Investigations of Seizure Propagation Directionality: Effects of Temperature and GABAergic Disinhibition

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Abbreviations:

Epileptiform Events (EEs): Field-recorded depolarizations associated with massive, synchronous neuronal activity.

Standard Directionality (SD): A measure of relative dominance between hemispheres.

Light-Referenced Directionality (LRD): A measure of relative dominance between hemispheres that incorporates the expectedly dominant hemisphere.

Interhemispheric Latency (IHL): The latency measured between the recognition of an EE in electrodes implanted in contralateral cortex.

Fraction Unilateral (FracUni): A ratio of the number of unilateral events compared to the total number of events (both unilateral and bilateral).

Bic Trials: Refers to experiments performed with the application of bicuculline.

CGP/Bic Trials: Refers to experiments performed with addition of both bicuculline and CGP.
Abstract

Epilepsy is one of the most common neurological disorders, affecting approximately 3% of the population. By understanding the mechanisms through which foci and seizure pathways develop, more precise therapies and fuller understandings of cortical connectivity can be developed. The studies in this paper examine hemispheric dominance, and seizure propagation in mouse in vitro cortical-slice preparations. The primary goals of these experiments are 1) to describe the relationship between inhibitory neurotransmitters and the development of stereotyped propagation 2) to describe how these biological phenomena relate to applied heat, and 3) to test the relevance of describing our experimental models as coupled oscillators.

We found that large-scale bath-temperature increase (~7°C) will globally amplify seizure genesis, and that small (<0.1°C) temperature differences between hemispheres can produce a significant effect on the relative dominance between hemispheres. However, this small amount of heat alone is not sufficient to significantly alter the rate of local epileptiform-like event (EEs) generation in cortex. While a dominant hemisphere almost always develops in a bicuculline treatment, heat effect which hemisphere becomes dominant. Non-dominant hemispheres have a significantly lower oscillatory rate after an hour of Bic treatment, suggesting that callosal projections are necessary for the emergence of this difference. GABA\textsubscript{B} was antagonized with CGP in an attempt reduce the disparity of oscillatory rates between hemispheres through the occlusion of feed-forward inhibition. While CGP was
shown to have negligible effects on directionality, it did surprisingly have the
significant effect of reducing the number of EEs generated. CGP also increased the
velocity at which EEs traveled, reduced the number of unilateral events, and reduced
the effect of small-scale local heat application on hemispheric dominance. While
precise mechanisms through which directionality and interhemispheric dominance
develop are unknown, these data give interesting insight into the effects that
functional GABA\textsubscript{B} currents have on the reduction of interhemispheric EE efficacy
and the early stages of the development of hemispheric dominance. Our analysis of
mono-hemispheric IHL and unilateral rates also give promising insight into the
attributes of a dominant hemisphere.

Describing our preparation as a pulse-coupled oscillator without an a priori
leader appears to be accurate, as hemispheres with drastically different oscillatory
rates can produce synchronous activity.
Introduction

Epilepsies are a collection of syndromes characterized by an increased presence or probability of synchronized neuronal activity known as seizures. The International League Against Epilepsy defines epilepsy as

“…1. At least two unprovoked (or reflex) seizures occurring more than 24 hours apart; 2. One unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk after two unprovoked seizures (at least 60%) occurring over the next 10 years; 3. Diagnosis of an epilepsy syndrome…” (Fisher et al., 2014)

While part 1 and 3 of the definition create diagnostic tools useful to medicine, the underlying mechanisms of the biological and physical phenomena related to the increased probability and occurrence of seizures are of more interest to our lab. The study of the mechanisms of epilepsy will allow for a more precise, biologically derived definition of epilepsy that can both stand alone from, and contribute to, the clinically relevant definitions. By further examining the underlying phenomena related to seizure initiation and propagation (ictogenesis) and the perturbations of the functional neuronal/biological factors leading to epilepsy (epileptogenesis), we not only perform an academic endeavor, but also a medical endeavor insofar as increasing the understanding of how epilepsy works lays groundwork that may lead to ways of disrupting aberrant activity and return neuronal networks to healthy conditions.
Symptomatic Classification

Without a doubt the most recognizable type of seizures are generalized tonic-clonic seizures, previously called “grand mal seizures”. These types of seizures fall under the category of seizures known as “generalized seizures” (Berg et al., 2010), which affect both hemispheres simultaneously upon ictogenesis. Seizures of this type were first acknowledged by the Babylonians who described a collection of symptoms consisting of loss of consciousness, convulsions, and lasting effects such as panic and fear after the “possession” (Wilson & Reynolds 1990). The Babylonian records go on to acknowledge that increased occurrence of seizures is associated with increased mortality, already recognizing a difference between acute seizures and epilepsy (Wilson & Reynolds 1990). Contemporary medicine recognizes that individuals diagnosed with epilepsy have a standardized mortality ratio of 3.6 when compared to the general population, and the ratio is even higher in children, who also are at the highest risk of sudden unexpected death in epilepsy (Nilsson et al., 1997). The Babylonians were unaware of the underlying neurological causes and outcomes of seizures and thus were unable to recognize other epilepsies that have different physical presentations from tonic-clonic seizures. The second main category of seizures, “focal seizures” (also known as partial or localized seizures), differ from generalized seizures because the regions affected by the aberrant activity are isolated to a specific region on the brain, whether that be a single hemisphere or a specific location within a hemisphere (Berg et al., 2010).
While the schema of generalized vs. focal seizures allows for useful descriptions of epilepsies, it is far from the only way to describe epilepsies; specifically, these categories only take into account the anatomy affected by the aberrant activity and disregard symptoms, causes, and neuronal activity associated with specific epilepsies. Absence seizures, historically called “petit mal” seizures, are a type of generalized seizure that can be almost completely asymptomatic. Absence seizures are characterized by short (< 20 second) lapses in consciousness that do not involve massive ballistic convulsions, but may contain automatisms such as lip smacking, myoclonic neck jerks, or relaxed muscles (Panayiotopolus 1999).

Focal seizures can lead to physical or psychological abnormalities but normally do not affect as many modalities as generalized seizures unless they progress into a secondary generalized seizure after an initial localized onset (Guerrini, Genton, 2004). Visually induced seizures, a common focal epilepsy, initiate in occipital cortex and will either remain there or progress into secondary generalized seizure. This progression can take anywhere between seconds and several minutes (Guerrini, Genton, 2004).

**Etiologic Classifications.**

Along with describing epilepsies by their physical effects, or locations of origin, they can also be classified by their causes. Examining epilepsies in this context can allow for more precise treatments and more directed research questions. Unconvinced by the lack of etiologic classifications in the International League
Against Epilepsies’ definition, Shorvon (2011) suggests a etiologically derived schema for classifying epilepsies. The schema is divided into 4 categories: 1) Ideopathic, genetic (either monogenic or multigenic) in origin without clear neurological pathologies, 2) Symptomatic, genetic or otherwise acquired where epileptogenesis is one of many symptoms of an underlying disorder, 3) Provoked, where seizures are mostly caused by a specific endogenous or exogenous factor (reflex epilepsies fall under this category), and 4) Cryptogenic, where the epilepsy is presumably symptomatic, but the underlying disorder is unidentified. The goal of assigning etiology to specific conditions is clinically relevant because it allows for a more direct approach to combating the aberrant activity, physiology, and anatomy. However, assigning a single etiology, or even expecting epilepsies to have a cause per se may not be the most accurate paradigm. Recognizing relevant precipitants that can singly or in conjunction increase the chance of seizures instead of looking for ‘causes’ may be both more interesting scientifically and more useful clinically.

**Precipitants**

Epileptic precipitants can be either endogenous or exogenous in origin and correlate with different types of epilepsy. Epilepsies can have well defined precipitants that fall under the categories proposed by Shorvon (2011), but often seizures are not always preempted by specific events (Frucht et al., 2000). Furthermore, certain factors such as stress and sleep loss can be a precipitant to many different types of epilepsy showing a looser connection between specific precipitants
and disorders than the four categories described in Shorvon (2011) would imply. In a study of 400 epileptic patients, 62% acknowledged specific precipitants, with stress (30%), sleep deprivation (18%), sleep (14%), illness/fever (14%), and fatigue (13%) being the five most common precipitants; menstruation was noted by 28% of women with temporal epilepsy (Frucht et al., 2000). It is important to note that just because 62% of patients reported precipitants does not suggest that the other 38% of patients do not have precipitative factors, but rather that they were unaware whether such factors existed.

**Disruption of the Blood Brain Barrier**

Disruption of the blood brain barrier (BBB) is a common precipitant that can arise endogenously due to epileptic activity itself (Ruth 1984, Van Vliet et al., 2014) or exogenously through trauma (Tomkins et al., 2008). It is well established that massive degradation of the blood brain barrier (e.g. stroke) is toxic to neuronal tissue (Clark et al., 1993). While the exact mechanisms that lead to epileptogenesis given mild BBB disruptions is still not completely understood, BBB disruption is a recognized icto-precipitant (Marchi 2007). Leaking of the BBB can remain heightened for over 3 weeks after the last seizure, and increased seizure frequency is associated with increased rates of leakage (van Vliet, Aronica Gorter 2014, Maggio et al., 2013). This can lead to a positive re-enforcement loop between seizures and blood brain barrier disruption (Maggio et al., 2013). Thrombin, a protease found in blood, has been shown to be associated with neurodegeneration and disruption of healthy
astrocyte glutamate uptake and is one of many invasive proteins found in blood that may participate in ictogenesis, and if presence is prolonged, epileptogenesis (Maggio et al., 2013).

**Febrile Seizure**

Febrile seizures, induced by fever or hyperthermia, are often associated with families, and are considered to be genetic in origin (Berg et al., 1999). Genetic origins are not ubiquitous, however, as extreme hyperthermic conditions have been shown to induce seizure in almost all immature rodents, regardless of genetic history (Holtzman et al., 1981, Jiang, Duong, Lanerolle 1999). Mutations to GABA<sub>A</sub> channels are a prominent single-gene mutation in individuals particularly susceptible to febrile seizure (Harkin et al., 2002). This is due to a temperature-dependent trafficking disruption of mutated subunits (Kang, Shen, Macdonald 2006). The relevance of minor heat increases (far below hyperthermic conditions), and their relation to large-scale GABA<sub>A</sub> and GABA<sub>B</sub> antagonism, are a focus of this paper and will be discussed in much more detail in following sections.

**Cortical Oscillations**

Neuronal oscillations are present in healthy cortical activity and are potentially necessary for synchronizing the relay of meaningful information in local, distal, and even contralateral cortex in (Engel et al., 1992). Individual cells or small groups of neurons may exhibit oscillatory activity in phase-locked response to regular
stimulus, such as outer hair cell in the cochlea (Santos-Sachi et al., 1992). However, these types of oscillations are not relevant to mass cortical synchrony as they rely on exogenous input to the system, rather than emergent electrical qualities of neuronal circuits (Cardin et al., 2009, Santos-Sachi et al., 1992). One interesting frequency band of oscillations is the gamma-range frequencies (20-80Hz). This range of frequencies has been associated with attention to a particular stimulus across multiple senses (Engel, Singer 2001). When functioning correctly, gamma oscillations allow for optimal synaptic communication between neurons by creating moments of activity associated with regular membrane potential oscillations that increase the chances of neurons “firing together.” Interestingly, while these oscillations are easily recognizable when examining populations of cells, they can often go undetected when recording from individual cells due to the relatively low chance of any individual cell firing during the population up-phase (Cardin et al., 2009). These oscillations are strongly mediated by GABAergic, parvalbumin-expressing interneurons (Traub et al., 1996). In fact, when genetically altered to express channelrhodopsin in parvalbumin-expressing cells, 40Hz light stimulation creates a sharp peak in field-potential power spectrums, meaning that parvalbumin-expressing cells are able to create a resonance at 40Hz (Cardin et al., 2009). On the contrary, channelrhodopsin expressing pyramidal cells respond optimally to 8Hz pulses, explaining how en masse, cortex can promote gamma range frequencies, but individually pyramidal cells fire during less than half of the up-phases.
Blumfield and McCormick (2002) showed that absence seizure-like oscillations (3-4Hz) could be initiated by electrical burst (200Hz) stimulus to corticothalamic tracts, while single shock stimulation presented sleep spindle-like oscillations (6-10Hz). When CGP (GABA\textsubscript{B} antagonist) was applied, spontaneous sleep spindle-like 6-10Hz oscillations were observed. When picrotoxin (GABA\textsubscript{A} antagonist) was applied, only 3-4Hz oscillations were present. This supports the hypothesis that “healthy” neuronal networks can be co-opted by stimulus and lead to aberrant activity, and that the epileptic circuits are not separate networks; on the contrary, normal neuronal circuitry is capable of producing seizures should any one of many systems fail to maintain a healthy equilibrium (Cardin et al., 2009).

**Seizure-Related Oscillations**

While seizures are often falsely conceived of as uncontrolled, chaotic activity, there are actually many predictable qualities of seizure both within and across patients. Individuals with focal pharmacoresistant epilepsy often present cortical oscillations at 2-50Hz, with particularly strong bands of activity at 3-10Hz during seizures, observed in both recordings with EEG and microelectrode arrays implanted in cortex near the expected seizures locus (Schevon et al., 2012). These oscillations only reach recognizable amplitudes during seizure activity when enough cortex is recruited and there is a large enough barrage of synaptic activity to render the depolarizations recognizable to MEAs and EEGs recordings. Schevon et al. (2012) concludes that what historically may have been considered a foci was in actuality a
volume that included both a small foci and larger surrounding early-recruited areas of cortex, and that precise seizure foci are difficult to locate because they may be much smaller than previously thought. As the seizure activity progresses away from the focus it moves in a step-wise motion where small regions of cortex are recruited to be part of the seizure with activity peaks at 0.5-2Hz. The oscillatory profile of recruitment has been shown to rely on the degradation of inhibitory signals, not a rhythmic burst of excitatory signals, as excitatory currents remain at similar levels before, during, and after recruitment, while inhibitory signals subside simultaneously with the activity associated with successful recruitment and propagation (Trevelyan et al., 2006). This is particularly relevant to my studies as the experiments reported on in this paper involve the disruption of inhibition, and not the increase of excitatory activity.

**GABA Receptors**

GABA is the primary inhibitory neurotransmitter. GABA\textsubscript{A} and GABA\textsubscript{B} receptors are found throughout the mammalian cortex (Bowery et al., 1987). In general there are higher concentrations of GABA\textsubscript{A} subtypes, but depending on the location, especially in deeper brain regions, GABA\textsubscript{B} can be more densely concentrated. GABA\textsubscript{A} receptors mediate their activity through an ionotropic channel selective to chloride (Cl\textsuperscript{−}) ions that directly inhibit neuronal activity either through hyperpolarization of neuronal membrane potential or through shunting inhibition (Smith et al., 1995). GABA\textsubscript{B}, a G protein-coupled receptor, is a metabotropic receptor
with sequence similarities to other metabotropic receptors, such as the metabotropic glutamate receptor, and is expressed both pre- and post-synaptically. (Kaupmann et al., 1997). Instead of a bound ligand (GABA) directly opening a channel, GABA_B leads to inhibition through downstream pathways. Through downstream closing of CA^{2+} channels (Mintz et al., 1993, Scholz et al 1991), pre-synaptic GABA_B will inhibit both GABA release and the release of other neurotransmitters (Wu et al., 1997). Post-synaptically, GABA_B mediates late inhibitory post-synaptic currents through the activation of GIRK channels, inwardly rectifying K+ channels (Blaxter et al., 1986). Because of the metabotropic nature of GABA_B signal transduction, the time-course of GABA_B inhibition is much longer than GABA_A IPSPs. GABA_B IPSPs peak at around 200ms and last for up to 5 seconds (Otis et al., 1993). GABA_A on the other hand has been shown to have a decay time-constant between <3ms and 3-30ms depending on the location and identity of the cells tested (Fisahn et al., 2001). It has also been shown that there was a large between-cell GABA_A decay time-constant variability, but very small within-cell variability (Eyre et al., 2012). GABA_C is a third type of GABA receptor. Like GABA_A, GABA_C is an Cl^{-} ionotropic receptor, but does not respond to bicuculline or baclofen, the archetypal GABA_A and GABA_B antagonists (Bormann, Feigenspan 1995). This third type of GABA receptor was not examined in our experimental design and will not be discussed further in this paper.
**Disinhibitory Models**

Healthy activity in cortex is maintained by a normal relationship between excitatory and inhibitory activity. By removing latent inhibition in cortex, normal levels of excitatory activity can accumulate and lead to EEs. Disinhibitory models often use some type of GABA$_A$ antagonist, with picrotoxin and bicuculline being the most common. Because GABA$_A$ is the most common inhibitory receptor in the cerebrum, by restricting its activity the primary inhibitory restraint within cortex is lost. Seizures develop within the first half-hour after exposure to suprathreshold concentrations of GABA$_A$ antagonists (PTX: $\sim$2.5µM, BIC: 4µM). Seizure dynamics and rates are not significantly affected by increasing concentrations beyond this point. Seizure induced *in vitro* have an extremely high velocity (~50-90mm/sec) (Chervin, Piers, Connors 1988), which is 100-200 times faster than clinically observed seizure propagation. (Trevelyan et al., 2006)

In clinical trials, epilepsies have been shown to progress not in a constant, unbroken wave, but in a stepwise pattern due to the inability to recruit cortex outside of the ictal penumbra. This penumbra is defined as the area directly surrounding the seizure (150-250 µM), with both increased excitatory and inhibitory synapse activity. Under normal inhibitory levels, the excitatory activity would be able to elicit massive amounts of activity in the penumbra, but because of the strong inhibitory restraint this does not occur. When the inhibition in the penumbra does break down, it is nearly simultaneous across all penumbral neurons, and strong phase-locked activity forms in
this region; the seizure has successfully recruited new territory (Trevelyan et al., 2006).

Importantly, disinhibitory models occlude feed-forward inhibition, at least with regard to GABA\textsubscript{A}. Thus in this model, the inhibition-dependent recruitment latency is greatly reduced, and the velocity of the events are expected to increase (Trevelyan et al., 2006). This is consistent with our findings in bicuculline models.

Previous work in this lab has found evidence of cross-callosal feed-forward inhibition arising due to local bicuculline application. In these experiments, concentrated bicuculline was injected into small areas of cortex via an extracellular electrode. Contralaterally, intracellular electrodes were able to record large inhibitory barrages that both preceded and followed EE onset (Walker et al., 2012). We believe that because the bicuculline was injected locally, and not bath applied, only one hemisphere was able to produce seizures, and that the contralateral side had sufficient inhibitory signals to squash any chance of contralateral seizure recruitment.
Figure 1: Evidence of Contralateral Pre-Ictal Inhibitory Barrages
The black trace is a field recording from the hemisphere in which bicuculline is injected. The blue trace is an intracellular recording contralateral to the bicuculline injection. Figure from Walker et al., 2012

Figure 2: Epileptiform-Induced Contralateral Inhibition is Present Without Contralateral Excitation
Evidence of both anticipatory and delayed IPSCs in relation to contralateral EE onset (time = 0). Figure from Walker et al., 2012
Excitatory Models

“0 Magnesium” is a popular excitatory model used to mimic epileptic events. This model utilizes traditional artificial cerebral spinal fluid without the addition of magnesium. With a low concentration of magnesium in the extracellular fluid, NMDA receptors lose their chronic Mg$^{2+}$ channel blockade and thus chronically depolarize cells. 0Mg$^{2+}$ preparations propagate through cortex about 10-100 times slower than disinhibitory models and at rates similar to those measured in clinical experiments (Schevon et al., 2012). In contrast to the disinhibitory models, 0Mg$^{2+}$ does not directly occlude any synaptic connectivity. This allows for a close examination of the interplay between inhibition and excitation that disinhibitory models do not, because a more-or-less functional penumbral inhibition remains present. Seizure events can be divided into three major periods: initiation, propagation and termination. It has been found that the initiation of seizures in 0Mg is due to the reduction of inhibitory activity, rather than purely increased excitatory. During propagation, inhibitory signals relax and allow for excitatory barrages to momentarily dominate. Termination ends the seizure when inhibitory restraint returns and quickly silences the propagation-related hyperexcitatory barrages. (Pinto et al., 2005). When GABA$_A$ antagonist picrotoxin was applied in low doses (<2.5μM), preictal barrages (measured intracellularly) consisted only of excitatory feed-forward synaptic release. Interestingly, these trials propagated at velocities similar to high doses bicuculline and PTX (Trevelyan et al., 2006).
Directionality and Related Factors

Before moving forward, we must first define a handful of terms specific to our research. Understanding “interhemispheric latency” (IHL) is necessary before any further discussion because it is the fundamental concept that allows us to analyze and describe bilateral EEs. IHL is defined as the time difference between the recognition of an EE by the two extracellular electrodes embedded in cortex in contralateral hemispheres. Events were considered bilateral if their IHL was less than 400ms. Given that the frequency of events is relatively sparse in most recordings (the onset of events lasts less than a second, and events are often more than 20 seconds apart), false positives derived from spontaneously close are rare, even in the most event-dense of recordings. False negatives due to slowly progressing events are nonexistent since the IHL of all bilateral EEs were always significantly under this cutoff. Traces were rechecked manually to locate false positives and negatives due to noisy recordings or small-amplitude events. By calculating IHLs, we are able to create both a continuous variable (how many milliseconds?) and categorical variables (Was this event bilateral? Which direction did it travel?), both of which were useful during analysis.

By counting how many events in a slice were unilateral (i.e. did not propagate across the callosum), we were able to calculate a “fraction unilateral” (FracUni) for every slice, which is simply defined as the number of unilateral events divided by the total events (the sum of unilateral and bilateral events). This allowed us to examine the efficacy of our different preparations in regards to quantifying the relative
connectivity between cortices (e.g. a completely non-efficacious connectivity, perhaps due to a severed callosum, would result in a FracUni of 1).

“Standard Directionality” (SD) is a measure of the percentage of bilateral events that originated in the same hemisphere across a given time period. Importantly, this measure allows us to determine which hemisphere becomes dominant and to what extent it becomes dominant. In a slice where 100% of bilateral events originate in hemisphere A, the directionality of this slice would equal 1. In a slice without a dominant hemisphere, where 50% of bilateral events originated in hemisphere A and 50% originated in hemisphere B, the directionality would equal 0.5

![Figure 3: Directionality Explained](image)

The recording represented on the left has a strong directionality, with the vast majority of events propagating from the hemisphere with the blue electrode to the hemisphere with the red electrode. In the recording represented on the right, an equal number of events propagated in either direction, and thus directionality is minimal. Another difference between the experiments is that the IHL is much lower in the first recording (~20ms), while the second recording has a much higher IHL (~100ms).
Recently we have realized that while this metric is extremely useful in slices without an implicitly biasing protocol, many of our experiments do introduce intentionally biasing aspects such as applied heat, applied light, or orientation. In these experiments, a new metric became necessary. We have called this “Bias-Referenced Directionality” (BRD). The specific biasing factor may also be named, as in Light-Referenced Directionality (LRD).

While traditional directionality is on a scale from 0.5-1, BRD is on a scale from 0.0-1 where a slice with 100% of bilateral events originating on the expectedly dominant hemisphere is given a directionality of 1, and a slice with 100% of bilateral events originating on the expectedly non-dominant hemisphere would have a directionality of 0.0. This allows us to not only tell how strong of a dominance is developed between hemispheres, but also to determine how strong of an effect the biasing factor has on hemispheric dominance.

Our fascination with directionality originated after examining the experiments presented in Walker et al., (2012) in which a strong left-to-right bias across many slices was observed. These experiments were conducted under the “Horizontal Central Light” preparation. The specifics of this design will be further explained in the methods section. What was particularly interesting was that the downstream hemisphere became the dominant hemisphere a significant amount of the time (sign test p < 0.05, n = 24). Two primary hypotheses generated were that a) the infrared light from the microscope that was left on during the experiment was potentially warming the solution and thus heating the downstream hemisphere generating a
robust affect and b) the slow rate of ACSF flow was not sufficiently dispersing waste material or ion efflux from action potentials, causing the downstream hemisphere to be slightly more depolarized and excitable due to excess K+ ions in the solution. By rotating the slice 90° in the bath, we were able to create a situation in which both hemispheres were symmetrical with regard to flow and light source. This vertical reorientation created a true negative control by removing the hemispheric bias intrinsic to the experimental design.

The temperature of the tissue in the light and dark hemisphere was then measured. It was found that in a vertical preparation, IR light did increase the temperature of the hemisphere to which it was applied within seconds after the application of light.

![Graph showing temperature changes](image)

**Figure 4: Light Increases Local Temperature**  
A rise in temperature (~0.1°C) was observed within seconds of the IR light being turned on.
Figure 5: Representative Hour-Long Bihemispheric Temperature
Over the course of an hour-long recording, the temperature probe on the IR-lit hemisphere consistently recorded a higher temperature than the dark hemisphere. It is important to note that the drift of ambient temperature was approximately a magnitude larger than the interhemispheric temperature difference, and yet the light:dark, warm:cool relationship remained constant throughout the experiment.

While the finding that a temperature difference existed was robust, the temperature difference in response to the light was miniscule. It was surprising that, while in support of our hypothesis, such a small temperature difference could result in such a powerful downstream effect such as the significant biases found in many of the horizontal recordings. We continued to examine the application of heat by introducing heated wired installed to hover just above the surface of the slice. In all 10 experiments conducted in this preparation, the heated hemisphere became the dominant hemisphere.
Because the temperature increase due to the heater (~3°C) was much larger than temperature increase due to the IR light (<0.1°C), and in conjunction with the strong directional bias on the heated side, we concluded that heat and not light per se was sufficient to create the strong left-to-right bias found in the horizontal recordings. Whether heat was acting alone, or if there were other factors involved – such as cortical plasticity – was still yet unanswered, and this has now become a focus of our work.

Figure 6: Heated Wire Causes a Large Increase in Cortical Temperature
When a microheater was used as the heat source, the difference between the warm and cool slices was ~3°C, a 30-fold increase from the IR light.
Figure 7: Heated Wire Leads to a Strong Hemispheric Bias
When the microheater was used, in 10 out of 10 experiments the heated hemisphere became the dominant hemisphere.

Coupled Oscillators as a Metaphor for Bi-Hemispheric EEs

Coupled oscillators are often used to describe how multiple oscillators with nearly harmonic relationships can entrain synchronicity; that is, synchrony without leadership is an emergent property of near-harmonic coupled oscillators (Sun et al., 2014). Our model, however, incorporates simultaneous potential leadership by either oscillator and does not require similar frequencies of oscillation.

In contrast to synchrony derived from near-harmonic relations, pulse-coupled oscillators, like that found in the cardiac pacemaker, are more relevant to our results. Pulse-coupled oscillators are usually driven by a leading oscillator. When it fires a pulse – due to a completion of an oscillation – it either pulls the following oscillator
closer to being in phase or initiates activity if a threshold is reached (Mirollo & Strogatz, 1990). Our model consists of 2 oscillators, standing in for the two hemispheres, which are able to oscillate at any frequency. Importantly there is no designated leader; at any moment either oscillator can send a pulse to the opposite oscillator. When an oscillator completes an oscillation, a pulse is generated and sent to the other oscillator. In regard to Mirollo & Strogatz (1990), our oscillators either reach threshold or fail to reach threshold; phases are not synchronized, and in fact frequency disparities were observed to increase over the course of the treatment.

In the simple model, we assume that the connectivity between oscillators is 100% in both directions. This represents an ‘ideal’ intact callosum, and will produce a FracUni of 0. In this situation the faster oscillator has complete control over the slower oscillator’s rate of activity; the intrinsic oscillatory rate of the slower oscillator is irrelevant because it is completely unable to determine the collective oscillatory rhythm due to the faster oscillator ubiquitously completing its oscillation first.

A noisy oscillation can also be generated through the application of a large amount of noise to the smooth cosine wave. This would create a relationship in which the slightly faster oscillator would infrequently receive a pulse from the on-average slower oscillator (Fig. 28Bi). This is more biologically relevant because in most recordings there are a non-zero number of events that originate on the non-dominant hemisphere.
Noisy oscillations are a rough replica of biologically observed directionality.

In a more nuanced model, the connectivity between oscillators is somewhere between 0-1, meaning that a pulse in one oscillator has a non-zero and non-definite chance of activating the other oscillator. In this model, the faster oscillator has control over the slower oscillator, but not complete control. Given the non-definite chance of activation, oscillators would sometimes complete an oscillation and the opposing oscillator would fail to receive a pulse. Assuming identical connectivity of 0.9 in both oscillators, a slightly slower oscillator would be able to initiate activity in the faster oscillator approximately 1 out of every 10 events, even without a noisy oscillation. Also, this model allows for a small number of unilateral events, a feature observed in experimentation that the simple model is unable to mimic. We can see how this begins to model a bi-hemispheric slice preparation.

A final criterion necessary to describe the observed activity is that the individual parameters – the oscillation rates of both oscillators and the connectivity
probability between the oscillators – must be changeable and responsive to the activity generated within the oscillators. In this model, there is no assumption that the connectivity between oscillators is identical in both directions. In response to the Mid-Cut results (Fig. 19), receiving a signal from the opposing oscillator would lead to a slight decrease in the local rate of oscillation, generating a positive reinforcement loop of increasing disparities of frequencies and increased dominance. Through this interaction, one oscillator will assume the role of leader; a directional, dominant/non-dominant relationship between oscillators will spontaneously emerge even if the oscillators exhibit identical parameters at the start of the simulation. This model mimics: the similar rates and connectivities between hemispheres in early time points of Bic experiments, the movement away from this similarity, the generation of unilateral events, the relative decrease in oscillatory rate due to the reception of seizures, and finally the increasingly dominant relationship between hemispheres leading to strongly established directionality.

It has been shown that inhibitory pulses are very useful in entraining synchrony in pulse-coupled oscillators (Ernst et al., 1995). Specifically they aid in synchronization due to the priming of synchronous up-phases that can then be taken advantage of by excitatory pulses. Pulse-coupled oscillators with inhibitory and excitatory pulses entrain phase relations with reduced latency than oscillators that only send excitatory pulses (Ernst et al., 1995). Because of the possibility of this inhibitory entrainment, we decided to examine the broad participation and the specific timing of inhibitory GABA_B activity in relation to the EE field-potential timing.
Materials and Methods

Mice

Black Swiss Webster mice were used in all the experiments found both in this paper. Both male and female mice (P18-24) were used for these experiments.

Slice Preparation

Mice were given a 0.1mL intraperitoneal injection of 120 mg/kg ketamine-10 mg/kg xylazine. After the mice had become unresponsive, they were decapitated and the brain was removed and placed in a beaker of “High Sucrose 1:3 ACSF” (in mM: 3 magnesium sulfate, 1 calcium chloride, 222 sucrose, 27.1 sodium bicarbonate, 1.5 sodium phosphate, 2.6 potassium chloride, 3 myo-inositol, 2 sodium-pyruvate, 0.4 ascorbic acid) cooled in an ice bath. The brain was then placed on a glass dish sitting on ice. The lateral millimeter of cortex was removed by a lateral sagital slice on both hemispheres by a razor blade. A coronal cut was performed at 6° from the vertical (rostral to caudal) to remove caudal occipital lobe and the cerebellum; most importantly, this cut allows the brain to be oriented correctly on the vibratome (Leica VT1000S). Once the brain had been prepared, the face of the third cut was glued to the table of the vibratome, ventral side facing away from the blade. 120mL of cooled High Sucrose 1:3 ACSF was then poured over brain such that it was fully immersed. The vibratome was set to cut 350µm slices. Five slices were saved and placed in a 34°C 1:3 ACSF bath (M: 3 magnesium sulfate, 1 calcium chloride, 126 sodium...
chloride, 26 sodium bicarbonate, 1.1 sodium phosphate, 25 glucose, 3 potassium chloride, 3 myo-inositol, 2 sodium-pyruvate, 0.4 ascorbic acid), with “slice #1” being the first slice with a visibly intact callosum. Slices 2-5 were used in electrophysiological recordings as slice #1 was often incapable of producing bilateral events. After all 5 slices were placed in the 34°C bath, the incubation chamber was placed on the bench top for an hour-long incubation period.

Figure 9: Schematic of Slice Preparation and Electrode Placement
Top Right: the angle (6°) from vertical that is used in making the cortical slices. Bottom Right: a cortical slice created for experimentation. Left: two electrodes are shown implanted in layer 2/3.
Figure 10: Complete Callosal Axons are Present in Slice Preparation
A neuron with an intact axon crossing from one hemisphere to the other. Evidence that the slicing protocol does not completely disrupt callosal projections.

Experimental Toxins

Bicuculline methiodide ordered from Enzo Life Sciences Cat. No: BML-EA149-0050

CGP52432 ordered from Sigma Life Sciences Lot # 0013M4737V

Two experimental solutions were derived from “2:1 ACSF” (in mM: 1 magnesium sulfate, 1.5 calcium chloride, 3.5 potassium chloride, 26 sodium bicarbonate, 1.1 sodium phosphate, 25 glucose, 3 potassium chloride, 3 myo-inositol, 2 sodium-pyruvate, 0.4 ascorbic acid); this solution will be referred to as “Bic” for the rest of this paper. To complete the first experimental solution (Bic), 20µM of bicuculline were added 2:1 ACSF. To complete the second experimental solution
(CGP/Bic), 20µM of bicuculline and 20µM CGP were added to 2:1 ACSF; this solution will be referred to as “CGP/Bic” for the rest of this paper.

Electrophysiological Recordings

After the incubation period, individual slices (2-5) were placed in the recording chamber, which was bathed in a constant flow of 34°C 1:3 ACSF. Slices were placed in either the vertical orientation or the horizontal orientation. The vertical orientation is defined as the slice being placed in the chamber such that the flow is parallel to the vertical axis of the cortex. This orientation allows for both hemispheres to be given identical interaction with both fluid and central light placement. In the horizontal orientation, the slice is placed such that the flow is parallel to the horizontal axis. This orientation creates an upstream and downstream hemisphere and thus does not treat the hemispheres identically.
The above diagram shows the horizontal slice orientation. The lower diagram shows the vertical slice orientation.

The slice was visualized on a monitor via a 20x (UMPlanFI 20x/0.50 w, Japan) or 40x Olympus objective (LUMPlanFL/IR, 40x / 0.80 w, Japan) and the program SimplePCI. The display was centered on the central fissure (Fig. 9 Left), just superior to the corpus callosum. Borosilicate capillary tubes (O.D.: 1.5 mm, I.D.: 0.86 mm, 10 cm length, Sutter Instrument) were pulled to a resistance of 1-4MΩ. The electrodes were filled with 1:3 ACSF and lowered into layer 2-6 of ACC. During the experiment, a constant pressure of 0.1-0.5psi was maintained in order to prevent clogging of electrodes. In experiments with intracellular recordings, a patch electrode was pulled to a resistance of 6-12MΩ and was lowered proximally to one of the extracellular electrodes; imaging of fluorescently stain neurons confirmed that
intracellular recordings were performed in Layer 5-6 of ACC (Fig. 31). After the electrodes were positioned, the bath was switched to the experimental bath solution Bic, CGP/Bic, or 0Mg. Recordings were initiated as soon as the valve to the experimental solution was opened. Two patch-clamp amplifiers model 2400 (A-M Systems Inc., Carlsborg, WA) were used to record from extracellular electrodes; the gain was set to 100 on the amplifier and digitized by a ITC-18 digitizer and USB-18 computer interface device (Instrutech). Finally, the digital signal was recorded at a sampling rate of 5kHz in Igor Software (Wavemetrics).

Corpus Callosotomies

Pre-Cut: The corpus callosum was severed by hand using a surgical scalpel blade #15 (EMS, Hatfield, PA) while the slice was in the incubation bath, just before it was placed into the experimental chamber. The cut was aligned with the central fissure to a) create a symmetrical cut b) cause minimal damage to cortex. The slice was examined visually in the incubation chamber and again at 20x magnification to ensure that the callosum was cut. If the experiments presented bilateral events, the data was thrown out as the callosum was partially intact. This experiment was performed to determine the endogenous rates of EE generation without cross-callosal interactions.

Mid-Cut: experiments were performed by lowering a sapphire blade (WPI, Sarasota, FL) attached to one of the electrode manipulators. The slice was laid atop a medium-hard vinyl sheet (Plastic Film Corp. of America,1.5H DPC .010 X 54”
4200105821) to allow for the sapphire blade to move freely through the slice and complete the callosotomy. In Mid-Cut experiments, the slice was bathed in BIC solution for an hour to allow for seizure pathways to develop, and the callosum was severed after 1 hour. The recording continued for another hour in order to record unilateral EEs; these rates were then compared with Pre-Cut experiments to determine whether or not functional connections between the hemispheres played a role in altering the endogenous rates of EE genesis. The slice was examined after the experiment to ensure that the callosum was fully severed. Like the Pre-Cut experiment, slices with incompletely severed callosum, or with bilateral events after the cut, were thrown out.

Light Placement

It is important to note how and when light was used in these experiments. When “light” is discussed to in this paper, we are referring to the light that is generated by the microscope to illuminate what is on the slide. An infrared filter along with a polarizing filter were applied to the light in order to create optimal visualization of the slice. Light was either left in a central position on the central fissure or lateralized with the intention of examining potential biases that the light generated when isolated to a single hemisphere. The lateral position was used in vertical orientation Pre-Cut, Mid-Cut, BIC, BIC/CGP, 0Mg, intracellular and extracellular recordings. Central light was used in horizontal recordings and vertical BIC recordings.
In “moving light” experiments, slices were bathed in Bic for 2 hours with a lateral light position. After the first hour of the experiment, the light was moved to the contralateral hemisphere.

*Bath Temperature*

In studies performed before 2014, bath temperature was held at ~27°C, which is approximately 7°C below physiological temperatures. These studies include the recordings in Walker et al., 2012, vertical Pre-Cut and Mid-Cut, and moving light. Since then we have been able to attain a more accurate temperature control through a heating apparatus that warms the bath and source tube directly instead of just the source tube. Since early 2014, all the experiments have been performed at 34°C, also known as “physiological temperature.”

*Data Analysis/Data Presentation*

After being stored in Igor binary format, data was analyzed with macros designed and written by Gloster Aaron used to detect EEs based on their absolute deflection and speed of deflection. If the events were recognized with an interhemispheric latency (IHL) less than 400 milliseconds, the events were considered bilateral. These automated processes were then double-checked visually to ensure that all events were included, and no non-events were included; manual adjustments to the time of event initiation were also performed to ensure accuracy.
Through these macros, IHL graphs, EEs counts, FracUni, event amplitudes, directionality, and BRD were generated to which statistical tests could be performed.

Intact Callosum experiments that presented a FracUni of 1.0 were not included in the data pool because this indicated that the callosum was most likely damaged during the preparation of the slice. Slices that presented less than 10 events over the course of an hour were not included because this indicated that the concentration of toxin in the bath was too low to surpass threshold concentrations for seizure activity, possibly due to a slow flow rate or an incorrect preparation of the toxin solution.

Statistical tests performed are assumed to be Student’s T-Tests with a significance threshold of 0.05 unless otherwise noted.

The plus-or-minus symbol (±) denotes standard error.
Results

Before discussing the more recent findings, the relationship between low-temperature and physiological-temperature preparations, and the different biases arising in the different slice orientations must be addressed. The horizontal-orientation experiments found in Walker et al. (2012) were expected to present an unbiased design in which neither hemisphere was significantly more likely to become the dominant hemisphere. This was not the case. Out of 24 experiments, 19 slices were led by the downstream hemisphere and only 5 were led by the upstream hemisphere (p = 0.002) (fig. 16).

As noted in the methods section, before 2014 the temperature of the bath was approximately 27°C, which is colder than the more physiologically relevant 34°C at which the bath is held now. We performed a collection of parallel experiments at 27°C and 34°C to test whether or not our conclusions based on the earlier, cooler recordings could be a relevant comparison to the physiological-temperature recordings. We hypothesized that the measurements of standard directionality (SD), light-referenced directionality (LRD), Fraction Unilateral (FracUni), interhemispheric latency (IHL), and the amplitudes of events would be insignificantly different between 27°C and 34°C trials. SD (p = 0.87) and LRD (p = 0.65) were found to be insignificantly different in 27°C and 34°C trials (fig. 12). It is important to note that we are able to reject the null hypothesis that light played no roll in affecting hemispheric dominance in 27°C (Sign-Rank Test p = 0.015) (fig. 12). The null
hypothesis was not rejected in 34°C (Sign-Rank Test p = 0.056). Consistent with our hypothesis, FracUni (p = 0.81) and IHLs (p = 0.37) were insignificantly different between 27°C and 34°C trials (fig. 13).

Figure 12: Standard Directionality and Light-Referenced Directionality are Unaffected by Temperature Differences.
Left: Standard Directionality was essentially identical (p = 0.87) in both 27°C (SD = 0.86± 0.03) and 34°C trial (SD = 0.85 ± 0.04). Right: LRD was insignificantly different between 27°C (LRD = 0.75 ± 0.07) and 34°C trials (LRD = 0.69 ± 0.09) (p = 0.65). The null hypothesis was rejected in 27°C (Sign-Rank p = 0.015), but was unable to be rejected in 34°C (Sign-Rank p = 0.056).
Figure 13: Fraction Unilateral and Interhemispheric Latency Are Unaffected By Temperature Differences.
Left: The difference between FracUni measured in 27°C (0.26 ± 0.065) and in 34°C experiments (0.28 ± 0.07) were insignificantly different (p = 0.81). Right: The IHL measured in 27°C experiments (IHL = 59 ± 9ms) was insignificantly different from 34°C (IHL = 71 ± 10ms) (p = 0.37).

As seen in figure 14, the average amplitude of EEs in 34°C (0.19 ± 0.021mV) was significantly lower in 27°C (0.30 ± 0.025mV) (p <0.01). This is consistent with the findings in Moser et al. (1993), which demonstrated that field-potential amplitude is inversely correlated with the temperature of cortical tissue. All events recorded within a given preparation were pooled together in this analysis because previous analyses (appendix #1) showed that no matter how the events were segregated – dominant vs. non-dominant, light vs. dark, amp1 vs. amp2 – there was no significant difference between electrodes.
Figure 14: Amplitude of Events are Significantly Reduced by an Increase in Temperature. The average amplitude of events in 27°C baths was 0.300 ± 0.025mV, which is significantly different from the average amplitude of events in 34°C 0.19 ± 0.021mV (p = 0.01).

We hypothesized that the number of EEs as 34°C would be higher than at 27°C because of the implications of the lateralized light/heat experiments. Consistent with this hypothesis, the number of EEs at physiological temperature (274 ± 48) was significantly higher than at 27°C (74 ± 14) (p < 0.01) (fig. 15). The data used in this analysis was restricted to slices that exhibited bilateral events (FracUni < 1) and had a non-negligible directionality (SD > 0.6) because these were the criteria used to define directional slices used in the original 27°C experiments.
Figure 15: The Number of Epileptiform Events is Significantly Increased in 34°C.
The number of EEs recorded in cold (#EEs = 74 ± 14) and physiological temperatures (#EEs = 274 ± 48) was significantly different (p < 0.001).

We were interested in what was causing the bias resulting in the downstream hemisphere’s dominance. To examine whether the direction of fluid movement over the slice played a role in the bias, we rotated the slice in the bath 90°; this created a situation in which the flow and the light (kept at the intersection of the central fissure and the corpus callosum) were oriented symmetrically with regard to both hemispheres. If there was a bias related to the direction of the flow, this alteration was expected to remove this bias. In 10 experiments, 5 demonstrated top-hemisphere dominance and 5 demonstrated bottom-hemisphere dominance (fig. 16). The
hypothesis that orientation played a role was supported by the results, and a true negative control was found.

Figure 16: Vertical Orientation Removes Bias found in Horizontal Orientation. The horizontal orientation showed a significant left-to-right bias ($p < 0.01$). The vertical orientation showed no bias between top and bottom.

We hypothesized that light was responsible for the genesis of this bias. To discern whether light placement was a factor that mediated the generation of the bias found in the horizontal preparations, we moved the infrared light laterally such that it was localized to a single hemisphere. These lateral light experiments reintroduced the bias found in the horizontal preparations. Out of 28 experiments, 23 were dominated by the “light” hemisphere and 5 by the “dark” hemisphere ($p < 0.001$) (fig. 17). The lateralized localization of light was sufficient to generate a significant bias.
Figure 17: Lateralized Light Reintroduces Bias.
When the IR light was moved laterally from the central fissure, a significant light-to-dark bias was introduced.

We next wanted to examine to what extent light and neuronal activity were responsible for a strong directionality found in these slices. How did directionality become stronger over the course of a recording? Are there endogenous properties and/or exogenous factors that create this strengthening of directionality? Are they both relevant, and if so, to what extent? Are they relevant at different times in the recording? In lieu of the ability to predict directionality with the lateralization of light (a proxy for heat), we hypothesized that if exogenous factors were the only relevant influences on directionality, then an “established” directionality would switch direction if the light were to change sides. The corollary hypothesis is that if neuronal activity plays a role in the establishment of directionality, switching the hemisphere
on which light was shone would have either minimal or no effect regarding the directionality of the slice.

In 7 Moving-Light experiments, the direction of seizure propagations did not change direction once (fig. 18). It should be noted that only one out of the seven slices had a dominant hemisphere on the first-hour dark side, an expected ratio of light-related dominance with regard to the previous experiments.

![Image of dominance experiment](image)

**Figure 18: Dominance is Unaffected by Switching the Placement of Light.**
After an hour of recording, the hemisphere on which the light was shone was switched. The propagation direction did not switch in a single recording (0 out of 7).

We were able to conclude from these results that while heat initially plays a role in the generation of hemispheric dominance, collosally-mediated activity is necessary for the establishment of directionality over time. A metaphor to represent this is that of an egg balanced carefully atop a chicken coop, perhaps with a tiny sail.
attached to the top. Without any wind, the egg may fall down either side with equal probability. A small breeze (exogenous application of heat) can begin the egg’s descent down a particular face of the roof (early directional EEs), but it is the topography of the roof (endogenous neuronal activity and connectivity) that removes the chance for the egg to alter its trajectory after it has been begun to fall (established directionality).

To continue to examine the mechanisms underlying how one hemisphere can significantly and predictably initiate seizures, we wanted to look more closely at the local oscillatory rates of hemispheres both in darkness, and when exposed to light. We hypothesized that an intact callosum was necessary for the creation of a disparity between hemispheric oscillatory rates. To test this, we compared the second hour of Pre-Cut with Mid-Cut preparations (fig.19). In Pre-Cut experiments, the light side had an average of $104.1 \pm 18.3$ events, while the dark side had an average of $91.0 \pm 21.3$ EEs per hour ($p = 0.61$). This creates a ratio of $1.15:1$ (Light:Dark). In Mid-Cut experiments, the average number of EEs after the severing of the callosum on the light and dark sides were $88.6 \pm 9.9$ and $53.1 \pm 10.1$ respectively ($p = 0.03$), with a ratio of $1.67:1$. Using the same source data, the number of EEs in the dominant and non-dominant hemispheres was measured. The number of events on the dominant and non-dominant hemisphere was $91.1 \pm 9.9$ and $50.6 \pm 9.0$ respectively ($p = 0.01$), with a ratio of $1.80:1$. Consistent with our hypothesis, hemispheric oscillatory disparities were not observed in Pre-Cut experiments, but were found in Mid-Cut experiments.
Figure 19: Reduction of Epileptiform Genesis in the Non-Dominant Hemisphere is Dependant on an Intact Callosum.

Top left: Average number of EEs across Pre-Cut (light/dark), Mid-Cut (light/dark), and Mid-Cut (Dominant/Non-Dominant). Top Right: Average and raw data points from Pre-Cut (light/dark). Bottom Left: Average and raw data points from Mid-Cut (light/dark). Bottom Right: Average and raw data points from Mid-Cut (Dominant/Non-Dominant).
In conjunction with the Moving-Light experiments, the Mid-Cut and Pre-Cut experiments support the hypothesis that an intact callosum plays an active role in the establishment of a directional slice. We were curious if a cross-collosal inhibitory signal, analogous to the feed-forward inhibitory ictal penumbra described in Schevon (2012), or the cross-callosal inhibitory currents described in Walker et al. (2012), was present in our preparation. It is well documented that bi-synaptic, contralateral feed-forward GABA_\text{A}- and GABA_\text{B}-dependent inhibitory projections exist in cortex (Palmer et al., 2012). However, whether these types of circuits play a role in the propagation of epileptic events or the refinement of ictal pathways needs to be studied more. While our findings imply that there is a process by which the dominant hemisphere drives the rate of bilateral ictal activity and instills contralateral depression, we know that this mechanism cannot depend on GABA_\text{A} because bicuculline has already blocked all GABA_\text{A} activity. Thus, broad GABA_\text{B} activation is a possible mechanism by which this disparity-amplifying plasticity is induced. The long-term inhibitory effects of GABA_\text{B} may slow the overall oscillatory rate of the non-dominant hemisphere.

We compared Bic and CGP/Bic experiments in order to examine the effect that GABA_\text{B} had in the establishment of directionality. If GABA_\text{B} activation alone were sufficient for the disparity between hemispheric oscillation rates, then the introduction of a GABA_\text{B} antagonist would be expected to reduce the strong directionality found in Bic experiments. Although this specific hypothesis was shown to be false, other significant differences between Bic and CGP/Bic applications were
found. Strong directionality persisted with the addition of CGP. In both of the
directionality metrics, the CGP/Bic experiments were insignificantly different from
Bic experiments.

Standard directionality in CGP/Bic conditions (SD = 0.81 ± 0.038, n=15) was
insignificantly different from Bic conditions (SD = 0.85 ± 0.037, n =16) (p=0.50)
(fig. 20). Experiments with FracUni = 1, or less than 10 EEs, were not included in
these data because, as noted earlier, this demonstrates an unhealthy slice, a damaged
callosum, or a subthreshold concentration of Bic, and an immeasurable directionality.

Figure 20: Standard Directionality is Unaffected by the Addition of CGP.
The difference in average SD between Bic-only (0.85 ± 0.037) and Bic/CGP (0.81 ±
0.038) was insignificantly different.
Light-Referenced Directionality of CGP/Bic conditions ($\text{LRD} = 0.53 \pm 0.091, n = 15$) was not significantly different from Bic conditions ($\text{LRD} = 0.70 \pm 0.088, n = 15$) ($p = 0.14$) (fig. 21). Although insignificant, it appears that $\text{GABA}_B$ antagonism slightly reduced the strong effect that heat plays in initiating the hemispheric disparities. The criteria for these data were identical to the criteria for SD ($\text{FracUni} \neq 1, \#\text{EEs} > 10$). We were unable to reject the null hypothesis for either Bic (Sign-Rank $p = 0.056$) or CGP/Bic (Sign-Rank $p = 0.53$).

Figure 21: Light-Referenced Directionality is Insignificantly Reduced with the Addition of CGP.
The difference between average LRD between Bic-only ($0.70 \pm 0.088$) and Bic/CGP ($0.53 \pm 0.091$) experiments was insignificantly different.
We hypothesized that the addition of CGP to Bic would increase the efficacy of transcallosal seizure propagations. With the disinhibition of both GABA\textsubscript{A} and GABA\textsubscript{B}, the feed-forward inhibitory barrage is expected to decrease and thus allow EEs to propagate with greater ease. This hypothesis was supported by the data (fig. 22). There was a significant decrease in the FracUni of Bic/CGP conditions (0.31 ± 0.091, n = 20) when compared to Bic conditions (FracUni = 0.48 ± 0.083, n = 22) (Rank-Sum Test \( p = 0.048 \)).

Figure 22: Fraction Unilateral is Significantly Reduced with the Addition of CGP.
The averages between Bic (0.48 ± 0.083) and Bic/CGP (0.31 ± 0.091) experiments were significantly different (Rank-Sum Test \( p = 0.048 \)).
There was slight increase in average amplitude of EEs in CGP/Bic preparations (0.23 ± 0.021mv) when compared to Bic preparations (0.19 ± 0.021mv), but this difference was insignificant (p = 0.27) (fig. 23). Again, all events – regardless of hemisphere, amplifier, or light placement (Index #1) – were included.

![Figure 23: Amplitude of Events are Unaffected by the Addition of CGP.](image)
The average amplitude of EEs in CGP/Bic and Bic recordings were insignificantly different.

The interhemispheric latency was expected to decrease with the addition of CGP because with reduced feed-forward inhibition, because the excitatory barrage in pre-ictal cortex at the border of recruited territories would be less hindered. This
hypothesis was supported by the findings (fig. 24). IHLs in CGP/Bic conditions were on average $34.0 \pm 6.3$ms ($n = 15$), while IHLs in Bic conditions were on average $71.5 \pm 9.8$ms ($n = 16$) ($p = 0.0025$ms).

**Figure 24: Interhemispheric Latency is Significantly Reduced with the Addition of CGP.**
The IHL for Bic/CGP ($34.0 \pm 6.3$ms) was significantly shorter than Bic ($71.5 \pm 9.8$ms).

We initially expected that the addition of a secondary disinhibitory antagonist would lead to greater overexcitation, and thus more EEs/hour. This was not the finding, and in fact the application of CGP reduced the number of events per hour by an order of magnitude (fig. 25). The number of EEs in the CGP/Bic and the Bic conditions were $25.0 \pm 3.9$ ($n = 18$), and $281.0 \pm 39.0$ ($n = 22$) respectively. The
difference between these two conditions was determined to be significant (Rank-Sum Test p < 0.0001).

![Graph showing number of events per hour for Bic and Bic/CGP experiments.](image)

**Figure 25: The Number of Epileptiform Events is Significantly Reduced with the Addition of CGP.**

Bic/CGP experiments (25.0 ± 3.9 EEs) had significantly fewer events than Bic experiments (281.0 ± 39.0 EEs).

**Pair-wise Analysis of Hemisphere-Specific IHL and Unilateral Events.**

We next wanted to see if there was a correlation between an increase of IHL and an increase in the number of unilateral events within individual slices, and if there was a hemisphere-specific correlation that gave insight into the “rules” of the dominant/non-dominant relationship. We divided the observed IHLs into domIHL and nondomIHL. domIHL is defined as the average IHL for bilateral events initiated in the dominant hemisphere, and nondomIHL is the average IHL for bilateral events
initiated in the non-dominant hemisphere. We also defined domUni and nondomUni to describe the number of unilateral events measured in the dominant and non-dominant hemispheres respectively.

Gloster Aaron performed these pair-wise analyses of the data I collected in the 34°C preparations. We hypothesized that there would be a correlation between hemisphere-specific IHLs and hemisphere-specific number of unilateral events, especially in the dominant hemisphere, because we believe that dominance is dependent on both local oscillatory rate (represented by number of unilateral events), and connectivity (represented by IHLs). When we compared the hemisphere-specific IHL and unilateral events of the Bic 34°C preparations, a significant correlation between the domIHL and domUni (p < 0.01), and between nondomIHL and bibdomFracUni (p < 0.01) was observed (fig. 26).

We also hypothesized that a correlation between dominant hemisphere IHLs and dominant hemisphere FracUni would exist because IHL and Frac Uni are believed to be dependent on a related underlying mechanism that dictates interhemispheric efficacy of seizure propagation. This hypothesis was supported by the results (fig. 27); a significant correlation (r = 0.7, p < 0.01) was found in the dominant hemisphere IHLs and FracUni.
Figure 26: Interhemispheric Latency and Rates of Unilateral Events are Correlated in the Dominant and Non-Dominant Hemisphere.
Mono-hemispheric IHL and the number of unilateral events are positively correlated in both the dominant and the non-dominant hemisphere. Top: Dominant Hemisphere Bottom: Non-Dominant Hemisphere.
Figure 27: IHLs and Mono-Hemispheric Fraction Unilateral are Correlated. Mono-hemispheric IHLs and FracUni are positively correlated in the dominant hemisphere.

Similarly, we defined domFracUni and nondomFracUni to describe the fraction of unilateral EEs on each hemisphere compared to the total number of EEs. The diagonal line stemming from the origin of these graphs represents a situation in which both hemispheres act identically; larger deviations from this line represent increasing disparities between hemispheres. In the FracUni representation, being above the line implies that the dominant hemisphere exhibited a greater number of unilateral events than the non-dominant hemisphere. In the IHL representation, being
above the line represents that the dominant hemisphere generated bilateral EEs with shorter IHLs than the non-dominant hemisphere.

It was hypothesized that a dominant hemisphere will possess a greater efficacy and have a faster oscillation than the non-dominant hemisphere. In these visualizations, IHLs represent a proxy for efficacy; mono-hemispheric FracUni represents a proxy for local oscillatory rate as the slower hemisphere will be unable to produce as many EEs as the faster hemisphere due to its local oscillation being constantly driven by the faster hemisphere (see: Coupled-Oscillator Model: page 23). Sadly, it is impossible to simultaneously measure IHL and the local oscillatory rate directly because measuring IHL necessitates an intact callosum, while local oscillatory rate necessitates a severed callosum. Because of this difficulty, we must look at the rate of oscillation through slightly obtuse measures. In these visualizations, we see that on average the dominant hemisphere had shorter IHLs (9 out of 12 trails) (sign-test p = 0.15) (fig. 28) and a greater number of unilateral EEs (10 out of 15 trials) (sign-test p = 0.30) (fig. 29) than the non-dominant hemisphere.
Figure 28: Pair-wise Comparisons of FracUni in the Dominant and Non-Dominant Hemispheres Suggest a Reduction an Increased Rate of Epileptiform Event Genesis in the Dominant Hemisphere. 
There are twice as many slices in which the dominant hemisphere produced a greater number of unilateral events, a proxy for local oscillatory rate, than the non-dominant hemisphere (10:5).

Figure 29: Pair-wise Comparison of IHLs of events generated in the Dominant and Non-Dominant Hemispheres Suggest an Increased Efficacy in Events Originating in the Dominant Hemisphere.
There were three times as many (9:3) slices in which the dominant hemisphere produced EEs with shorter IHLs than those originated in the non-dominant hemisphere. Because of the relatively low number of trials, more experiments are necessary before these trends can be confidently called insignificant.
**Intracellular Analysis**

Intracellular recordings were performed in Bic and CGP/Bic vertical preparations. However, because of the small number of useable recordings, they, and possible future directions stemming from these preliminary data, will be covered in the discussion section.
Discussion

*Temperature plays a significant role in the generation of epileptiform-events.*

Both bath-level and hemisphere-level temperature alteration were positively correlated with EE activity. The average number of EEs in slices that exhibited bilateral EEs increased significantly from 74 to 274 (p < 0.01) when the bath-applied heat increased from 27°C to 34°C. Average amplitude of EEs also dropped significantly from 0.30mV to 0.19mV (p < 0.01). While these two metrics were different between the cool and physiological baths, all other extracellular measures were extremely similar; FracUni (p = 0.81), IHL (p = 0.37), SD (p = 0.82), and LRD (p = 0.65) all showed insignificant differences. Because of this I am confident that general trends can be compared across observations derived from cool and physiological baths. Further, these trends of overall increases in activity due to temperature increases give support to the heat-based effects of the more fine-tuned trends found in lateralized light experiments.

Laterized white-light experiments, the lateral heated-wire experiments, lateralized IR experiments, and bath temperature increase demonstrate that the application of heat to the slice preparation will result in an increase in EE generation. Not only do large temperature increases alter large-scale neuronal activity, very small temperature increases (<0.1°C) are able to greatly alter interhemispheric dominance relationships. While large-scale temperature increases lead to large-scale EE generation, small-scale, local (<0.1°C) temperature increases lead to insignificantly
small differences in relative activity between isolated heated and non-heated hemispheres.

Mid-Cut experiments give important insight to the interaction between applied heat and cross-callosal connectivity. After an hour of activity, the dominant hemisphere exhibits a much higher rate of EE genesis than the nondominant hemisphere after the callosotomy. This result is regarded in contrast to the Pre-Cut experiments in which bilateral events were impossible, and no bilateral EE-dependent plasticity can take place; the ratio of EEs generated in the light and dark hemispheres was much smaller than the ratio of EEs generated post-callosotomy in the Mid-Cut experiments. While influenced by heat, the intrinsic qualities of interhemispheric connectivity are what lead to the increasing differences between hemispheric oscillations.

Although Pre-Cut experiments involving direct heat (Δ3°C) increases were not performed, I conjecture that there would have been a much larger difference in hemispheric rates of EE generation, even without the hour-long establishment of directionality. This supposition is derived from the comparisons between 27°C and 34°C experiments that demonstrated that large amounts of heat alone, when applied to both hemispheres, can increase epileptic activity, and that lateralized heat application (Δ3°C) can lead to 100% bias (10/10 warmed side leads). This strong bias found in Heated-Wire experiments is expected to be due to an increased rate of EE generation and not increased connectivity, as FracUni was unaffected by changes in bath temperatures.
**Standard Directionality is unaffected by GABA\textsubscript{B} antagonism.**

With the addition of CGP, we were able to study details that escaped us when only bicuculline was used. Our primary hypothesis, that the establishment of directionality relied upon GABA\textsubscript{B}-dependent feed-forward inhibition, was demonstrably unsupported. Regardless of the GABA\textsubscript{B} antagonism, strong directionality was still established. CGP application was insignificantly correlated with a reduction of LRD ($p = 0.14$). Although effect of light on the LRD of Bic was insignificantly different from the null-hypothesis (Sign-Rank $p = 0.056$ $n = 14$), I think that had there been as many trials as the identical test at 27°C ($n = 24$), the null hypothesis would have been rejected. Should this have been the case, it would mean that the application of CGP (Sign-Rank $p = 0.53$) greatly reduces the ability of light to affect which hemisphere gains dominance. As this is so far just a hypothesis, more studies examining the effect of CGP on BRD must be undertaken.

The difference between the SD measure in both Bic and CGP/Bic trial was insignificant ($p = 0.50$), meaning that regardless of what led to the hemisphere gaining dominance in the first place, once the dominance was established, the relative ability between dominant and non-dominant hemispheres to initiate and send EEs across the callosum was essentially identical. That is, GABA\textsubscript{B} activity, or lack thereof, does not affect the relative dominance once a dominant and nondominant hemisphere have been established.
While we did not perform tests specifically examining monosynaptic transcallosal efficacy, we believe that examining certain extracellular results will give useful insight about the relative efficacy of transcallosal projections. FracUni can be conceived as one outcome of transcallosal efficacy because if there is a low cross-callosal efficacy, there is a higher chance that EEs will fail to propagate across the callosum, resulting in a heightened FracUni. We found that there was a significant decrease in FracUni in CGP/Bic trials compared to Bic trials (Rank-Sum Test $p = 0.048$). This finding supports the hypothesis that additional disinhibition due to the second toxin results in less inhibitory restraint leading to a reduction in the ability to contain seizures within individual hemispheres.

IHL is another measurement that relates to the efficacy of cross-callosal connectivity. There was a significant drop in IHL from 71.5ms to 34.0ms when CGP was applied alongside bicuculline ($p = 0.003$). This latency is most likely dependent not only on the callosal connections, but also on countless synapses in both hemispheres. IHL was hypothesized to decrease due to the increased disinhibition because disinhibition results in more excitable neurons and a faster recruitment (Schevon et al., 2012). To narrow down where this latency develops, we could utilize protocol similar to Schevon et al. (2012) in which the ictal penumbra was well localized by micro-electrode arrays and $\text{Ca}^{2+}$ imaging. Would the direct targets of callosal axons elicit a slower response in normal (non disinhibited) situations? Or is this reduced velocity not noticeable until substantial amounts of neurons are in play?
Of course, propagation speed down the callosal axons themselves would not be expected slow due to GABAergic inhibition because axonal propagation is not affected by synaptic inhibition.

*Lower IHLs and lower FracUni are observed in dominant hemispheres.*

Fig. 30 represents the distance of each pair-wise measure from the unity lines of fig. 28, 29 (distance = √((x₂-x₁)²+(y₂-y₁)²) ) by plotting the distances of mono-hemispheric IHLs on the y-axis and FracUnis on the x-axis for each slice. Because of this figure we have begun to isolate broad rules that define what types of traits dominant hemisphere can exhibit. A theoretically maximally dominant hemisphere would have both high efficacy (low domIHL) and high oscillatory rate (high donUni) relative to the non-dominant hemisphere. However there is something slightly paradoxical about testing this hypothesis with these metrics because a maximally high efficacy would imply that there should be no unilateral events; albeit, this is why the Pre-Cut and Mid-Cut experiments were necessary in measuring true hemispheric oscillatory rates.

We noticed that there were no slices in which the dominant hemisphere has both a lower domUni and greater domIHL than the non-dominant hemisphere (fig. 30). This would represent a hemisphere with relative low efficacy and slow oscillations, and it appears that it is impossible to establish dominance with this profile. There is no a priori reason why these attributes must not exist in the dominant hemisphere, and it is very interesting to us that this profile was never
observed. Some slices have relatively large domIHL (read: low efficacy), but compensate with a relatively large domUni (read: fast oscillation). Similarly, some slices present a relatively low domUni, but compensate with a relatively short domIHL. At first glance it would seem that the top-right quadrant would be filled with points because this represents simultaneous high efficacy, high-rate oscillation slices. However, this quadrant might be relatively empty because slices with simultaneously maximal efficacy and oscillation rate have a low chance of producing unilateral events due to their high efficacy. It follows that a slice with maximal dominance is expected to reside along the y-axis, and above the x-axis.

Figure 30: No Slice Contained a Dominant Hemisphere that Presented both a Lower Fraction Unilateral and Larger Average IHL than The Non-Dominant Hemisphere.

We hypothesize that no data points exist in the bottom left quadrant because simultaneous low efficacy and low oscillation prohibit a hemisphere from obtaining dominance. Low efficacy can be compensated for by high rate of oscillation, while low rates of oscillation can be compensated for by high efficacy. Simultaneously high efficacy and high rate of oscillation slices are underrepresented in the top-right quadrant of this graph as an artifact of measuring oscillation via monohemispheric unilateral rates.
Intracellular hyperpolarizations and burst activity demand further experimentation.

Intracellular patch-clamp recordings were performed in layer 5-6 pyramidal cells (Fig. 31). These recordings were performed in V-clamp with $V_m = -55\text{mV}$. Early events were analyzed in order to test whether or not GABA$_B$ currents were present during the critical phase (first 10 minutes of seizure activity) in the determination of the dominance relationship, and if so, what their temporal relationship to the massive depolarization found during EEs was. In particular, we were interested to determine if we could replicate the anticipatory and delayed contralateral IPSCs found in Walker et al., 2012 (Fig. 1, 2) in a bath application of Bic instead of a local injection. While there were not enough successful intracellular recordings to make useful conclusions, a few events that were recorded present tantalizing results that call for further examination of intracellular dynamics during EE activity. EEs elicited such strong excitatory activity in the patched cells that the voltage clamp was broken and action currents were recorded (Fig. 32). In the CGP/Bic trials, extracellularly recognized EEs were accompanied by bursts of action currents that were seemingly distinct from the intracellular responses recorded in Bic trails. The number of action currents and the length of depolarization/burst were much longer in CGP/Bic trials than in the Bic trials. A few intracellular traces are presented below, but broad conclusions will not be drawn from them because the number of useable recordings was too low.
Figure 31 Biocytin-Stained Neuron in L5/6
This L5/6 neuron stained in biocytin is representative of the location of intracellular recordings in found in this paper. The central fissure can be seen bordering the right side of the image, and the corpus callosum can be seen bordering bottom of the image.
**Fig 32: CGP/Bic Trials Appear to Present an Extended Depolarization Associated with Contralateral Epileptiform Events.**

Red = CGP/Bic; Blue = Bic: In the few traces we collected, it appears that both the number of EEs and the length of the EE-related depolarization are larger in CGP/Bic than in Bic. Arrows indicate the initiation of the EE, as observed in field recording.

When the initial events were averaged together, there appears to be evidence of less inhibitory restraint in CGP/Bic trials, which can be seen in the upward deflection in the opposite-side Bic trace when compared to CGP/Bic and same-side Bic trace. “Opposite side” is meant to denote that the EE was initiated on the opposite hemisphere to the intracellular electrode, while “same side” means that the EE was initiated on the same hemisphere. The variance of magnitude of events was large, and the number of event was small (n < 4). Therefore, the average waves are not necessarily reliable insofar as they may not represent reliable descriptions of a broadly applicable, stereotyped intracellular response to epileptic activity. To clarify
the presence of inhibitory activity preceding contralateral EE generation, future experiments would benefit from inhibition of v-gated Na⁺ channels so that IPSCs and EPSCs are not hidden by massive action currents.

Figure 33: Contralateral Pyramidal Cells May Experience GABA₉ Dependent Inhibitory Activity That was Not Observed in Ipsilateral Bic, or Contralateral CGP/Bic Trials.

Preliminary data regarding bath-application evidence of inhibitory barrage shows that there may be a delayed inhibitory signal present in contralateral Bic (grey trace) hemispheres that is occluded when CGP is applied. The arrow represents the initiation of the EEs, as observed in the field recording.
**Conclusion**

Under bicuculline treatment, intact-callosum cortical slices will generate a dominant/non-dominant relationship between the hemispheres regardless of external factors. The chances that either hemisphere will become dominant are essentially identical, as described in Vertical Center-Light experiments; the “rules of the game” necessitate a “winner” and even though the differences in oscillatory rate, efficacy etc. between the hemispheres are miniscule, a dominant hemisphere will develop. When cooled to 27°C, slices produce significantly less EEs with greater amplitudes, but all other measures were insignificantly different from slices in 34°C. Interestingly, introducing very small asymmetrical stimulus (lateralized 0.1°C increase) will create predictable outcomes and large-scale dominance biases as seen in the Vertical Lateralized-Light experiments. It is important to note that a 0.1°C increase in temperature has no significant observed effect on local oscillatory frequency – as observed in Pre-Cut experiments – underscoring the necessity of functional connectivity for the dominant/non-dominant relationship to develop, and thus the disparity of oscillatory rates – found in Mid-Cut experiments – depend on an intact callosum. These hemispheric biases are hypothesized to be dependent on long-term feed-forward inhibition, as it was found that the non-dominant hemisphere had a significantly reduced oscillatory frequency when compared to the dominant hemisphere after an hour of Bic treatment (Mid-Cut). While the antagonism of GABA$_B$ had no effect on directionality, it did significantly increase the velocity of
EEs, further substantiating the role that GABA$_B$ plays in the inhibitory restraint known to exist in cortex during seizure propagation (Schvon et al., 2012). The findings imply that GABA$_B$ may be involved in the early stages of the development of dominance although the finding did not reach significance. Strangely, GABA$_B$ antagonism reduced the overall number of EEs. Inhibitory restraint maintains a barrier around a seizure and slows the rate at which cortex is recruited by a seizure; how the increased disruption of this restraint leads to fewer EEs is unknown and requires further experimentation to clarify this seemingly contradictory bimodal effect of GABA.

Finally, through the analysis of mono-hemispheric IHL and unilateral events, we have been able to define “rules” that describe what types of attributes are present in dominant hemispheres. Broadly, hemispheres with higher efficacies and quicker oscillations are more likely to become dominant, and not a single slice existed in which the dominant hemisphere had simultaneously larger monohemispheric IHLs and less unilateral events. By understanding the “rules” by which hemispheric dominance, seizure foci, and ictal pathways form, we will be able to make more accurate predictions about potential ways of disrupting these processes, and perhaps completely occlude what allows these aberrant circuits to develop in the first place.
Appendix 1:
When segregated by either light placement or dominance, there was no significant difference between the Amplitude of events recorded in either Bic or CGP/Bic.
References:


Guerrini, R., & Genton, P. (2004). Epileptic Syndromes and Visually Induced


