Decreased Hyperpolarization-activated Currents in Layer 5 Pyramidal Neurons
Enhances Excitability in Focal Cortical Dysplasia

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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 KH₂PO₄, 23 choline HCO₃, 0.4 ascorbic acid, 0.5 CaCl₂, 3.5 MgCl₂ and 25 D-glucose (Tanaka et al. 2008). The solution was bubbled with 95% O₂/5% CO₂ 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 NaHCO₃, 2.0 Ca²⁺, 2.0 Mg²⁺, 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<sub>A</sub> 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 \( N-(3\text{-}(\text{triethylammonium})\text{propyl})-4\text{-}(4\text{-}(p\text{-diethylaminophenyl})\text{butadienyl})\text{pyridinium dibromide} \) (RH 414). Individual slices were stained with 30 \( \mu \text{M} \) 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 \( \mu \text{A} \) in amplitude with duration of 190 \( \mu \text{s} \). 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 $\Delta F$ 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 ($-76.5 \pm 1.0$ mV, $n=24$) than sham-operated animals ($-73.8 \pm 0.9$ 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: $77.4 \pm 4.2$ MΩ, $n=24$; sham-operated: $64.9 \pm 3.7$ 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: $-79.1 \pm 1.4$ mV; sham-operated: $-78.5 \pm 0.81$ mV; $R_{in}$: lesioned: $92.3\pm6.5$ MΩ; sham-operated: $85.6 \pm 5.3$ MΩ), suggesting that the initial differences arose from alterations in $I_h$ expression (Fig. 1A 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 $2.26 \pm 0.3$ (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 I₉ in neurons from lesioned animals results in increased intrinsic excitability of L5 pyramidal cells.

Voltage-clamp analysis of I₉

Somatic voltage-clamp recordings were performed to examine I₉ 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 I₉ 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 \( \mu m \)
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 \((\text{EPSP}_5/\text{EPSP}_1)\) 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).

Ih and spread of activity

Ih 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 Ih 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 Ih 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. 8B. 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. 8C. Response half-widths were significantly increased when the $I_h$ blocker was present at all stimulation intensities (Fig. 8D). 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\textsubscript{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\textsubscript{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\textsubscript{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 Ih 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 Ih. 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 Ih 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 Ih activation. Temporal EPSP summation and intrinsic excitability was increased in neurons near the freeze lesion. These differences became non-significant following application of the Ih blocker ZD7288. Furthermore, we demonstrated a role for Ih in constraining network activity, finding that this effect was reduced in dysplastic cortex.

Ih changes in epilepsy

Alterations in Ih 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 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.
Ih has a well-characterized role in regulating dendritic excitability. Ih 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 Ih 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 Ih 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, Ih 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 Ih 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 $I_h$ could also 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 to the increased network excitability.
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FIGURE LEGENDS

FIG. 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.

FIG. 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.

FIG. 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.

FIG. 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.

FIG. 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.

FIG. 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.

FIG. 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.

FIG. 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.

FIG. 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.

FIG. 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).
Lesioned 100 ms 200 pA

Sham-operated

B

Voltage (mV)

Voltage (mV)

Sham  Lesioned

200 pA

100 ms

-50 mV

-130 mV

A
Sham-operated

Lesion

EPSP5/EPSP1

Control

- Sham-operated
- Lesion

+ZD7288

2mV

100ms

*
Effect of LTG on Area Lesion Vs. Control

Effect of LTG on Amplitude Lesion Vs. Control

A

B

LTG in Control

LTG in Lesion

Diodes Activated (% Control)

Stimulus Intensity (μAmps)

Control (n=14)
Lesion (n=13)

Amplitude (% Control)

Stimulus Intensity (μAmps)

Control (n=13)
Lesion (n=13)