 1989). L1 interneurons display a diversity of firing patterns (Hestrin and Armstrong 1996Zhou and Hablitz 1996). Furthermore, L1 interneurons process information differently than cells in the

other cortical layers (Armstrong-James et al. 1992). We therefore examined the effect of I_h blockade on L1 interneurons. These cells were characterized by dense, laterally projecting dendritic arbors. Only cells with physiological characteristics consistent with those previously reported for L1 interneurons were included (Hestrin and Armstrong 1996; Zhou and Hablitz 1996). These included high input resistance, narrow spike width, and large after-hyperpolarizations following individual APs (Fig. 4A). As with L5 interneurons, L1 interneurons exhibited small amplitude I_h currents, with little or no I_h active at resting membrane potentials (Fig. 4 B&C). Small voltage sags were seen upon hyperpolarization, as observed above in L5 interneurons (Figure 4C). As described for L5 interneurons, evoked epileptiform events in L1 interneurons were characterized by depolarizations 10-30 mV in amplitude and 100-250 ms in duration. Unlike, L5 interneurons, these depolarizations had one to four overlying APs, but did not exhibit overlying bursts following intense stimulation (Fig. 4D). As with L5 pyramidal neurons and interneurons, ZD-7288 application significantly increased the response area (Fig. 4E) (Control: 14287.09 ± 1761.5 mV*ms; ZD 7288 30800.84 ± 3352.76 mV*ms, t-test $p<0.05$, n=11). We did not observe a significant effect of ZD-7288 on the number of APs in L1 interneurons during epileptiform events (Control: 0.7 ± 0.47 spikes; ZD 7288 1 ± 0.53 spikes, t-test $p>0.05$, n=10). We also evoked epileptiform events in L1 interneurons with stimulation in L5. As with proximal stimulation, distal stimulation significantly increased the area of evoked epileptiform events in L1 interneurons (Control: 28752.38 ± 6980.82 mV*ms; ZD 7288 55818.74 ± 7259.42 mV*ms, t-test $p<0.05$, n=5) but did not significantly increase the number of overlying APs (Control 0.4 ± 0.24 spikes; ZD 7288

0.8 ± 0.37 spikes, t-test $p>0.05$, n=5). This further suggests that I_h inhibition selectively increases the output of excitatory neurons within the neocortex.

I_h inhibition enhances epileptiform discharges via increased network activity

Synaptic activity onto cortical pyramidal neurons is modulated by I_h. Specifically, I_h reduces the time course of EPSP decay from distal inputs and limits the ability of EPSPs to summate (Berger et al. 2001; Magee 1999). It is therefore possible that the increase in epileptiform activity we observed following I_h inhibition is due to postsynaptic changes in the intrinsic properties of the recorded neuron, rather than increased network excitability. To test this hypothesis, we recorded from L1 interneurons, which displayed small amplitude I_h currents under voltage-clamp conditions. Neurons were held at -60 mV where activation of I_h would be minimized. Under these conditions, stimulation evoked long duration inward currents in L1 interneurons. Bath application of ZD-7288 significantly increased the response area (Fig. 5B) (Control: 9183.46 ± 6661.65 pA*ms; ZD 7288 17224.28 ± 7644.6 pA*ms, t-test $p<0.05$, n=7). Similar results were obtained in L5 pyramidal neurons. Bath application of ZD 7288 significantly increased the response areas (Control: 71509.27 ± 27757.5 mV*ms; ZD 7288: 149584.60 ± 55628.8 mV*ms, t-test $p<0.05$, n=6) (Fig. 5 C&D. These data suggest that the increased area of epileptiform events seen following I_h inhibition is not due to postsynaptic effects in the recorded cell. The effect of I_h inhibition is due to an increase in network excitability, perhaps due to prolongation of activity in local axon collaterals.

$GABA_B$ inhibition and I_h

Epileptiform discharges are often followed by pronounced afterhyperpolarizations attributable to either Ca^{2+}-dependent K^+ conductances (Hablitz 1981) or $GABA_B$ receptor-mediated synaptic potentials (Scanziani et al. 1991; Sutor and Luhmann 1998). When recording from L1 interneurons, a late hyperpolarizing component to the epileptiform response was observed (amplitude 4.53 ± 1.0 mV, n=4). . As shown in Fig. 6A, the amplitude of the late response was voltage-dependent and reversed upon hyperpolarization. Under voltage clamp conditions, a late, long-lasting outward current was observed following the initial inward current (Fig. 6B). We hypothesized that this late response was mediated by $GABA_B$ receptors. To test this, we bath applied the selective $GABA_B$ receptor blocker SCH 50911 (Bolser et al. 1995) (2μM). As shown in Fig. 6C, late response amplitude was significantly reduced by SCH 50811 (90.41% ± 13.7, t-test, $p<0.05$). The enhancement of epileptiform activity following I_h inhibition masked this late inhibitory response (Fig. 6B). The increased duration of the epileptiform events following I_h inhibition outlasted the period during which the late, hyperpolarizing response would normally occur. Voltage clamp recordings revealed that the late, outward current was still present, but occurring in the midst of an increased inward current (Fig. 6C). Together, these data suggest that the normal dampening effect of I_h on epileptiform events may allow $GABA_B$ mediated hyperpolarizations to effectively curtail paroxysmal acyivity.

Enhancing I_h reduces epileptiform activity

The anticonvulsant drug lamotrigine has previously been shown to enhance I_h (Poolos et al. 2002) and recent work from our lab has demonstrated that augumenting I_h with lamotrigine significantly reduces evoked network activity (Albertson et al. 2011).

We tested the hypothesis that enhancing I_h by bath applying lamotrigine would reduce epileptiform events in L5 pyramidal neurons. Lamotrigine (20 μM for at least 20 min) significantly reduced (Figure 7A) the number of superimposed APs (Control 9.9 ± 2.13 Spikes; Lamotrigine 7.7 ± 1.87 spikes, t-test $p<0.05$, n=10) and significantly decreased the amplitude (Figure 7C) of the underlying depolarization (Control: 50.64 mV ± 1.72mV; Lamotrigine 46.04 ± 2.49mV, t-test $p<0.05$, n=10). Paradoxically, lamotrigine also significantly increased the duration of the epileptiform events (Figure 7D) (Control 341.15 ± 60.71 ms; ZD 7288: 482.82 ± 70.79, t-test $p<0.05$, n=10).

DISCUSSION

In this study, we examined the influence of I_h on BIC-induced epileptiform events in the neocortex. I_h inhibition with ZD-7288 was observed to increase the area of paroxysmal depolarizations in L5 and L2/3 pyramidal neurons as well as the number of superimposed APs in L5 pyramidal neurons. When recordings were obtained from L5 and L1 GABAergic interneurons, stimulation evoked smaller depolarizations associated with few or no APs. In the presence of ZD-7288, the number of APs was not increased in interneurons. Under voltage-clamp conditions which precluded activation of I_h, ZD-7288 still produced an increase in paroxysmal activity. This suggests that, at least in part, enhancements in epileptiform activity were due to increased network activation and not a change in the intrinsic properties of the recorded cell. In addition, the increase in magnitude of the epileptiform responses following I_h inhibition masked a slow $GABA_B$-receptor mediated hyperpolarization observed in L1 interneurons. Pharmacologically upregulating I_h with the anticonvulsant lamotrigine reduced the amplitude and number of spikes in epileptiform events, but paradoxically also increased their duration.

The *in vitro* disinhibition model of epilepsy is a well-established model of in vitro network hyperexcitability which produces reliable and reproducible epileptiform discharges (Gutnick et al. 1982) with a characteristic pattern of propagation through the cortical mantle (Alefeld et al. 1998). Neocortical disinhibition results in synchronized epileptiform discharges by enhancing late polysynaptic EPSPs with AMPA and NMDA receptors both contributing to generation of epileptiform activity (Lee and Hablitz 1991; Hwa and Avoli 1992). Use of this acute model avoids potentially confounding changes in HCN expression and neuroanatomical changes that may follow repetitive behavioral seizures in chronic in vivo epilepsy models such as the kainate (Huang et al. 2009) and pilocarpine models (Jung et al. 2010). In the present study, $GABA_A$-receptor blockade allowed us to see specific effects of HCN channel inhibition on both polysynaptic excitatory events and slow, $GABA_B$-receptor mediated events.

HCN channels and excitation of pyramidal cells and inhibitory interneurons

HCN channel inhibition significantly increased the area of evoked epileptiform events in both pyramidal cells and interneurons. Since responses were evoked in the presence of bicuculline, the change is likely produced by an increase in excitatory input. I_h significantly decreases EPSP summation in pyramidal neurons (Williams and Stuart 2000; Berger et al. 2001, 2003). Removing this dampening influence increases the excitability of pyramidal neurons within the neocortical network, enhancing recurrent excitation and paroxysmal activity. Evoked paroxysmal activity likely activates numerous excitatory neurons within the network. As such, individual neurons were likely seeing multiple EPSPs with many of them summating. I_h inhibition probably enhanced this summation within pyramidal neurons increasing the number firing action

potentials. HCN channel's effect on intrinsic membrane properties strongly influences the shape of EPSPs (Williams and Stuart 2000). This increase in excitability could be amplified by associated changes in intrinsic excitability.

In L5 pyramidal cells, I_h inhibition produces a hyperpolarization of the resting potential (Berger et al. 2001; Day et al. 2005; Breton and Stuart 2009; Chen et al. 2010). An increased R_{in} is also observed making it difficult to predict the net effect on intrinsic excitability of L5 neurons (Dyhrfjeld-Johnsen et al. 2009). Despite the membrane hyperpolarization, depolarizing current pulses evoke significantly more APs in HCN1 null mice (Huang et al. 2009) and in several models of epilepsy where HCN expression is reduced (Shah et al. 2004; Jung et al. 2007; Albertson et al. 2011)

The observed changes in the dynamics of the polysynaptic events may be due to similar effects of HCN channel blockade on intrinsic membrane properties. However, we still observed the effect in interneurons which express extremely low levels of Ih (Figure 3B). Furthermore, when interneurons and L5 pyramidal neurons were held at -60 to precluded HCN channel opening prior to wash-in of ZD-7288, the effect was still observed (Figure 5) suggesting Ih inhibition increased epileptiform events by influencing excitatory neurons other than those from which we were recording. While these data suggest that Ih inhibition increases the excitability of the network either directly or indirectly contacting the cell, it does not exclude the possibility of intrinsic changes in the cell itself also influencing the shape of epileptiform events.

GABAergic neurons are hypothesized to play a crucial role in epileptogenesis. Specifically, altered function of inhibitory circuits may contribute substantially to the hyperexcitability observed in human cases of epilepsy (Cepeda et al. 2007). Interneurons

are important for synchronizing populations of hippocampal excitatory neurons (Cobb et al. 1995), and activation of inhibitory neurons can drive hippocampal seizure like activity (Fujiwara-Tsukamoto et al. 2010). Additionally, interneuron cell loss may directly contribute to hippocampal epileptogenesis (Sloviter 1987; Magloczky and Freund 2005). Given this, changes in cellular properties that differentially effect inhibitory and excitatory network elements could strongly contribute to epileptiform events. By observing the effect of HCN channel inhibition during epileptiform events in both interneurons and pyramidal neurons, we were able to compare the consequences of HCN channel loss in these two cell types. While ZD-7288 greatly increased the excitatory drive impacting both cell types, it differentially affected the output of pyramidal neurons and interneurons. ZD-7288 increased the duration of epileptiform events universally, but only in excitatory, layer 5 pyramidal neurons did this result in more spikes. These results suggest that during epileptiform events induced in the presence of bicuculline, loss of HCN channels selectively increases the output of excitatory neurons. This selective increase may cause an imbalance of excitation and inhibition providing an additional mechanism for increased network excitability.

The selective effect of HCN channel inhibition on excitatory neurons is most likely due to differing expression of HCN channels. HCN1 channels have primarily been reported in hippocampal and cortical neurons (Lorincz et al. 2002; Piskorowski et al. 2011) and studies characterizing the inhibitory role of Ih on individual cellular excitability have primarily been conducted in pyramidal neurons (Magee, 1999;Berger et al. 2001). Additionally, recent work characterizing a role for HCN channels in regulating presynaptic release probability showed that Ih selectively acted at synapses onto

pyramidal neurons (Huang et al. 2011). However, HCN channels have been reported within several types of interneurons (Santoro et al. 2000; Aponte et al. 2006; Kilb and Luhmann 2000; Lujan et al. 2005) and enhancement of I_h with the antiepileptic drug lamotrigine has been shown to increase spontaneous GABA release (Peng et al. 2010). Despite this, a similar role for Ih in regulating the excitability of inhibitory neurons has not been described. Our data (Figures 2 & 3) suggest that within layer one and layer five interneurons, there is very little total Ih and this Ih is not activated until significantly hyperpolarized membrane potentials. We did not observe a consistent effect of Ih on the membrane potential of either class of interneurons. Therefore, blockade of Ih may only have the opportunity to influence the excitability of pyramidal neurons. Loss of Ih, like that occurring in epilepsy, may selectively increase the excitability of pyramidal neurons contributing to an imbalance in excitation and inhibition. This may become especially apparent during epileptiform events when neurons are receiving many closely spaced inputs.

Epileptiform events were often followed by a late hyperpolarizing response (Figure 6). While we observed these responses in both layer one interneurons and layer five pyramidal neurons, they were most consistently observed in layer one interneurons. The specific $GABA_B$ receptor blocker SCH 50911 blocked this hyperpolarizing response in layer one interneurons. The response disappeared following HCN channel inhibition (Fig. 6B); however, ZD-7288 did not block the outward current (Fig. 6B) mediating this hyperpolarization, rather HCN channel blockade increased excitatory drive to the point that it overcame the GABAB-mediated inhibitory drive. These data suggests that I_h may decrease the time course of epileptiform events and allow late rebound $GABA_B$ mediated

hyperpolarizing events. Masking of this $GABA_B$ mediated inhibition may contribute to increased excitability in cases of reduced I_h. $GABA_B$ knockout animals exhibit spontaneous seizures (Schuler et al. 2001), $GABA_B$ blockade in vivo results in hippocampal epileptiform discharges (Leung et al. 2005), rats with kainate induced temporal lobe epilepsy have fewer $GABA_B$ receptors (Straessle et al. 2003), and early-life seizures reduce the amplitude of inhibitory $GABA_B$ responses (Qu et al. 2010). Given this, an additional mechanism by which HCN channel loss contributes to hyperexcitability may be its indirect influence on post-epileptic membrane potential by mitigating the effect of $GABA_B$.

Lamotrigine is a commonly used anticonvulsant which has previously been shown to increase the function of HCN channels (Poolos et al. 2002). In concert with the function of Ih in constraining cellular excitability I_h enhancement with lamotrigine decreases pyramidal neuron excitability and inhibits temporal summation (Poolos et al. 2002). We therefore hypothesized that enhancing Ih would reduce the magnitude of epileptiform events, in contrast to the magnifying effect of inhibiting Ih. Lamotrigine significantly reduced the amplitude of evoked epileptiform events in layer five pyramidal neurons as well as the number of overlying action potentials. These data further suggest that Ih modulates the output of cortical excitatory neurons during epileptiform events. Together our data show that reduced Ih increases excitatory output while enhanced Ih decreases excitatory output. We have previously shown that lamotrigine is less effective in a model of malformation epilepsy which exhibits reduced HCN channels (Albertson et al. 2011), suggesting that in cases of epilepsy, the ability of Ih to reduce excitatory output is impaired. Somewhat paradoxically, lamotrigine also increased the duration of the

epileptiform events. This could be a secondary effect of the reduction in the underlying depolarization of epileptiform events leading to reduced or delayed potassium channel opening.

In summary the data presented here show that in addition to its role in the reduction of normal neuronal network excitability, Ih may constrain the severity of epileptiform events. Loss of HCN channels, as occurs in a variety of epilepsy models, may therefore contribute to increased seizure severity. Specifically, our data show that Ih inhibition increases the duration of evoked epileptiform events seen from layer five pyramidal neurons, layer five interneurons, and layer one interneurons. Interestingly, while HCN channel inhibition increased the overlying APs observed in pyramidal neurons, this was not observed in interneurons suggesting that HCN channel inhibition may selectively increase excitatory activation during epileptiform network events. We also observed that increased network activity contributed to the increases severity of events rather than changes in the intrinsic properties of the neurons from which we recorded. This increased network activity masked a late GABAB response. Finally, enhancing Ih with the anticonvulsant lamotrigine reduced the amplitude and spike number of evoked events further suggesting that Ih reduces the severity epileptiform network activity.

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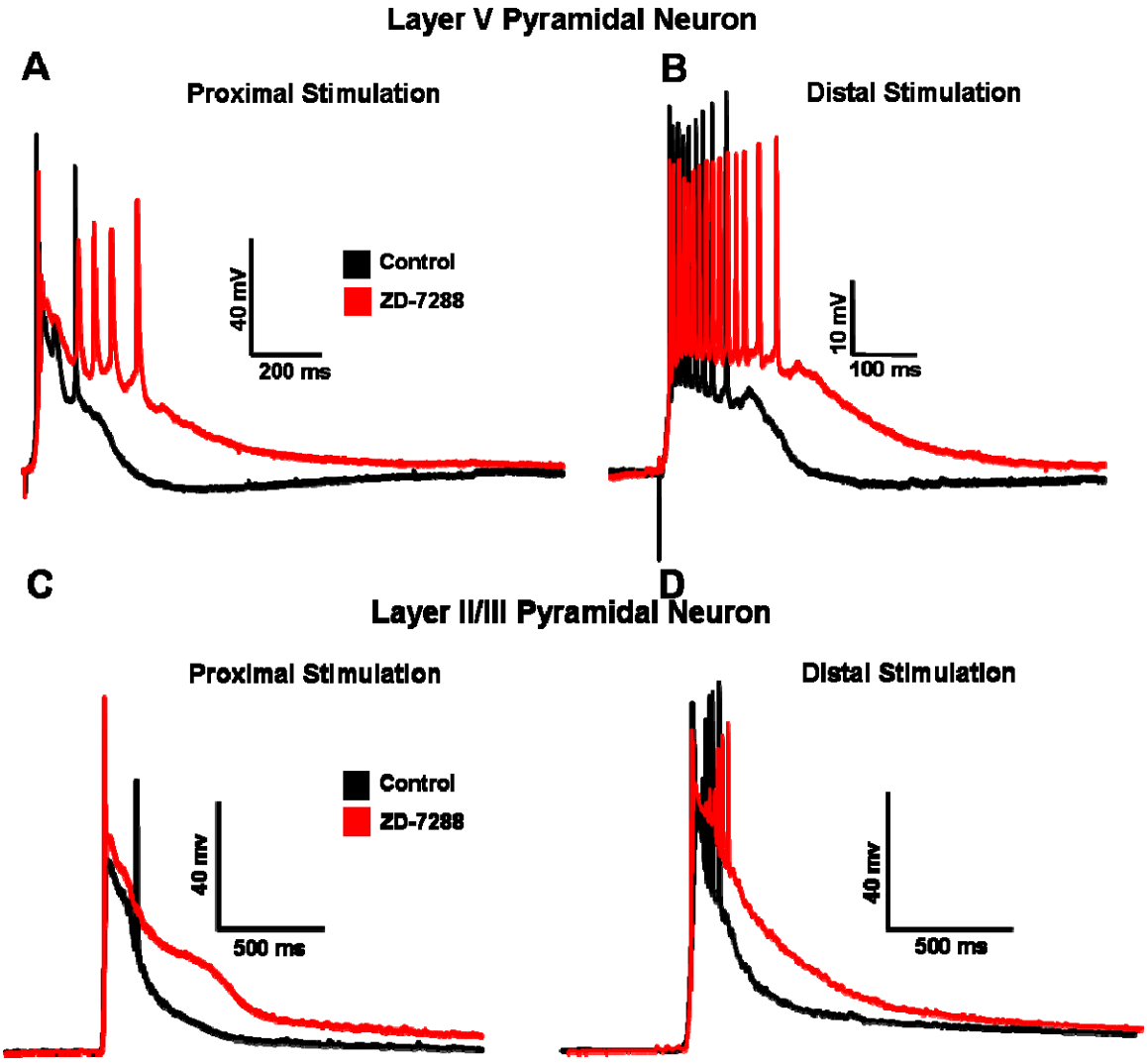

Figure 1. Ih Inhibition Increases the Severity of Epileptiform Events in Layer V and Layer 2/3 Pyramidal Neurons. A. Evoked epileptiform event in a layer five pyramidal neuron before (black) and after (red) ZD 7288 following proximal stimulation B. Evoked epileptiform event in a layer five pyramidal neuron before (black) and after (red) ZD 7288 following distal stimulation. C. Evoked epileptiform event in a layer five pyramidal neuron before (black) and after (red) ZD 7288 following proximal stimulation. D. Evoked epileptiform event in a layer five pyramidal neuron before (black) and after (red) ZD 7288 following distal stimulation.

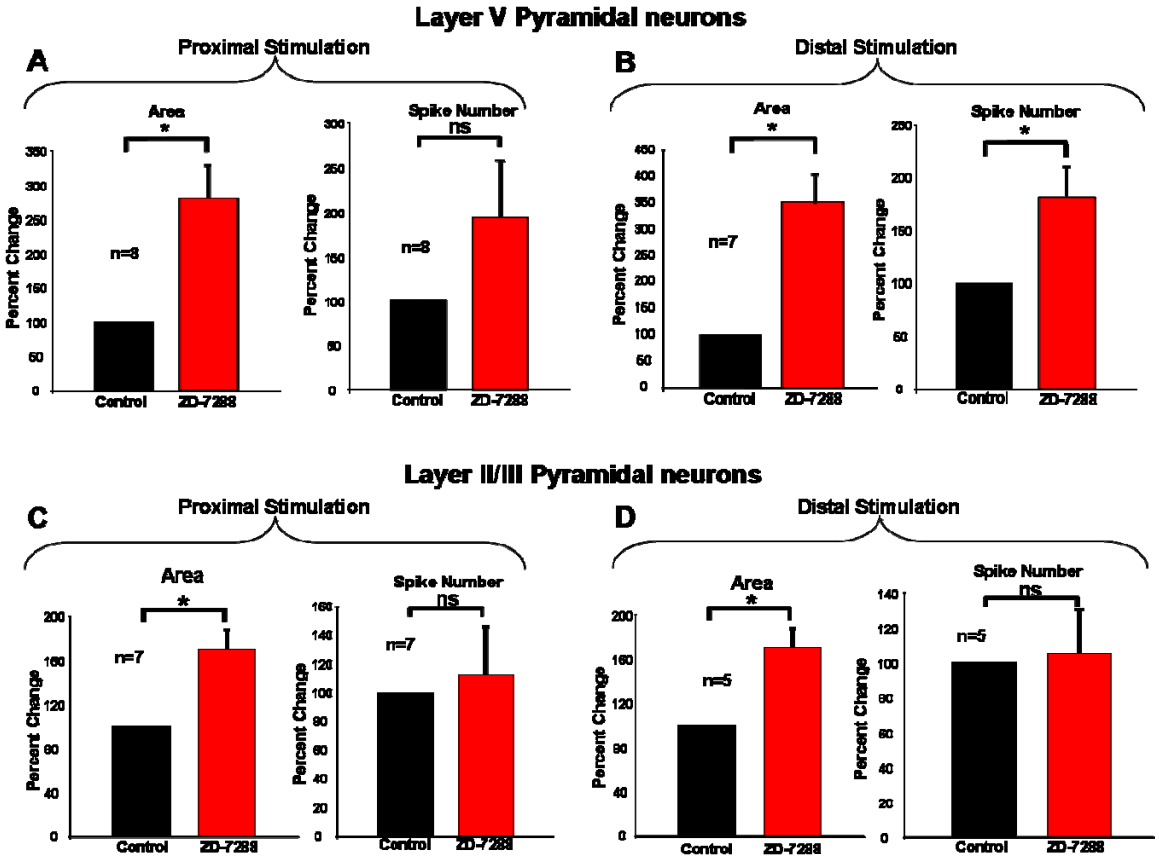

Figure 2. Ih Inhibition Increases the Severity of Epileptiform Events in Layer V and Layer 2/3 Pyramidal Neurons. A. ZD 7288 significantly increases the area of epileptiform events in layer 5 pyramidal neurons but not the number of overlying spikes following proximal stimulation. B. ZD 7288 significantly increases the area of epileptiform events in layer 5 pyramidal neurons and the number of overlying spikes following proximal stimulation. C. ZD 7288 significantly increases the area of epileptiform events in layer 2/3 pyramidal neurons but not the number of spikes following proximal stimulation. D. ZD 7288 significantly increases the area of epileptiform events in layer 2/3 pyramidal neurons but not the number of spikes following distal stimulation.

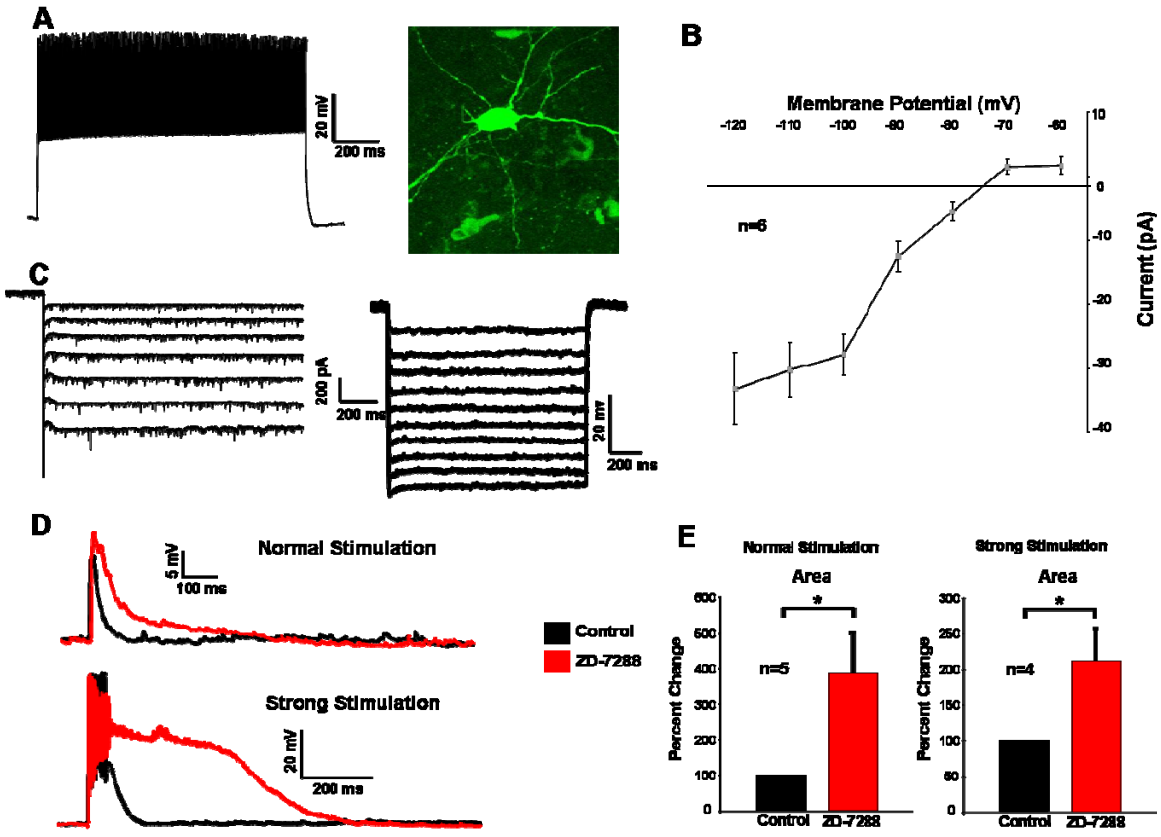

Figure 3. Ih Inhibition Increases the Severity of Epileptiform Events in Layer V Interneurons. A. Typical spike pattern and morphology and spike pattern of layer five interneurons used for study. B. Current voltage relationship showing low levels of Ih in layer five interneurons. C. Ih and associated voltage "sag" typical of layer five pyramidal neurons. D. Epileptiform event in a layer five interneuron before (black trace) and after (red trace) ZD 7288. Lower traces represent typical responses following greatly increased extracellular stimulation intensity. E. ZD 7288 significantly enhances the severity of epileptiform events in layer five interneurons.

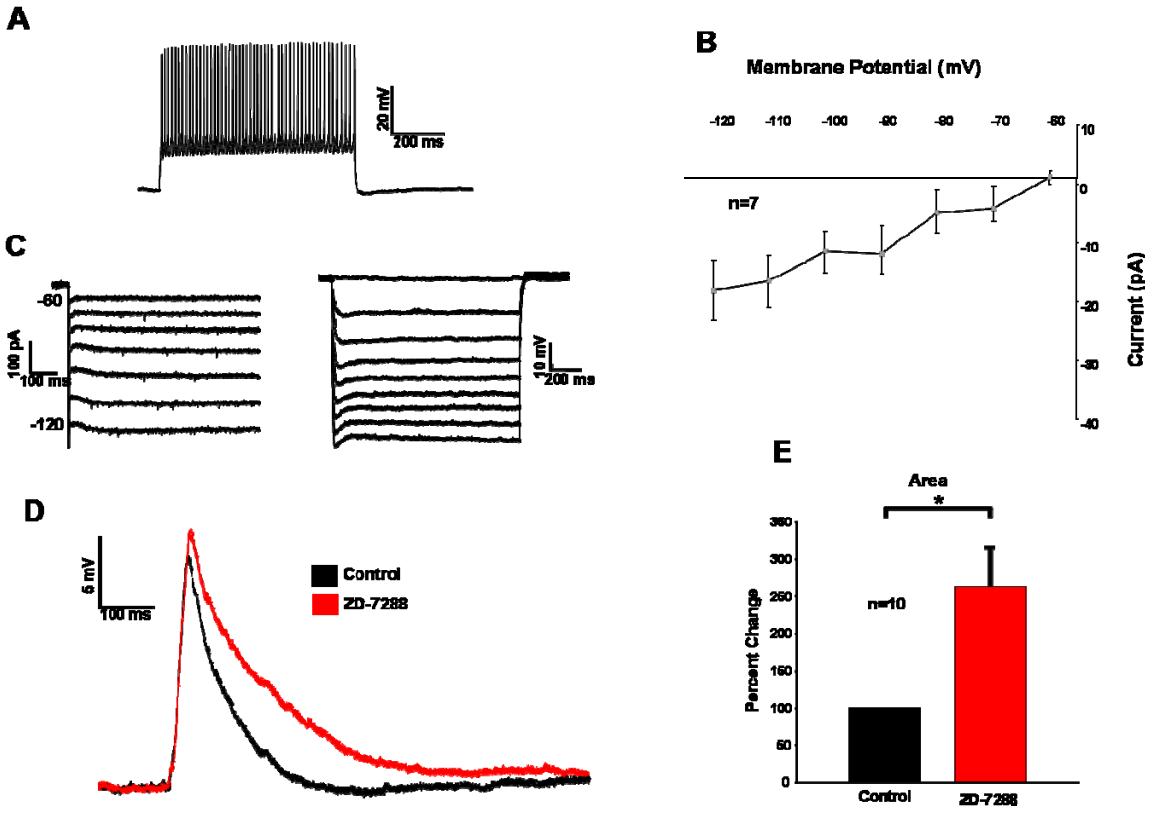

Figure 4. Ih Inhibition Increases the Severity of Epileptiform Events in Layer I Interneurons. A. Typical spike pattern of layer one interneurons used for the study. B. Current voltage relationship showing low levels of Ih in layer one interneurons. C. Ih and associated voltage "sag" typical of layer one interneurons. D. Epileptiform event in a layer one interneuron before (black trace) and after (red trace) ZD 7288. E. ZD 7288 significantly enhances the severity of epileptiform events in layer one interneurons.

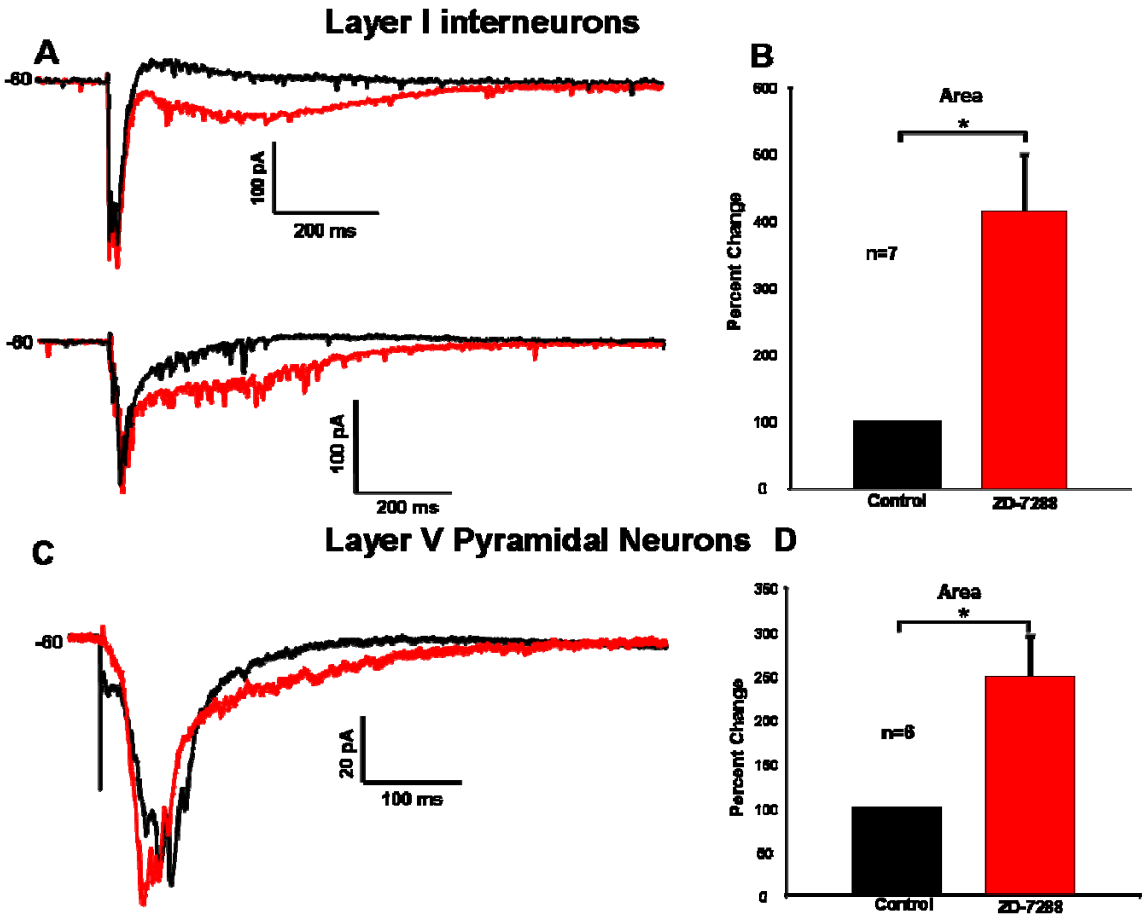

Figure 5. Ih Inhibition Increases Epileptiform Events Severity by Enhancing Network Activation. A. Epileptiform event in a layer one interneuron voltage-clamped at -60 mV before (black traces) and after (red traces) ZD 7288. B. ZD 7288 significantly increases the severity of epileptiform events in layer one interneurons clamped at -60 mV to preclude HCN channel opening. C. Epileptiform event in a layer five pyramidal neuron voltage-clamped at -60 mV before (black trace) and after (red trace) ZD 7288. C. ZD 7288 significantly increases the severity of epileptiform events in layer five pyramidal neurons voltage-clamped at -60 mV to preclude HCN channel opening.

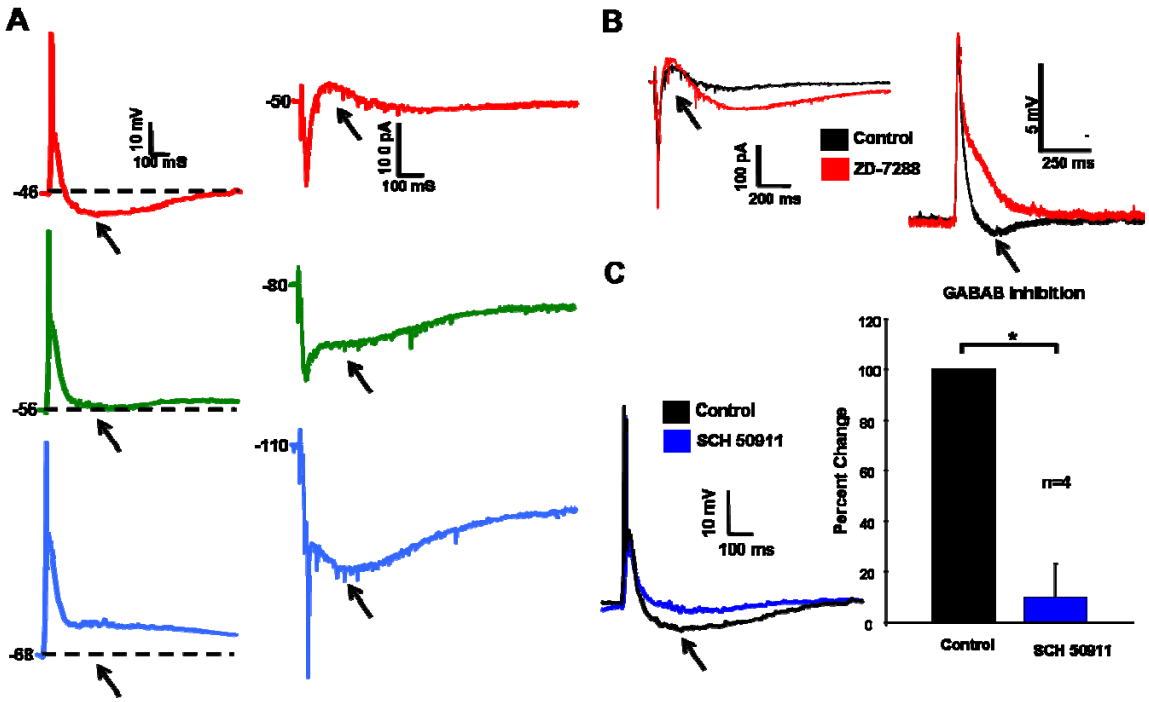

Figure 6. Increases Epileptiform Event Severity Masks a Late GABAB Mediated Hyperpolarization. A. Epileptiform events in layer one interneurons were followed by a slow hyperpolarizing event visible in both current and voltage clamp conditions. The amplitude and polarity of this event was voltage dependent. B. ZD 7288 mediated enhancement of epileptiform network events masked the hyperpolarizing event without blocking the current underlying it. C. The specific GABAB Blocker SCH 50911 almost completely blocked the hyperpolarizing events.

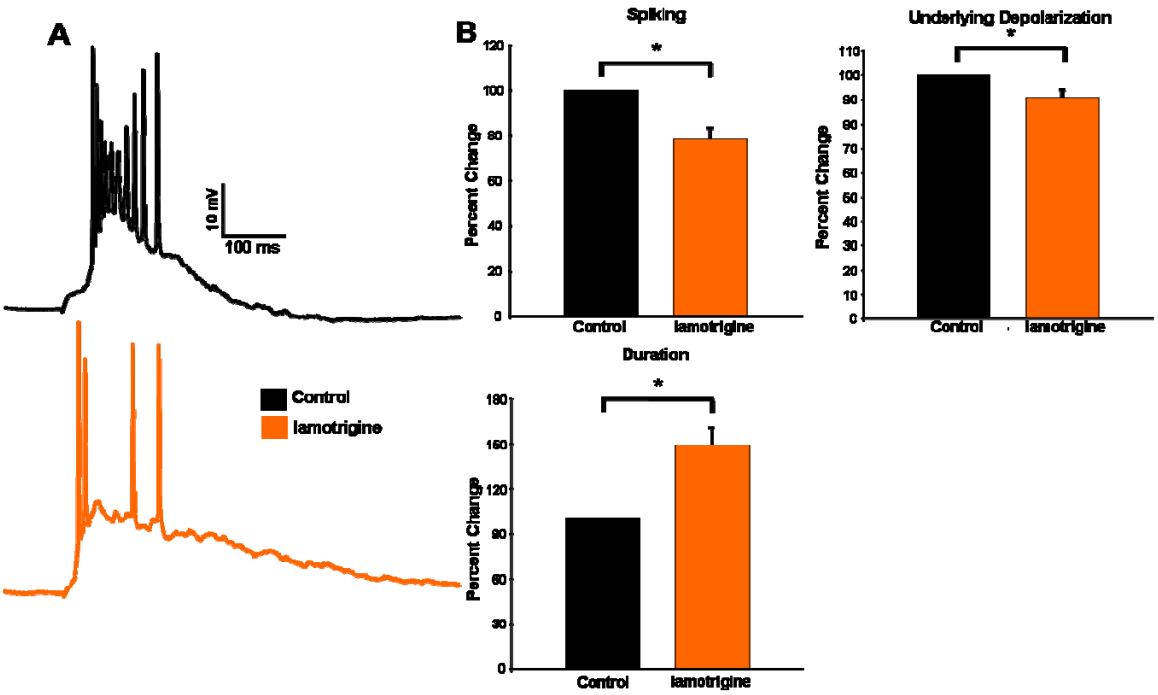

Figure 7. Ih Enhancement Reduced the Severity of Epileptiform Events in Layer One Interneurons. A. Epileptiform event in a layer five pyramidal neuron before (black trace) and after (orange trace) lamotrigine. B. Lamotrigine significantly decreased the amplitude of the underlying depolarization as well as the number of overlying APs in epileptiform events measure in layer five pyramidal neurons. Paradoxically it also increased the duration of these events.

RATS WITH CORTICAL MALFORMATIONS HAVE REDUCED IH IN LAYER V INTERNEURONS

by

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In preparation for *Epilepsia*

Format adapted for dissertation

ABSTRACT

Cortical malformations are associated with severe pharmacoresistant epilepsy. Rats with cortical freeze lesions are a well established model of malformation epilespy. While a variety of factors contribute to malformation associated hyperexcitability, reduced hyperpolarization activated non-specific cation (HCN) channels likely contribute significantly. HCN channels pass a non-inactivating cationic current following membrane hyperpolarization. Typically expressed in the apical dendrites of cortical and hippocampal pyramidal neurons, channels active at rest decrease neuronal membrane excitability and limit excitatory post synaptic potential (EPSP) summation. We have recently demonstrated that HCN channels and I_h are reduced in cortical pyramidal neurons of rats with freeze lesions. This loss likely increases network excitability. Here we demonstrate that HCN channels are also lost in fast spiking layer five interneurons from rats with freeze lesions. While this loss of HCN channels does not affect single EPSPs, it does increase summation of EPSPs onto interneurons. HCN channel inhibition has no effect on synaptic transmission in rats with freeze lesions. Loss of HCN channels does not increase summation of EPSCs in interneurons voltage clamped at -60 mV to preclude channel opening. Furthermore, we did not observe an increase in miniature EPSP frequency, amplitude, or paired pulse ratio in these interneurons. These data indicate that HCN channels are not presynaptically active at excitatory synapses onto these interneurons. Finally we observed a decrease in spike frequency following depolarizing in current injection after HCN channel inhibition and interneurons from freeze lesioned rats had decreased baseline spiking.

INTRODUCTION

Cortical dysplasia is associated with severe pharmaco-resistant epilepsy (Guerreiro 2009; Leventer et al. 2008). Regions of cortical dysplasia are often surgically resected in hopes of reducing seizures, though can have limited efficacy (Krsek et al. 2009; Sisodiya 2000). The epileptogenic nature of dysplastic tissue in cortical malformations is poorly understood. The tissue is characterized by intrinsic hyperexcitability (Guerreiro 2009), abnormal cell types (Cepeda et al. 2003), and gliosis (Guerreiro 2009). Many of the features of human cortical dysplasia, including dislamination and gliosis, are reproduced in rats with cortical freeze-lesions (Dvorak et al. 1978). Rats with freeze lesions develop a small sulcus along the surface of the normally smooth rat brain (Dvorak and Feit 1977; Dvorak et al. 1978; Hablitz and Defazio 1998). The cortical tissue adjacent to the lesion is hyperexcitable and rare spontaneous epileptiform events have been observed (Jacobs et al. 1996; Hablitz and Defazio 1998; Jacobs et al. 1999). Rats with freeze lesions have a reduced threshold for hyperthermia induced seizures (Scantlebury et al. 2004). The hyperexcitable tissue near the freeze lesion is characterized by a variety of neuronal changes including decreased $GABA_A$ binding sites (Zilles et al. 1998), decreased glial glutamate transporters (Campbell and Hablitz 2008), decreased astrocytic inwardly rectifying K^+ currents (Campbell and Hablitz 2008), and increased NR2B containing NMDA receptors (DeFazio and Hablitz 2000). We have recently shown that pyramidal neurons near the freeze lesion have reduced hyperpolarization-activated non-specific cation (HCN) channels and their associated current I_h (Albertson et al. 2011).

HCN channels are unique channels that pass a non-inactivating, cationic current upon membrane hyperpolarization below approximately -60 mV (Robinson and

Siegelbaum 2003). They are coded by 4 genes (HCN1-4) (Santoro et al. 2000) and are expressed primarily in the apical dendrites of hippocampal and cortical pyramical neurons (Notomi and Shigemoto 2004). Importantly, a small amount of current is fluxed at resting membrane potentials which reduces neuronal input resistance and depolarizes the resting membrane potential (Magee 1998). Inhibition of HCN channels increases the intrinsic excitability of neuronal membranes (Magee 1998). The dendritic expression of HCN channels also serves to normalize the time-course of distally evoked EPSPs and limits temporal summations of EPSPs (Williams and Stuart 2000). HCN channel inhibition with low concentrations of the antagonist ZD 7288 allows EPSP summation to reach membrane potentials significantly closer to threshold (Berger et al. 2001).

Given the HCN channel's role in decreasing cellular excitability, it is not surprising that the channel is almost universally reduced in models of epilepsy. The kainate (Shin et al. 2008) and pilocarpine (Jung et al. 2007) models of temporal lobe epilepsy as well as the amygadalic kindling model (Powell et al. 2008) all have reduced HCN channels. Furthermore, excised tissue from human epilepsy patients has indications of reduced I_h (Wierschke et al. 2010). HCN1 knockout animals have reduced seizure thresholds (Huang et al. 2009) and HCN2 knockout animals exhibit an absence epilepsy pheonotype (Ludwig et al. 2003). The spontaneously epileptic *Wag/rig* strain of rats also has reduced I_h (Strauss et al. 2004). Finally as mentioned, our lab has shown rats with freeze induced cortical malformations have reduced HCN channels in layer five cortical pyramidal neurons (Albertson et al. 2011).

The role of HCN channels in neuronal excitability has primarily been examined in pyramidal neurons; however, HCN channels are also expressed in interneurons. HCN

channels have been identified in the axons of hippocampal interneurons (Lorincz et al. 2002) and layer one cortical interneurons in young rats express I_h (Wu and Hablitz 2005). HCN channel inhibition changes the firing properties hippocampal basket cells (Aponte et al. 2006). Furthermore, HCN channels inhibition increases release at inhibitory synapses onto granule cells in hippocampus (Aponte et al. 2006). It is unknown if HCN channels influence the intrinsic and integrative properties of interneurons in the same manner as excitatory pyramidal neurons.

Our lab has recently shown that loss of HCN channels not only enhances individual neuronal excitability, but also network excitability (Albertson et al. 2011). Key to understanding how this effect on network excitability occurs is understanding the role of HCN channels in both inhibitory and excitatory network elements. This paper examines the effects of HCN channel loss on the integrative and intrinsic properties of fast spiking basket cells in layer 5 of the neocortex in control rats and rats with freeze lesions. Reduced I_h was observed in rats with freeze lesions. HCN channel inhibition causes a small but significant increase in summation. Rats with freeze lesions had a greater final EPSP area than control rats. We did not observe a pre-synaptic influence of HCN channels onto interneurons. Interestingly, we also observed a decrease in spiking following ZD 7288 and decreased baseline spiking in freeze lesioned rats.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* with protocols approved by the University of Alabama at Birmingham Institutional Care and Use Committee. We

made every effort to minimize pain and discomfort. Focal freeze lesions were induced in postnatal day 1 Sprague-Dawley rats as previously described (Albertson et al. 2011). In brief, newborn rat pups were anesthetized by hypothermia, and a small incision was made in the scalp. A 2mm copper rod cooled to approximately -50ºC was placed on the surface of the skull for 3 seconds. After the scalp was sutured, the animals were warmed and returned to their home cages. Rats were allowed to recover for 20 to 27 days before recordings were made.

Preparation of acute neocortical brain slices

Rats were anesthetized with isoflurane and decaptiated. The brain was quickly removed and placed in ice-cold oxygenated (95% O2/5% CO2, pH 7.4) cutting solution consisting of (in mM): 135 N-Methyl-D-glucamine, 1.5 KCl, 1.5 KH2PO4, 23 choline HCO3, 0.4 ascorbic acid, 0.5 CaCl2, 3.5 MgCl2 and 25 D-glucose (Tanaka et al. 2008). 300 μM thick coronal brain slices were cut with a vibratome (Microm, Waldorf, Germany). Slices were obtained from an area of somatosensory cortex containing the microgyrus in freeze lesioned animals, and a corresponding region in control animals. The slices were rested for 40-60 minutes at 37ºC in oxygenated recording solution containing (in mM) 124 NaCl, 2.5 KCl, 10 D-glucose, 26 NaHCO3, 2.0 $Ca2^{+}$, 2.0 Mg^{2} and then kept at room temperature. For recording, individual slices were transferred to a recording chamber and continuously perfused (4ml/min) with oxygenated recording solution kept at 32 ± 1ºC.

Whole Cell Recording

Neurons were visualized with Leica DM LFSA (Leica Microsystems Wetzlar GMBH,Wetzlar, Germany) microscope equipped with Nomarski optics, a 40X water

immersion lens, and infrared illumination. Layer five interneurons were identified by their round shape, size, absence of prominent apical dendrite, distance from the pial surface, intrinsic properties, and spiking properties. Additionally, some cells were labeled with intracellular biocytin to confirm their identity. Labeled cells were processed as previously described (Zhou and Hablitz 1996).

Whole cell recordings were obtained from visually and physiologically identified fast spiking basket cells in layer five of the neocortex. Signals were acquired with an Axopatch 200B amplifier (Scientifica, Uckfield, United Kingdom) controlled by Clampex 8.0 software via a Digidata 1322A interface (Molecular Devices). Responses were low pass filtered at 5 kHz, digitized at 5 kHz, and analyzed offline with Clampfit 8.0 software. Patch electrodes with an open tip resistance of 2-5 MΩ were pulled from borosilicate glass tubes. Tight seals (>2 GΩ) were obtained between the patch electrodes and the neurons before using suction to break into whole cell mode. Only recordings with a series resistance of less than 25 MΩ were used for the study and recordings in which a large increase in series resistance was observed were excluded. Patch electrodes were filled with an internal solution containing (in MM): 125 K-gluconate, 10 KCl, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP, 0.5 EGTA, and had an adjusted pH and osmolarity of 7.3 and 290, respectively. $GABA_A$ receptors were inhibited with bicucline methiodide (10μM; Sigma Aldrich, St. Louis, MO) in all experiments. Synaptic responses were evoked with a concentric bipolar stimulating electrode (FHC, Bowdoin, ME) positioned within several hundred micrometers later to the interneuron of interest. EPSPs were evoked with 100 μS pulses of 20-200 μA in amplitude. Traces are shown as the average of 5 consecutive responses. For measurement of miniature EPSCs, tetrodatoxin (0.5 μM;

Tocris Bioscience, Ellisville, MO) was washed in for the duration of the experiment following neuronal identification.

Data Analysis

Data is either shown as dots representing every individual data point or as means ± the standard error. Statistical analysis was carried out with either a one tailed Students *t*-test or with a two-way ANOVA. $P<0.05$ was considered significant.

Drugs

Drugs were stored in frozen stock solution and dissolved in the recording solution prior to each experiment. Bicuculine methodide was present at all times in all conditions. After recording control responses, ZD-7288 (20μM) (Tocris Bioscience, Ellisville, MO) was washed in to block HCN channels for ten minutes. For experiments with tetrodatoxin, cells were identified by their spike patter, then tetrodatoxin was washed in for three minutes before continuing the experiment.

RESULTS

I_h is reduced in rats with cortical dysplasia

Interneurons help regulate network activity (Cobb et al. 1995; Pouille and Scanziani 2001), therefore changes in their intrinsic function or integration of inputs could drastically influence network behavior. Differential influence of HCN channel loss on inhibitory vs. excitatory cell types might contribute to epileptic pathology. HCN channel immunoreactivity is not overtly apparent in the interneurons within the neocortex, however it is present in hippocampal interneurons (Lorincz et al. 2002). We have recently shown that HCN channels are reduced in animals with freeze lesions(Albertson et al. 2011). Reduced interneuron I_h in freeze lesioned rats may suggest

a common mechanism for I_h loss. We therefore tested the hypothesis that I_h is reduced in fast spiking interneurons in rats with freeze lesions.

We obtained somatic whole cell patch clamp recordings from visually and physiologically identified interneurons in layer five of the neocortex in control rats and rats with freeze lesions. In freeze lesioned rats, interneurons were recorded from the hyperexcitable zone immediately adjacent to the freeze lesion (Jacobs et al. 1999). Only interneurons with physiological properties consistent with fast spiking basket cells were used. These included rapid non-accomodating spike trains upon current injection (Figure 1A), input resistances around 200 MΩ, and narrow spike widths (Kawaguchi and Kubota 1993; Kawaguchi and Kondo 2002). Pyramidal neurons near freeze lesions have hyperpolarized resting membrane potentials and increased input resistance, likely due to loss of HCN channels (Albertson et al. 2011). Fast spiking interneurons near the freeze lesion had slightly, but significantly hyperpolarized membrane potentials (Control: -65.97mV ± 1.45, n=33; Lesion: -72.81 mV ± 1.72, n=10; $p<0.05$, *t*-test) (Figure 1B) relative to control neurons, but did not differ significantly in somatic input resistance (Control: 213.6 MΩ ± 14.17, n=18; Lesion: 226.5 MΩ ± 12.78, n=10; $p<0.05$, *t*-test) (Figure 1B). Negative current injections in pyramidal neurons results in a hyperpolarization of the membrane potential followed by a depolarizing "sag" of the membrane potential. This is caused by activation of the inward I_h current (Berger et al. 2001). At the offset of the negative current pulses a a rebound "hump" in the opposite direction as HCN channels close is observed (Berger et al. 2001; Albertson et al. 2011). We observed small sag and rebound responses in layer five basket cells from control animals (Figure 1C black trace) upon hyperpolarizing (-50pA through -250 pA) current

injections. Sag responses (Control -250 pA: 1.59 mV ± 0.19, n=18; Lesion -250 pA: 0.87 mV ± 0.21, n=9; $p<0.05$ 2 way ANOVA) were significantly reduced in interneurons from freeze lesioned rats and rebound (Control -250 pA: 1.13 mV ± 0.1, n=16; Lesion -250 pA: 0.23 mV ± 0.07, n=9; $p<0.05$ 2 way ANOVA) responses were virtually absent (Figure 1C&D).

HCN Channel Inhibition does not Influence EPSP/EPSC amplitude onto L5 Interneurons

HCN channel inhibition has been reported to increase EPSP amplitude in pyramidal neurons (Magee 1998), however, I_h primarily influences the integrative properties of multiple synaptic inputs (Magee 1999). Recent evidence has suggested that in some excitatory cell types, presynaptic HCN channels influence both evoked and spontaneous release (Huang et al. 2011). We therefore tested whether HCN channel inhibition influenced excitatory post-synaptic potential (EPSP) amplitude. Responses were evoked with a single extracellular shock near the cell from which we were recording before (Figure 2A Black Trace) and after ZD 7288 (Figure 2B Red Trace). We did not observe a significant influence of HCN channel inhibition on EPSP amplitude (Control:4.68 mV ± 0.48 n=5; ZD 7288: 3.80 mV ± 1.14, n=5; *t*-test, $p<0.05$) (Figure 2C Left). We also tested whether HCN channels influenced glutamatergic currents by voltage clamping the same cells at -60 and evoking responses as before. As with post-synaptic potentials, we did not see a significant influence of HCN channel inhibition on post-synaptic currents (Control: 145.4 pA ± 26.19 n=5; ZD-7288: 130.5 pA ± 35.83, n=5; $p>0.05$, *t*-test) (Figure 2C Right).

HCN Channel Loss and Synaptic Integration in Fast Spiking Interneurons

HCN channel expression along the long apical dendrites of pyramidal neurons and perkinje cells filters dendritic EPSPs and limits synaptic integration (Magee 1999; Angelo et al. 2007). Loss of HCN channels in models of epilepsy significantly increases EPSP summation (Shin et al. 2008) allowing synaptic inputs to more effectively drive excitatory neurons toward action potential threshold. We also recently observed this effect of HCN channel loss on pyramidal neurons from animals with freeze lesions (Albertson et al. 2011). Increased effectiveness of synaptic input may contribute to the hyperexcitability observed in epilepsy. However, if loss of HCN channels in inhibitory interneurons similarly increased effectiveness of EPSP summation, it could match and offset the increased effectiveness of summation in excitatory pyramidal neurons.

To examine this, we evoked five EPSPs in layer five fast spiking basket cells at a frequency of 25 hertz (Hz). Stimulation in control animals resulted in minimal, sublinear EPSP summation (Figure 3A Black Trace) in control animals. HCN channel inhibition caused a slight but significant increase in summation (Control: -0.13 ± 0.22, n=11; ZD 7288: 0.37 ± 0.07, n=11, p-value < 0.05, *t*-test) (Figure 3A Red trace left). The results are shown in Figure 3C. Summation was measured as the amplitude of the first EPSP subtracted from the peak amplitude reached by the 5^{th} EPSP divided by the amplitude of the first EPSP [$(EPSP_5\text{-}EPSP_1)/EPSP_1$]. We next examined summation in fast spiking interneurons from lesioned animals. As before, a train of five pulses delivered at 25 Hz resulted in minimal, sublinear EPSP summation (Figure 3B Black Trace). We did not observed a significant difference in summation between control and lesion animals (Control: -0.13 ± 0.22, Lesion: -0.07 ± 0.15, n=6, p-value > 0.05, *t*-test) (Figure 3C). In contrast to control neurons, HCN channel inhibition with ZD-7288 did not significantly

increase summation (Lesion: -0.07 ± 0.15, n=6; ZD 7288: -0.71 ± 0.66. n=6; p-value > 0.05, *t*-test) (Figure 3B Red Trace). These data are shown in Figure 3C. This is consistent with our observation of reduced interneuronal I_h in animals with freeze lesions. We also quantified the area for the final EPSP. ZD-7288 significantly increased the area of the final EPSP in control animals (Control: 4621 mV/ms ± 467.7, n=11; ZD 7288: 7930 mV/ms± 579, n=11; p-value < 0.05, *t-test*) (Figure 3D) but did not significantly influence the final EPSP area in rats with freeze lesions (Lesion: 6432 mV/ms ± 732.2, n=6; ZD 7288: 7085 mV/ms ± 1306, n=6, p-value > 0.05, *t*-test) (Figure 3D). The final EPSP area was also significantly increased interneurons from rats with freeze lesions when compared to control animas (Control: 4621 mV/ms ± 467.7, n=11; Lesion: 6432 ± 732.2 n=6, p-value < 0.05, *t*-test) (Figure 3D).

Train responses were next evoked in interneurons voltage clamped at -60. This holding potential limits HCN channel opening (Robinson and Siegelbaum 2003) and therefore should also limit voltage and HCN channel dependent changes in other currents during the experiment. Any effect on integration observed under these conditions is likely mediated by the surrounding network and not changes intrinsic to the cell from which we are recording. As in current clamp, evoked EPSCs exhibited sublinear summation in both control and lesion animals (Figure 4A&B Black traces). HCN channel inhibition did not significantly influence EPSC summation in either control (Control: -0.16 ± 0.26, n=8; ZD 7288: 0.20 ± 0.11, n=8, p-value>0.05, *t*-test) interneurons or interneurons from animals with freeze lesions (Lesion: -0.22 ± 0.13, n=6; ZD 7288: -0.54 ± 0.39, n=6; p-value> 0.05, *t*-test (Figure 4A&B Red traces). These data are shown in Figure 4C. These results suggest that the influence of HCN channels

inhibition on summation is due to a postsynaptic effect. We did not observe a significant increase in the area of the final EPSC area in both control and freeze lesioned animals. Summation in voltage clamp resulted in a slight increase in the holding current from before the first EPSC to before the final EPSC. Interestingly, HCN channel inhibition significantly increased this change in holding current in control animals (Control: 5.49 pA ± 1.26, n=8; ZD 7288: 11.90 pA ± 2.26 n=8, p-value > 0.05, *t*-test) (Figure 3A red trace). We did not observe a similar effect in interneurons from freeze lesioned rats (Lesion: 5.49 pA ± 1.26, n=8; ZD 7288: 7.55 pA ± 2.90, n=5, p > 0.05, *t*-test) (Figure 3B red trace). These data are shown in Figure 3F. The increase in the holding current during EPSC summation prior to drug wash-in was not significantly different between control and lesion animals (Control: 5.49 pA ± 1.26, n=8; Lesion: 7.55 pA ± 2.90, n=5; p-value > 0.05, *t*-test). Since this was observed in neurons voltage clamped at -60 mV it is likely that this effect is due to a change in the excitability of the surrounding network.

HCN channels do not Influence the Presynaptic Release Properties of Excitatory Synapses onto Layer Five Fast Spiking Interneurons

Recent evidence has suggested that at excitatory neuron terminals within entorhinal cortex, presynaptic HCN channels serve to inhibit release by increasing resting state Ca^{2+} channel inactivation (Huang et al. 2011). HCN channel inhibition also changes miniature inhibitory post-synaptic currents in hippocampus (Aponte et al. 2006). Given this, and given the increased change in holding current observed during summation of EPSCs in voltage clamped interneurons, we examined a potential role of pre-synaptic HCN channels onto layer five interneurons. We did not see a significant influence of HCN channel inhibition on mEPSC interevent interval: (Control: 0.24 sec ± 0.072, n=7;

ZD 7288: 0.25± 0.07, n=7, p-value > 0.05, *t*-test) (Figure 5A&C) or mPSC amplitude (Control: 30.33 ± 1.78 pA, n=7, ZD 7288: 28.15 pA ± 2.36, n=7, p-value > 0.05, *t*-test) (Figure 5B&C). These data are shown in Figure D. We also examined release probability by quantifying the paired pulse ratio of the first and second EPSCs in the 25Hz trains discussed already. We did not observe a significant effect of HCN channel inhibition on paired pulse ratio at 25Hz (Control: 1.2 ± 0.21, n=8; Lesion: 1.19 ± 0.28, n=8, p >0.05, *t*-test)(Figure 5F). Together these data suggest that there is no significant effect of presynaptic HCN channels at excitatory synapses onto fast spiking layer five basket cells. These data are in agreement with previous work suggesting that presynaptic HCN channels in cortical pyramidal neurons is synapse specific (Huang et al. 2011).

Loss of HCN Channels Reduces Interneuronal Spiking

HCN channel inhibition significantly increases spike frequency following current injection in both dendritic and somatic membranes (Magee 1998). Membrane excitability is similarly increased in animal models of epilepsy with reduced HCN channels (Albertson et al. 2011; Jung et al. 2007). Increased membrane excitability paired with increased summation of excitatory inputs onto excitatory neurons may significantly contribute to hyperexcitability observed in epilepsy. Interestingly, loss of HCN channels decreases spike frequency following current injection in hypothalamic neurons (Aponte et al. 2006), increases action potential initiation theshold hippocampal basket cells (Aponte et al. 2006), and decreases spontaneous firing frequency in stratum oriens interneurons (Maccaferri and McBain 1996). An increase in membrane excitability in excitatory neurons paired with a decrease in membrane excitability following universal

HCN channel loss in cases of epilepsy could contribute to an imbalance of excitation and inhibition.

We therefore tested the influence of HCN channels inhibition on spiking by injecting steadily increasing current amplitudes into layer five fast spiking basked cells and counting the number of spikes in 300 ms. We found that HCN channels inhibition significantly decreased spike number (Control 500 pA: 70.8 spikes ± 10.57, n=5; ZD 7288 500 pA: 57 spikes ± 4.51 n=5, p-value < 0.05, two-way ANOVA) (Figure 6A & B). We also found that interneurons in the hyperexcitable zone in rats with freeze lesions had a significantly reduced spike frequency (Control 500 pA: 70.8 spikes ± 10.57, n=5; Lesion 500 pA: 56 spikes ± 3.92, n=8; $p < 0.05$, two way ANOVA) (Figure 6B) very similar to that observed in control rats in the presence of ZD 7288. HCN channel inhibition in rats with freeze lesions further decreased spike frequency (Lesion 500 pA: 56 spikes ± 3.92, n=8; ZD 7288 42.2 spikes ± 6.102, n=5, $p < 0.05$, two way ANOVA) (Figure 6B). These data are in contrast to our observation in pyramidal neurons in rats with freeze lesions in which HCN channel loss increases spike frequency (Albertson et al. 2011).

DISCUSSION

In this study we examined whether loss of cortical HCN channels in animals with freeze lesions occurred in interneurons in addition to pyramidal neurons. We found that fast spiking interneurons in the hyperexcitable region adjacent to the freeze lesion had reduced sag and rebound potentials, indicative of reduced HCN channels and I_h. We determined that loss of these channels did not influence baseline synaptic transmission. Evoked EPSP and EPSC amplitude was unaffected by HCN channel inhibition. We

found a small but significant effect of HCN channel inhibition on integration of EPSPs onto fast spiking interneurons. ZD 7288 also increased the area of the final EPSP. ZD-7288 had no significant effect on integration in animals with freeze lesions, consistent with the observation of significantly reduced I_h. Furthermore, animals with freeze lesions had significantly greater final EPSP areas than control animals, mimicking the influence of HCN channel inhibition. When neurons were voltage clamped at -60 mV, we did not observe an effect of ZD 7288 on summation or final EPSC area in either control or freeze lesioned rats. Interestingly, ZD 7288 increased the amount of underlying current between the baseline holding current and the final EPSP in control animals, but not in lesioned animals. We did not observe an effect of HCN channel inhibition on mini EPSC frequency, amplitude, or paired pulse ratio. Finally, we found that HCN channel inhibition decreased spiking in response to current injection. Freeze lesioned animals had a similar reduction in spiking prior to HCN channel inhibition.

Rats with freeze lesions are a well established model of malformation epilepsy (Hablitz and Defazio 1998; Jacobs et al. 1996). While, they do not exhibit behavioral seizures per se, they exhibit reduced seizure threshold (Scantlebury et al. 2004) and have a region of extreme cortical hyperexcitability (Hablitz and Defazio 1998). Understanding the mechanism of malformation associated hyperexcitability is crucial to advancing anticonvulsant therapy. Our lab has recently demonstrated that I_h is reduced in layer five pyramidal neurons near the malformation in freeze lesioned rats (Albertson et al. 2011). In this paper we have demonstrated that HCN channels are similarly reduced in nearby fast spiking interneurons. To our knowledge, this is the first evidence of interneuronal I_h

being down regulated in a model of epilepsy. This suggests that loss of HCN channels may be similar between cell types in epilepsy.

We have also recently shown that the increase in neuronal excitability observed in excitatory cell types following HCN channel loss translates to an increase in network excitability (Albertson et al. 2011). A key to understanding how loss of HCN channels increases network excitability is understanding the role of I_h in individual network elements including specific subclasses of interneurons. Imbalance of excitation and inhibition contributes to aberrant network activity (Yizhar et al. 2011) as well as epileptic hyperexcitability (Gutnick et al. 1982) and dysfunction of interneurons has been implicated as a potential contributor to epileptic hyperexcitability (Gill et al. 2010). Inhibitory post-synaptic currents are altered (Zhou et al. 2009) in the irradiation model of cortical dysplasia and inhibitory terminal number is altered (Zhou and Roper 2010). There is also evidence of dysfunctional inhibition in humans with cortical dysplasia (Andre et al. 2010). The work detailed here takes the first step toward better understanding the functional consequence of HCN channel loss in interneurons by examining both neuronal spiking and synaptic integration following pharmacological inhibition of HCN channels in control rats and rats with cortical dysplasia.

While neocortical interneurons do not typically stain for HCN channels, hippocampal interneurons are immunoreactive for HCN channels (Lorincz et al. 2002). Furthermore, varying levels of interneuronal I_h have been identified in some populations of interneurons (Wu and Hablitz 2005; Aponte et al. 2006). To our knowledge we are the first lab to characterize I_h within fast spiking interneurons within layer five of the cortex. Interneuron HCN channels may serve a completely different function in interneurons as

they lack the long apical dendrites of pyramidal neurons (Markram et al. 2004). However, HCN channels in purkinje neurons which express HCN channels in a non-polarized fashion along their unique dendrites serve a similar function as in pyramidal neurons in that they constrain integration of synaptic inputs (Angelo et al. 2007). We observed a similar influence of I_h on synaptic integration within layer five interneurons. HCN channels may have a common role in dendritic filtering among cell types. Interestingly, the effect of HCN channel loss on synaptic inhibition was not nearly as large as that observed previously in pyramidal neurons (Shin et al. 2008; Albertson et al. 2011; Huang et al. 2009) and freeze lesioned rats with decreased I_h did not exhibit significantly increased baseline summation. These data suggest that loss of HCN channels may differentially influence excitatory and inhibitory network elements. Universal loss of HCN channels may therefore universally increase EPSP summation, but to a much greater degree in excitatory principle neurons. This may contribute to an imbalance of excitation and inhibition, and may be a potential mechanism by which HCN channel loss increases network excitability.

Interestingly, we also observed a change in the integration of EPSCs in neurons that were voltage clamped at -60 mV following HCN channel loss. EPSC integration is associated with an increase in the holding current between the baseline and the final EPSC. This increase was amplified in rats following HCN channel inhibition. We did not observe a similar effect in rats with freeze lesions. Because in these experiments interneurons were clamped at a potential which likely precluded channel opening (Berger et al. 2001) it seems likely that this effect is due to increased network excitability. One potential mechanism is increased presynaptic release of glutamate following HCN

channel inhibition. HCN channel inhibition increases release probabiltiy at pyramidal neurons synapses in entorhinal cortex (Huang et al. 2011). However, when we examined release probabilty at excitatory synapses onto interneurons, we did not see an increase in either mini- EPSC frequency or paired pulse ratio. These data suggest that loss of HCN channels does not change release at this synapse. This is in agreement with previous work suggesting that pre-synaptic HCN channels are expressed in a synapse specific manner (Huang et al. 2011). The influence of HCN channel inhibition on EPSC integration likely occurs via an increase in the excitability of surrounding, unclamped neurons. HCN channel inhibition may increase the excitability of surrounding neurons so much that 25 Hz stimulation causes action potentials. Loss of HCN channels increasing summation to the point of action potential firing at frequencies not typically associated with sufficient summation has been observed in the HCN1 knockout mice (Huang et al. 2009). These action potentials may mediate additional glutamate release onto the neuron from which we were recording.

HCN channels within interneurons have been strongly implicated in the control of GABA release. As mentioned. within the hippocampus, HCN channel inhibition decreases the frequency of mEPSCs (Aponte et al. 2006). Additionally, immunogold labeling reveals HCN channels in cerebellar basket cell terminals (Lujan et al. 2005), HCN channel inhibition decreases the rate of spontaneous inhibitory currents in amygdala (Park et al. 2011) and hippocampus (Peng et al. 2010). Whether, the I_h we observed within fast spiking basket cells of layer five interneurons alters GABA release will be a topic for future studies.

Typically, loss of HCN channels is associated with increased spiking in response to depolarizing current pulses (Huang et al. 2009). Interestingly, in our recordings, we found the opposite to be true in fast spiking cortical interneurons. Depolarizing current pulses significantly reduced the number of spikes during depolarizing current pulses. Furthermore, animals with freeze lesions in which we have observed reduced interneuronal I_h have reduced baseline spiking following current injection. Interestingly, their spike rate is further reduced following I_h inhibition. This is the only instance in any of our experiments in which HCN channel inhibition had any effect on interneurons from freeze lesioned animals. It likely represents residual I_h as interneurons near the lesion, while expressing significantly reduced I_h were not devoid of it altogether. HCN channel inhibition has previously demonstrated to reduce spike frequency in other non-pyramidal cell types including hypothalamic GnRH neurons (Chu et al. 2010). Our results are therefore in concert with these data and may suggest a common mechanism. Loss of HCN channels in excitatory pyramidal neurons leading to increased membrane excitability while concurrent loss in interneurons leading to decreased excitability may be a potential contributor to the network hyperexcitability observed in rats with cortical malformations.

In summary we have observed that HCN channel loss in rats with cortical malformations is not restricted to L5 pyramidal neurons, but also occurs in adjacent interneurons. The functional consequences of that loss may differ between excitatory and inhibitory cell types. We observed an increase in summation of EPSPs in interneurons following HCN channel inhibition; however it was not as robust as that which has been observed in pyramidal cell types (Huang et al. 2009; Shin et al. 2008; Albertson et al.

2011). Furthermore, interneurons from rats with malformations exhibiting reduced I_h did not have increased baseline EPSP summation. These data suggest that while I_h may serve a similar dendritic filtering role in interneurons, the channel's influence over pyramidal neurons is much greater. We did not observe an effect of HCN channels on single EPSPs or EPSCs. We also did not observe any evidence of pre-synaptic HCN channels at excitatory synapses onto fast spiking layer five interneurons. Finally, we observed a decrease in membrane excitability of interneurons from both freeze lesioned animals and following HCN channel inhibition. This is in contrast to the increase observed in excitatory pyramidal neurons.

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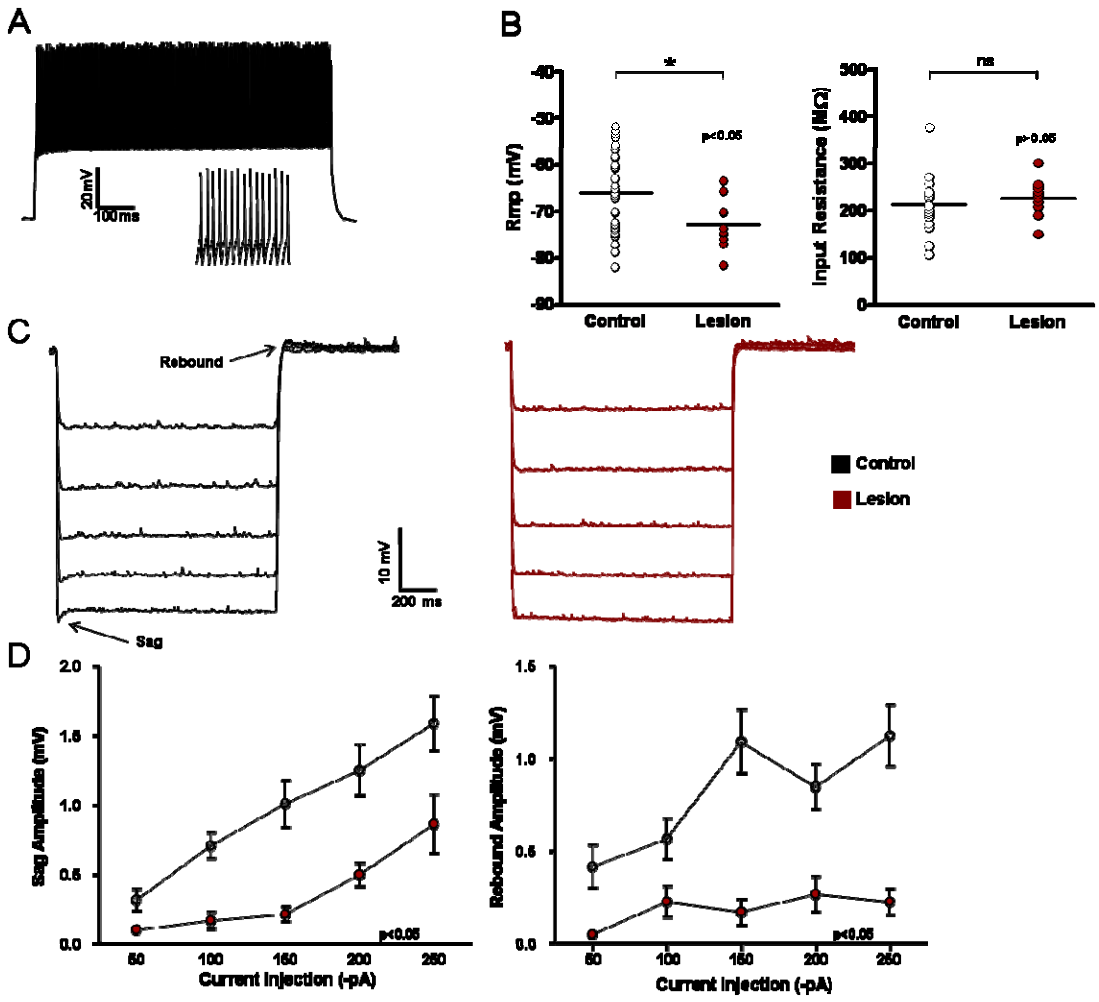

Figure 1. I_h Associated "Sag" and "Rebound" Voltages are Reduced in Interneurons from Animals with Transcortical Freeze Lesions. A. Typical spiking response from a fast spiking layer five interneuron. B. Fast spiking interneurons from freeze lesioned animals and slightly hyperpolarized membrane potentials, but did not have significantly different input resistances. C. Typical "sag" (arrow) and "rebound" (arrow) responses in response to hyperpolarizing current pulses from control animals (black trace) and animals with freeze lesions (red trace). D. Quantification of voltage "sag" (left) and voltage "rebound" (right) in interneurons from control and freeze lesioned rats.

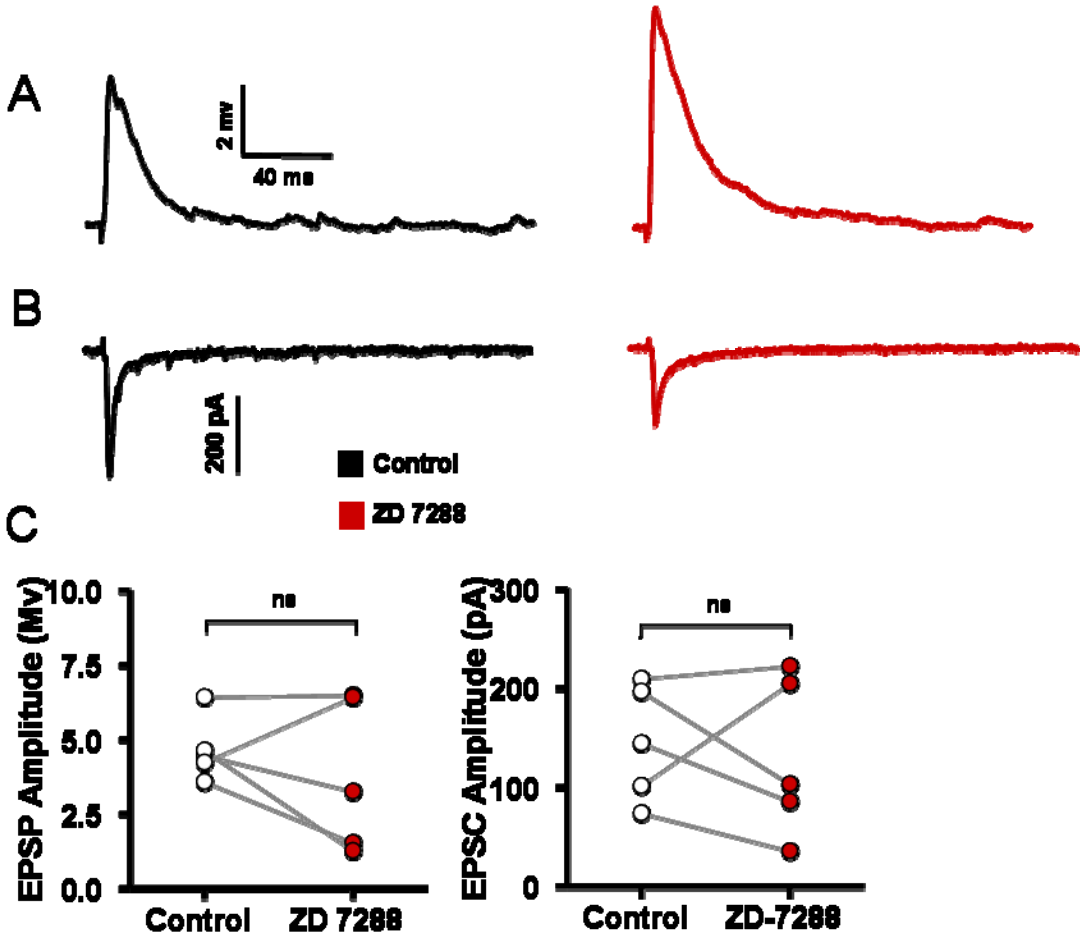

Figure 2. HCN Channel Inhibition Does not Significantly Influence Single EPSP in Layer V Fast Spiking Interneurons. A. Typical interneuronal EPSP from a control (black) and freeze lesioned rat (red). B. Typical interneuronal EPSC from a control (black) and freeze lesioned (red) rat. C. Quantification of EPSP & EPSP amplitudes before (clear dots) and after (red dots) ZD 7288.

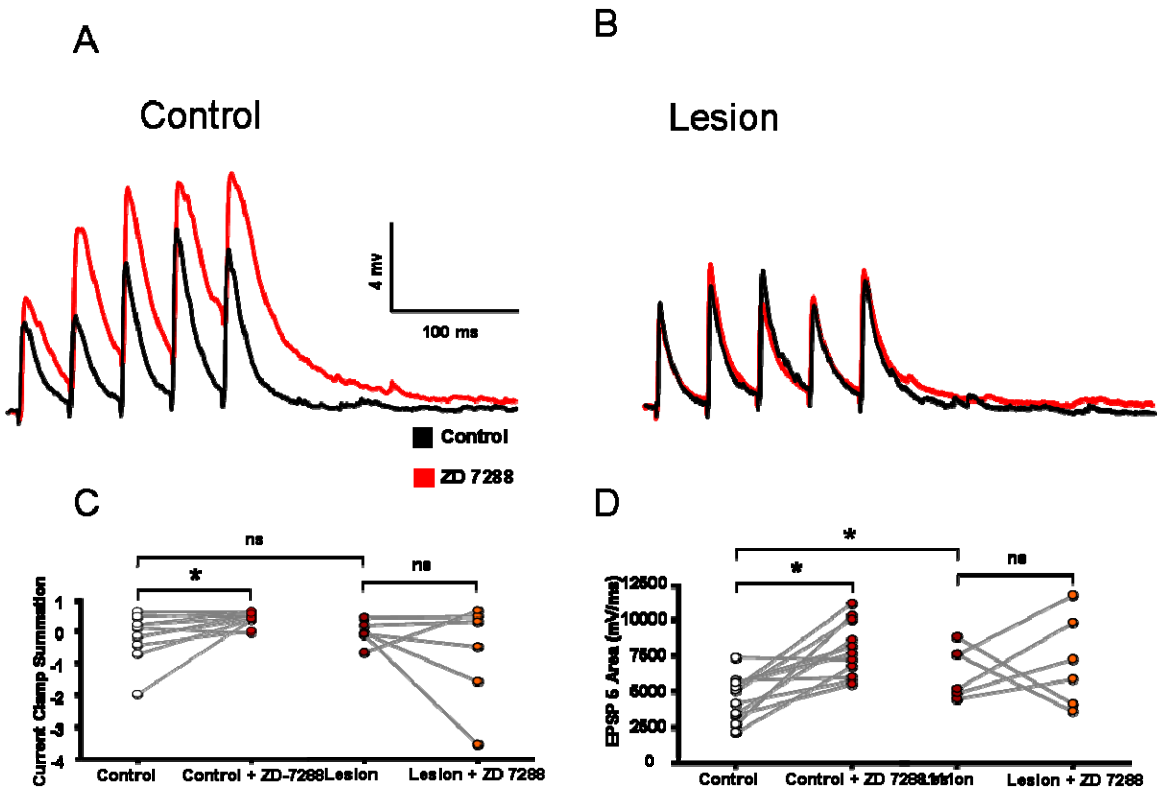

Figure 3. HCN Channel Inhibition Significantly Increases EPSP Summation in Layer V Fast Spiking Interneurons. A. Evoked EPSPs in interneurons at 25 Hz before (black) and after (red) ZD 7288 in control animals. B. Evoked EPSPs in interneurons at 25 Hz before (black) and after (red) ZD 7288 in lesioned animals. C. HCN channel inhibition significantly increases interneuronal summation in control animals, but not freeze lesioned animals. There is not difference in pre-drug summation between control and freeze lesioned rats. D. HCN channel inhibition significantly increases the area of the final EPSP in control but not freeze lesioned rats. Freeze lesioned rats have a significantly greater final EPSP area compared to control rats.

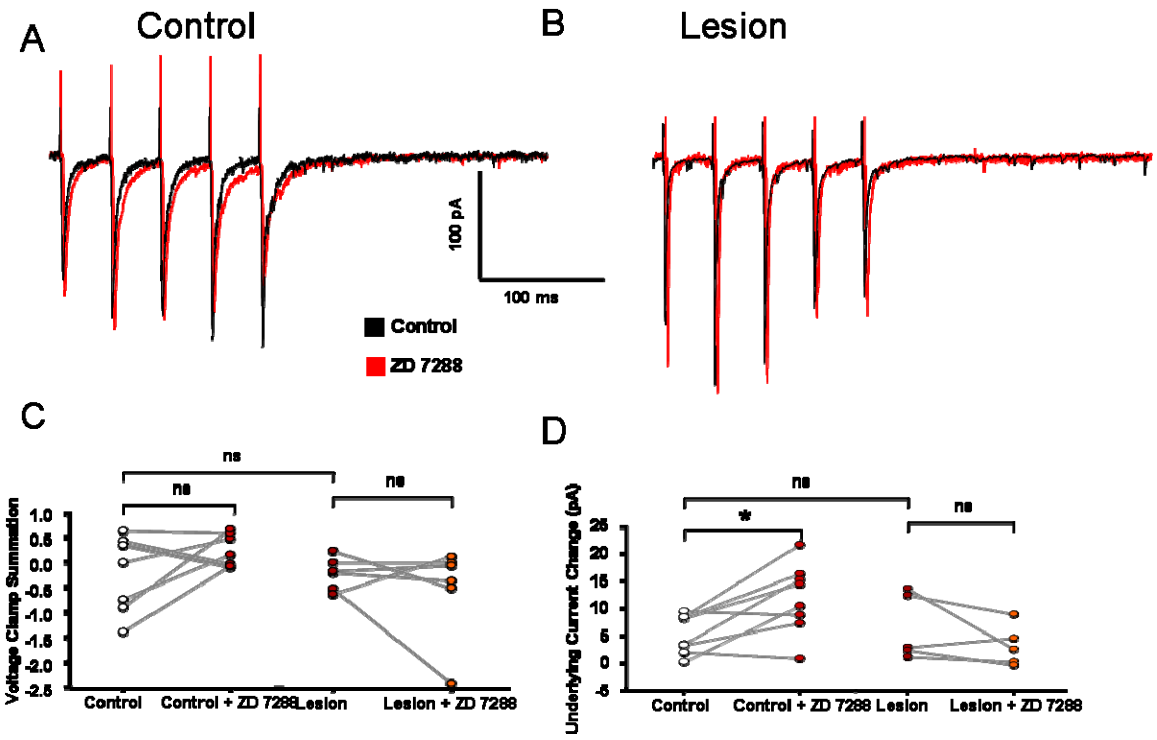

Figure 4. HCN Channel Inhibition does not Increases EPSC Summation in Layer V Fast Spiking Interneurons. A. Evoked EPSCs in interneurons at 25 Hz before (black) and after (red) ZD 7288 in control animals. B. Evoked EPSCs in interneurons at 25 Hz before (black) and after (red) ZD 7288 in lesioned animals. C. HCN channel inhibition did not increases interneuronal summation in control animals or freeze lesioned animals. There is no difference in pre-drug summation between control and freeze lesioned rats. D. HCN channel inhibition significantly increases the increase in underlying current during summation in control but not freeze lesioned rats. Freeze lesioned rats did not have a significantly greater increase in underlying current compared to control rats.

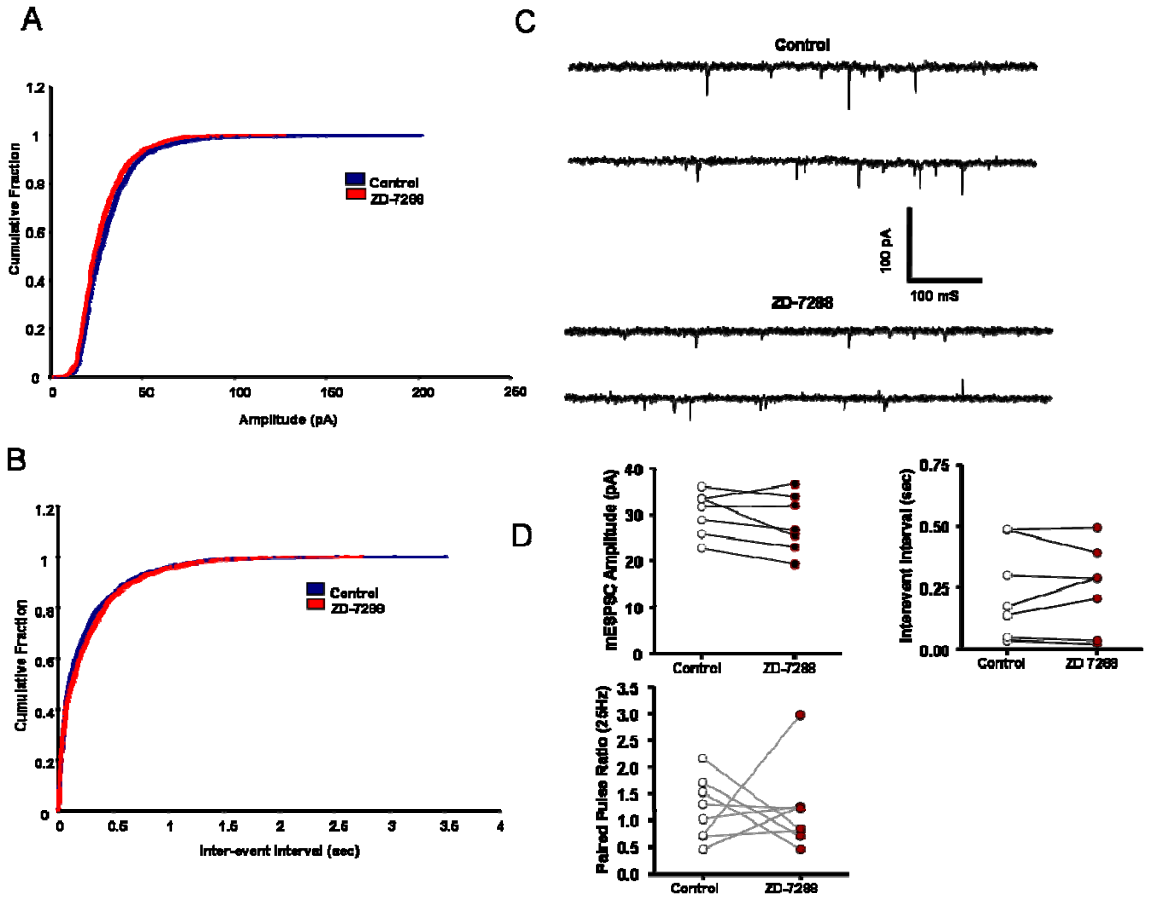

Figure 5. HCN Channels do not Signficantly Influence Release at Excitatory Synapses on to Layer Five Interneurons. A. Cumulative distribution plot for mEPSC frequency before (Blue) and after (Red) ZD 7288. B. Cumulative distribution plot of mEPSC amplitude before (Blue) and after (Red) ZD 7288. C. Example of typical mEPSC activity in before and after ZD 7288. D. HCN channel inhibition did not increase mEPSC frequency, amplitude, or paird pulse ratio.

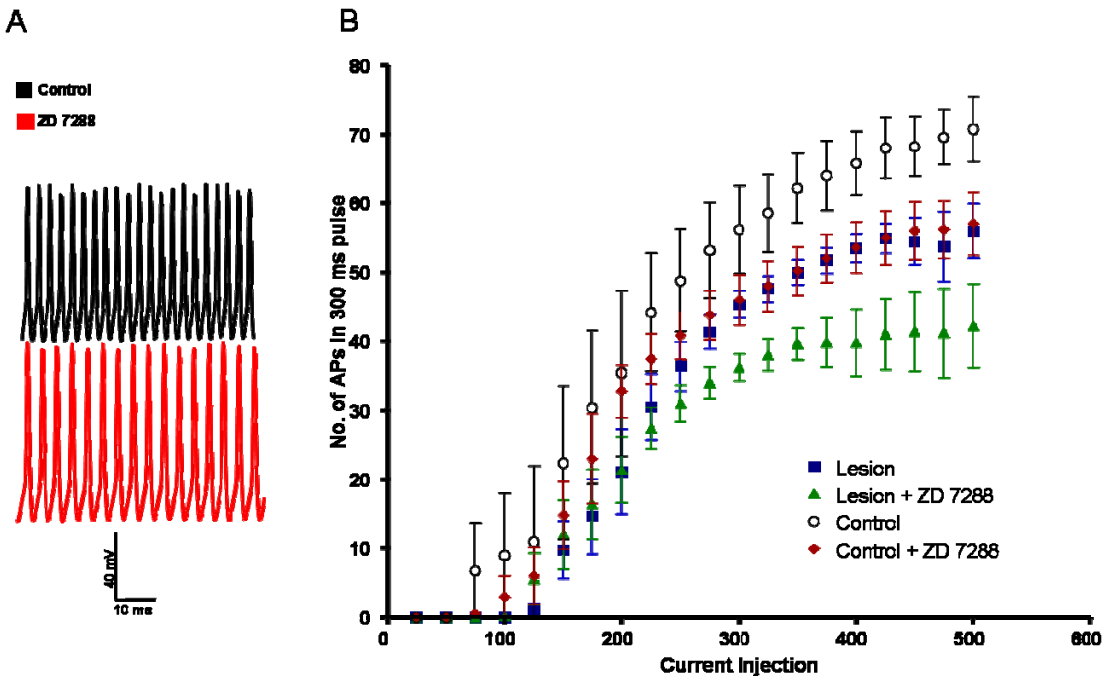

Figure 6. HCN Channel Inhibition Significantly Decreases the Firing Frequency of Fast Spiking Layer V Interneurons. A. Example spike patter in response to current injection before (black) and after (red) ZD 7288. B. HCN channel inhibition significantly decreased spike frequency in control animals. Lesion animals had significantly lower pre-drug spike frequency. ZD 7288 further decreased spike frequency in interneurons from animals with freeze lesions.

CONCLUSIONS AND SUMMARY

The broad goal of this dissertation was to better understand how HCN channel expression or lack thereof influenced neocortical network behavior. This goal was motivated primarily by two factors. The first was that in the healthy neocortex, HCN channels active at resting membrane potentials have a profound impact on the excitability and behavior of individual pyramidal neurons (Williams and Stuart 2000). It seems likely that this impact on individual neuronal behavior translates to an impact on network behavior. Second, HCN channels are widely down-regulated in models of epilepsy (Jung et al. 2007; Shin et al. 2008). Loss of HCN channels is associated with increased intrinsic excitability and summation of excitatory synaptic inputs (Huang et al. 2009). It is therefore our hypothesis that increased neuronal excitability that follows HCN channel loss in epilepsy contributes to some of the network hyperexcitability seen in epilepsy. We focused our efforts on a potential contribution of HCN channel loss in rats with freeze lesions, a model of cortical malformation epilepsy.

These goals were addressed using several techniques. The first was whole cell patch clamp recording before and after pharmacological inhibition of HCN channels examining synaptic and intrinsic properties of pyramidal neurons and interneurons. Understanding the specific influence of HCN channels on both excitatory and inhibitory elements of the neocortical network is crucial to more broadly understanding the channel's role in network behavior. Whole cell recordings were performed in both healthy rats and rats with focal cortical dysplasia. The second technique was examination

of network activity across large sections of neocortex from healthy rats and freeze lesioned rats using voltage sensitive dyes. This technique allowed us to see the broad, network level effects of HCN channel loss. Finally, we studied large epileptiform network events in interneurons and pyramidal neurons using whole cell patch clamp recordings. This technique allowed us to evaluate both a potential influence of HCN channels on epileptic activity itself as well as its influence on poly-synaptic network events in individual cell types.

Broadly, our findings are as follows: Using voltage sensitive dye imaging, we found that HCN channels in healthy rat neocortex limit the duration of non-epileptic activity. Pharmacological inhibition of HCN channels resulted in voltage sensitive dye responses with significantly increased half-widths. Enhancing I_h channels with the anticonvulsant lamotrgine significantly dampened network events. We found that rats with cortical malformations had significantly reduced I_h as well as increased intrinsic excitability and EPSP summation. This finding was correlated with a large increase in baseline network activity seen with voltage sensitive dye imaging. Additionally, HCN channel enhancement was significantly less effective at limiting network events in freeze lesioned rats. Together these data suggest that HCN channels serve in normal cortex to limit network activity and loss of the channel, as is observed in rats with cortical dysplasia, may contribute to epileptiform hyperexcitability

We next found that HCN channels greatly limit the severity of epileptiform network events. The area of epileptiform events, initiated with strong intracortical stimulation in disinhibited cortex, was significantly increased following HCN channel inhibition. We observed this increase in excitatory pyramidal neurons and inhibitory

interneurons. Interestingly, while the effect of HCN channel loss on response area was ubiquitous among cell types, its effect on spiking was not. HCN channel inhibition significantly increased spiking in layer V pyramidal neurons, but not layer 2/3 pyramidal neurons or interneurons. These data suggest that loss of HCN channels may selectively drive increased output from layer V pyramidal neurons during epileptiform events.

Finally we found that I_h loss observed in layer V pyramidal neurons in the freeze lesioned rats is mirrored by a concomitant decrease in I_h within nearby layer five fast spiking interneurons. These data suggest that HCN channel loss may be universal in models of epilepsy rather than cell-type specific. Interestingly, as in pyramidal neurons, HCN channel loss is associated with increased summation; however, this increase is not nearly as robust as that observed in pyramidal neurons (Shin et al. 2008; Huang et al. 2009). Also, in contrast to pyramidal neurons in which HCN channel loss increases membrane excitability (Magee 1998), HCN channel loss in layer five fast spiking basket cell was associated with decreased spiking following positive current injection. Freeze lesioned rats already expressing reduced I_h in interneurons also had reduced spiking. The lesser effect of HCN channel loss on summation in interneurons paired with an apparent decrease in membrane excitability further suggests that HCN channel loss selectively increases the output of the excitatory part of the network while have a minimal effect on the inhibitory part. HCN channel loss in numerous models of epilepsy may contribute to an epileptic imbalance of excitation and inhibition. Furthermore, these data suggest that in the healthy neocortical network, HCN channel's expression may help regulate the balance of excitation and inhibition.

The observation that I_h is reduced in rats with freeze-lesions is consistent with work showing that HCN channels are widely down-regulated in models of epilepsy. Progressive, but persistent downregulation of dendritic HCN channels is observed in the rat pilocarpine model of epilepsy (Jung et al. 2007). This is associated with increased input resistance and dendritic excitability, similar to our observation in pyramidal neurons in rats with freeze lesions. Reduced I_h and increased EPSP summation has also been observed in the kainic acid model of epilepsy (Shin et al. 2008). Perinatal seizures induced by hypoxia are also accompanied by downregulation of I_h (Zhang et al. 2006). All three of these models are dependent on an initial seizure event. The freeze lesion model however does not exhibit behavioral seizures. Therefore, the present study indicates that reductions in I_h associated with increases in neuronal excitability and EPSP summation may be a pervasive finding in many types of epilepsy. It is interesting to consider potential roles for HCN channel loss in initial epileptogenesis vs. post-epileptic hyperexcitability. Our finding of reduced I_h in the freeze lesion model raises further questions about the mechanism of HCN channel loss. What is intrinsic to developmental cortical malformations that causes downregulation I_h? Is this a distinct mechanism from that which causes reduced I_h in post epileptic models like the kainate model? A single seizure episode can decrease I_h (Derchansky et al. 2004); however, long-term downregulation of I_h occurs independent of repeated seizure activity (Jung et al. 2007). The freeze lesion model does, however, exhibit increased synaptic activity (Jacobs and Prince 2005) and this may be a potential driver for I_h loss. High frequency stimulation is known to downregulate I_h in pyramidal neurons (Campanac et al. 2008) and activation of AMPA and NMDA receptors alters trafficking of HCN1 channels (Noam et al. 2010). It

is unknown if observed decreases in I_h in the freeze lesion model is activity dependent or secondary to the initial cortical injury.

Intracortical stimulation in slices stained with a voltage sensitive dye elicits synchronous, horizontally restricted, areas of activity in the neocortex (Yuste et al. 1997; Bandyopadhyay and Hablitz 2006). HCN channel inhibition significantly increased the half-width of voltage sensitive dye recordings in rat neocortex. A similar increase in half-width has previously been described for distally evoked EPSPs in single cortical neurons following HCN channel inhibition (Williams and Stuart 2000). It is tempting to hypothesize that the effect of ZD 7288 on network activity observed with voltage sensitive dye imaging is simply due to a net increase in the time constants of EPSPs. We observed that the anticonvulsant lamotrigine significantly reduced voltage sensitive dye signals. Lamotrigine pharmacologically upregulates I_h (Poolos et al. 2002). In keeping with our observation that rats with freeze lesions have reduced I_h, lamotrigine was significantly less effective at reducing network signals from freeze lesioned rats. We did not observe a similar decrease in the effectiveness of ZD 7288 to increase voltage sensitive dye response half width. The hyperexcitable nature of dysplastic cortex, the low concentration of ZD 7288 (10 μM) used, remaining I_h (we observed a reduction in I_h in freeze lesioned rats, not a total loss), and variability in the nature of hyperexcitable network events may mask subtle alterations in the ability of ZD 7288 to enhance network activity.

Together the data from this study suggest that HCN channels are reduced in excitatory neurons in a model of cortical dysplasia. This is associated with increased EPSP summation and membrane excitability. Furthermore, the data suggest that

increased neuronal excitability associated with channel loss translates to an increase in network excitability. The study does not address how individual neuron types within the neocortical network are affected by HCN channel loss. Further, it does not address how epileptiform events themselves are influenced by HCN channels.

Our work showing that epileptiform events in acute slices are constrained by HCN channels is consistent with the observation that HCN channels serve to limit network activity. This is further supported by our data showing that voltage clamping neurons at membrane potentials which preclude HCN channel opening does not block the increase in the area of epileptiform events following HCN channel inhibition. These experiments were conducted in an in vitro disinhibition model using bicuculine mediated to allow extracellular stimulation to evoke inter ictal events in neocortical slices. This is a well established model of *in vitro* network hyper excitability (Campbell and Hablitz 2005; Gutnick et al. 1982; Salgado-Commissariat and Alkadhi 1997). This model has an additional advantage of allowing us to examine the specific effects of HCN channel inhibition on run-away excitatory synaptic transmission. This is in contrast to the voltage sensitive dye study which examined the role of HCN channels in network activity with intact inhibition. It also avoids confounding protein-level, and neuroanatomical changes which may follow repetitive behavioral seizures like those observed in the chemical models of epilepsy.

Since epileptiform responses were evoked in the presence of bicuculine, the increase in duration likely represents an increase in the level of excitatory activation. As discussed, I_h significantly decreases the excitability of pyramidal neurons (Magee 1998; Poolos et al. 2002). Concurrent increases in the excitability of all the pyramidal neurons

within the neocortical network likely drives the increased intensity of epileptiform events following HCN channel inhibition. Stimulation in these experiments probably results in activation of many of excitatory neurons within the network. As such, individual units see multiple EPSPs with the summation greatly enhanced following HCN channel inhibition. It is our hypothesis that this drives many more neurons to threshold, the net effect of which is increased excitatory drive. Taken in context with our work using voltage sensitive dye imaging, these data suggest that HCN channel loss not only increases normal network hyperexcitability, but also increases the severity of network events that become epileptic.

Interestingly, we saw a different influence of HCN channel inhibition on epileptiform events observed from the perspective of pyramidal neurons verses interneurons. HCN channel loss selectively increased the number of spikes overlying epileptiform depolarizations in pyramidal neurons, but not in interneurons. This is potentially important as it suggests that HCN channel loss may selectively increase output from excitatory neurons. Altered function of inhibitory circuits may contribute substantially to hyperexcitability observed in human cases of epilepsy (Cepeda et al. 2007). Interneurons are important for synchronizing populations of hippocampal excitatory neurons (Cobb et al. 1995), and activation of inhibitory neurons can drive hippocampal seizure like activity (Fujiwara-Tsukamoto et al. 2010). Interneuron cell loss may also directly contribute to hippocampal epileptogenesis (Sloviter 1987; Magloczky and Freund 2005). Given this, changes in the cellular properties that differentially effect inhibitory and excitatory network elements could strongly contribute to epileptiform events. A selective increase in excitatory drive following HCN channel loss in cases of

epilepsy may be a potential mechanism by which HCN channel pathology contributes to epilepsy.

The preferential effect of HCN channel inhibition for excitatory neurons may be due to differing expression of HCN channels. HCN1 channels are primarily express in hippocampal and cortical pyramidal neurons (Lorincz et al. 2002) and the role of I_h in reducing individual neuronal excitability has primarily been conducted in pyramidal neurons (Magee 1999; Berger et al. 2001). However, HCN channels or I_h have been reported in several types of interneurons (Santoro et al. 2000; Aponte et al. 2006; Wu and Hablitz 2005) and HCN channel inhibition increases release from inhibitory terminals within the hippocampus (Peng et al. 2010). Recordings in this study revealed very little total I_h in either layer one interneurons or fast spiking layer five interneurons, especially at resting membrane potentials. Therefore, HCN channel loss may selectively influence excitatory network elements simply because there is greater HCN channel density within excitatory network elements.

The observation that HCN channel inhibition differentially influenced excitatory and inhibitory network elements raised additional questions. The first was whether HCN channel loss occurred in both inhibitory and excitatory neurons in models of epilepsy, or if loss was selective for pyramidal neurons. The second was whether HCN channel loss would have the same influence over synaptic integration and intrinsic excitability in excitatory and inhibitory neurons. An un-equal increase in neuronal excitability following HCN channel loss in inhibitory vs. excitatory neurons could contribute to the increased network excitability observed in cases of HCN channel loss.

The final study detailed in this dissertation found that, despite having initially low levels of I_h in normal rats, fast-spiking interneurons from freeze lesioned rats had reduced I_h. To our knowledge, this is the first evidence of interneuronal I_h being reduced in a model of epilepsy. Loss of I_h may therefore be a common feature of all neuron types in cases of epilepsy. We also found that HCN channel inhibition slightly but significantly increased summation in layer V interneurons. We did not observe a similar effect in interneurons from rats with freeze lesions, consistent with the observation that interneuron I_h is significantly reduced in these animals. The effect of HCN channel loss on temporal summation was not less robust than that observed in pyramidal neurons in the freeze lesion model or the kainate model (Albertson et al. 2011; Shin et al. 2008). This is especially apparent when compared to the increase in summation observed in HCN1 knockout animals (Huang et al. 2009). We also did not observe a difference in baseline EPSP summation in interneurons from control rats and rats with freeze lesions. This is likely due to the small effect HCN channel inhibition has on EPSP summation in interneurons as well as remaining I_h in freeze lesioned animals.

It is interesting that we observed an increase in summation following HCN channel inhibition. It suggests that HCN channels may filter dendritic inputs similarly in both interneurons and pyramidal neurons. However, the difference in the magnitude of the effect on summation between pyramidal neurons and interneurons further suggests that universal HCN channel loss may more selectively increase output from excitatory cell types. Furthermore, we observed that HCN channel loss decreased action potential firing in response to depolarizing current pulses in layer five interneurons. This is contrast to what has been observed following HCN channel loss in excitatory neurons

(Jung et al. 2007; Magee 1998) including pyramidal neurons from rats with freeze lesions (Albertson et al. 2011). HCN channels decreasing spike frequency has been observed before in hypothalamic GnRH neurons (Chu et al. 2010) and may represent another mechanism by which universal loss of HCN channels may selectively increase excitatory drive.

Together these data suggest that HCN channels have a strong role in limiting network excitability and that HCN channel loss may contribute to hyperexcitability observed in cortical dysplasia. Rats with freeze lesions have reduced I_h in both interneurons and pyramidal neurons. This is correlated with an increase in network excitability. HCN channel loss significantly increases both normal network excitability as well as epileptiform network events. Finally and perhaps most interestingly, universal HCN channel inhibition or loss may selectively drive increased output from excitatory network elements, contributing to an imbalance of excitation and inhibition.

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APPENDIX

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF RENEWAL

DATE: March 16, 2011

TO: JOHN JOSEPH HABLITZ, Ph.D.
SHEL-1014 2182
FAX: (205) 934-6571

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Neocortical Epilepsy During Development
Sponsor: NIH
Animal Project Number: 110408136

As of April 27, 2011, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	A	30
Rats	B	200

Animal use must be renewed by April 26, 2012. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 110408136 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

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