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Potential Treatments for Malformation Associated Epilepsy

Olivia M. Bowles
Virginia Commonwealth University

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POTENTIAL TREATMENTS FOR MALFORMATION ASSOCIATED EPILEPSY

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by
Olivia M. Bowles
B.S. Loyola University Maryland, 2014

Director: Kimberle Jacobs, Ph.D.
Associate Professor
Department of Anatomy and Neurobiology

Virginia Commonwealth University
Richmond, Virginia
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"No hi ha dolor, no hi ha guany"

Table of Contents

List of Figures

List of Abbreviations and Definitions

°C -------- degrees Celsius

aCSF ---- artificial cerebrospinal fluid

AED ----- antiepileptic drug(s)

AMPA -- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, a subtype of ionic glutamate receptor

AP-------- action potential

APV------ D(-)-2-Amino-5-phosphonopentanoic acid, an antagonist for the NMDA subtype of ionic glutamate receptors

bLED ---- blue LED

CNS ----- central nervous system

ChR ----- Channelrhodopsin

Cre ------ Cre recombinase (enzyme)

DIC ------ Differential interference contrast (optical microscopy illumination technique)

DNQX -- 6,7-Dinitroquinoxaline-2,3-dione, an AMPA receptor antagonist

EPSC ---- excitatory postsynaptic current

FL -------- freeze-lesion

FS -------- fast spiking

GABA -- γ-aminobutyric acid

GBP------ Gabapentin

GPCR---- G-Protein Coupled Receptor

IPSC ----- inhibitory postsynaptic current

LTS ----- low threshold spiking

mGluR -- metabotropic glutamate receptor

mGlu5 -- metabotropic glutamate receptor (homomeric) consisting of only subunit 5

mGlu1 -- metabotropic glutamate receptor (homomeric) consisting of only subunit 1

mM ------ millimolar

mOsm --- milliosmoles

MTEP --- 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine, an mGluR5 selective antagonist

NMDA -- N-methyl-D-aspartate, one subtype of ionic glutamate receptor

P --------- postnatal day

PMR ----- paramicrogyral region

PV ------- parvalbumin

SS -------- somatostatin

µm ------- micrometer

µM ------- micromolar

VIP------- vasoactive intestinal peptide

ABSTRACT

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By: Olivia M. Bowles

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Anatomy and Neurobiology at Virginia Commonwealth University

Virginia Commonwealth University, 2016.

Advisor: Kimberle M Jacobs, Ph.D.
Associate Professor, Department of Anatomy and Neurobiology

Epilepsy has been previously attributed to either increased excitation or decreased inhibition. With this closed frame of mind, modern medicine has been unable to develop a permanent treatment against the mechanisms of epilepsy. In order to treat patients with intractable seizures, especially those caused by developmental malformations, it is essential to understand the entirety of mechanisms that could possibly play a role in the abnormal cortical function.

One such developmental malformation is known as polymicrogyria. Epileptogenesis occurs in an area laterally adjacent to this malformation known as the paramicrogyral region (PMR). Past studies have narrowed down the potential cause of this increased network excitation to a certain type of inhibitory interneuron, the somatostatin (SS) interneuron. Additionally, previous studies have shown an increase in the mGlu5 receptor on this interneurons in the PMR region only and not in control tissue, meaning that targeting these receptors as treatment will not affect normal functioning tissue. These results lead to our hypothesis: blockade of the mGluRs will decrease the

activity of SS interneurons and thereby prevent the generation of epileptiform activity and increased SS output in malformed cortex.

Utilizing the freeze-lesion model for microgyria in transgenic mice expressing Channelrhodopsin optogenetic channels in SS interneurons, we assessed the contribution of these SS interneurons in four different animal groups: control or PMR treated with either Gabapentin, a current AED (antiepileptic drug), or MTEP, an mGlu5 receptor antagonist. We tested the effects of these two drugs on SS interneuron output to determine whether they decrease the over activation in the PMR that has been previously studied. The following study revealed no correlation between Gabapentin-treated animals and a decrease in epileptiform activity. Additionally, no significant difference was seen between the MTEP-treated groups in the protocols that were measured.

Chapter 1

Introduction to Epilepsy, Interneurons, Optogenetics, mGluRs, and Possible Treatments

Polymicrogyria (PMG) causes intractable epilepsy, or epilepsy not permanently treatable with medication. In order to properly treat intractable seizures, one must understand the underlying mechanisms to target. My project focuses on a specific receptor, the mGlu5 receptor, as the key in reducing the epileptiform activity seen in our freeze-lesion mice that mimic the hyperexcitablilty and histopathology of PMG. This project will aid in the search for a specific treatment for intractable seizures as opposed to patients having to result to surgery or a continuous rotating drug regimen.

1.1 Epilepsy – general characteristics

Epilepsy is a very common disorder that affects around 50 million people worldwide or 1-2% of the world's population (WHO, Epilepsy et al. 2005, Varvel, Jiang et al. 2014) It has known to be recorded as "the falling disease" as early as 1060 BC (Varvel, Jiang et al. 2014). In addition, seizures were thought to be the work of demons rather than a neurological condition (Varvel, Jiang et al. 2014). In 400 BC, Hippocrates was one of the first people to characterize epilepsy as a disorder of the brain, but he was not taken seriously. It was not until the early 1900s the first antiepileptic drug (AED) was introduced (Varvel, Jiang et al. 2014). Today, it is characterized as a chronic neurological disorder with characteristic seizures, or abnormal electrical discharges, that can cause changes in emotional and motor activity (Fisher and Saul 1997, Fisher, Arzimanoglou et al. 2014). Epilepsy can also cause cellular and molecular changes in between seizure activity (Elger 2005). A seizure is

characterized as a synchronous over-activation of one or many synaptic pathways (Varvel, Jiang et al. 2014). A consistently lowered threshold of one of these pathways may produce epilepsy (Varvel, Jiang et al. 2014). Usually, this uncontrolled electrical activity is the cause of too little inhibition or an increase in excitation within the cortical network (Davies 1995).

As discussed by Fisher et al (2014), these seizures either involve specific systems of the brain, as in the case of partial seizures, or they can be in a restricted area and eventually spread, leading to the involvement of multiple cortical and subcortical subunits. An epileptic seizure must have the presence or signs of symptoms related to excess neuronal activity, or synchronous activity in the brain as demonstrated on electroencephalogram or EEG (Fisher, Arzimanoglou et al. 2014).

Treatment with AEDs or surgery is at least partially effective in approximately 2/3 of epilepsy patients (Fisher and Saul 1997). When AEDs are ineffective, not possible, or not desired, surgery is the last resort. For AEDs with known mechanisms, they target ion channels and postsynaptic receptors to enhance the brain's ability to limit the spread of seizures (Alexander & Godwin, 2006; Fisher & Saul, 1997). AEDs have three different modes of action, they either facilitate GABA transmission through multiple mechanisms, they can block voltage gated ion channels which reduces excitatory transmission, or they have an unknown/other mode of action (Davies 1995). All AEDs treat epilepsy after it is diagnosed, but there are no successful strategies for prevention of epilepsy for those at risk (Varvel, Jiang et al. 2014).

In more than 50% of surgery cases, however, seizures are not eliminated completely or they are not even significantly reduced (Palmini, Gambardella et al.

1994, Olivier, Andermann et al. 1996). If the seizure has no focal region, or a specific area where the seizure originates, then surgery is not an option (Fisher and Saul 1997). Ben-Ari stated that understanding and treating seizures caused by developmental malformations requires knowledge of cortical networks and cellular mechanisms in order to determine the mechanisms that are absent or enhanced. With this knowledge, it is possible to understand the underlying mechanisms of the hyperexcitability that are seen (Ben Ari 2006).

Even though some patients are treated with AEDs, 40% of patients have seizures that are drug resistant or intractable (Fisher and Saul 1997, Alexander and Godwin 2006). The reasons for this intractability are not fully known, but approximately 25% of the intractable seizures are caused by malformations of cortical development (MCDs), according to Leventer (2008). Classification of MCDs is based on the developmental steps of cell proliferation, neuronal migration, and cortical organization (Barkovich, Guerrini et al. 2012). These classifications span three different groups. Group I constitutes malformations secondary to abnormal neuronal and glial proliferation or apoptosis. Group II includes malformations that are secondary to abnormal neuronal migration. Finally, Group III contains all the malformations secondary to abnormal migrational and post migrational development, as the process of cortical organization begins before the termination of neuronal migration. Lastly, there are still MCDs that are not classified into groups (Kuzniecky 2015). Of the 25% of intractable seizures attributed to MCDs, 50% of those are diagnosed in children (Leventer, Guerrini et al. 2008). In addition to the high prevalence in children, as a whole, approximately 75% of patients diagnosed with a

MCD will have epilepsy (Leventer, Guerrini et al. 2008). Thus there is a strong connection between these errors in development and cortical hyperexcitability.

Epilepsy associated with MCDs comes about as a result of the abnormal presence or absence of neurons or by the faulty positioning of cortical neurons (Leventer, Guerrini et al. 2008, Varvel, Jiang et al. 2014). This misplacement or absence can result in an imbalance between the excitatory and inhibitory neuronal systems which would normally control this epileptiform activity and prevent these spontaneous events from occurring (Leventer, Guerrini et al. 2008). In addition, malformations associated with errors in development during the formation of the cortical plate have an effect on maturation (Squier and Jansen 2010). Developmental malformations are therefore the focus of our epilepsy studies because the biology underlying the transition of a normal brain to a brain with epilepsy must differ from the biology driving seizures in the epileptic brain (Varvel, Jiang et al. 2014).

1.2 Malformations – Polymicrogyria

Developmental malformations are more common than previously realized in the past, due to the recent advancements in technology such as magnetic resonance imaging or MRI. In fact some malformations, particularly PMG, in some cases, require the highest current resolution of MRI (at 7T) in order to be identified (De Ciantis, Barkovich et al. 2015).

One such malformation is known as PMG. PMG is a developmental malformation that is present when there are multiple small convolutions on the surface of the brain (Kuzniecky 2015). PMG is often associated with type II lissencephaly, or "smooth brain" where there is an absence of normal convolutions (Stouffer, Golden et

al. 2015). The distribution of PMG greatly varies from diffuse, symmetrical, bilateral, asymmetrical or unilateral (Kuzniecky 2015). With the small convolutions come underlying laminar abnormalities. Takano discusses PMG is also one of the most common MCDs and has different types, unlayered and 4-layered. We focus on the 4-layered type. In four-layered PMG, the cortex consists of a molecular layer (1st layer) and two neuronal layers underneath (Takano 2011). In between the two neuronal layers is an intermediate layer that contains few cells and many fibers (Takano 2011).

It has been previously reported that approximately 85% of patients diagnosed with polymicrogyria have seizure disorders (Leventer, Guerrini et al. 2008). As mentioned in Leventer et al. (2008), PMG is also a very common additional component with other disorders such as chromosomal deletion syndromes, metabolic disorders, and multiple congenital anomaly syndromes. Additionally, PMG , unlike other MCDs has non-genetic causes that are recognized (Stouffer, Golden et al. 2015). The incidence of epilepsy with PMG is very high (Kuzniecky, Andermann et al. 1993); the susceptibility of seizures most often peaks during brain growth and synaptogenesis during childhood in humans, suggesting that the immature brain is the focus for the initiation of the epilepsy (Rakhade and Jensen 2009, Takano 2011). The seizures associated with PMG tend to be intractable in about 50% of the cases of patients, and also tend to show up in childhood (Leventer, Guerrini et al. 2008). This is important because seizures can be caused by different factors depending on the age of a person (Fisher and Saul 1997). The young brain is different from the adult brain in many ways; the young brain is not a smaller adult brain. We cannot get the

information we need from adult brains in order to elucidate the underlying mechanisms of developmental epilepsy (Ben Ari 2006).

In our lab, we use the freeze-lesion (FL) model due to its ability to accurately replicate the histopathology and hyperexcitability of the microgyria associated with seizures. We specifically look for mechanisms in development because we want to ultimately identify the one(s) that produce the onset of this hyperexcitability.

Freeze lesion model of microgyria

PMG has been modeled by a number of insults to the neonatal cortex, including direct ibotenic acid injection, stabbing punctures, and most commonly, a neonatal transcranial FL. In the normal developing brain, the cortical layers form in an inside-out progression, with the bottom layers forming first and the upper layers following. The model used for experiments described in this dissertation is the neonatal FL, performed on postnatal day (P) 1 in mouse. Lesions are done on P1, as opposed to P0, because lesions at P1 are more likely to cause the chronic hyperexcitability (Jacobs, Hwang et al. 1999). The lesion produces a focal loss of neurons within the cortical plate at the time of the lesion. At P1 in mouse, this is some of layer IV, and all of layers V and VI. The superficial layer neurons will migrate into the cortical plate on subsequent days. For this model, mice are anesthetized and a frozen probe is placed onto the skull overlying somatosensory cortex for a few seconds, creating a focal loss of the neurons present in the cortical plate at that developmental age (Jacobs and Prince 2005) (**Figure A**). Thus, this FL process mimics a fetal stroke or direct injury resulting in focal loss of neurons. In addition, this FL model shows epileptiform activity consistently and is therefore a useful model for

study of the underlying mechanisms of epileptogenesis associated with polymicrogyria (Jacobs, Hwang et al. 1999).

It has been shown that these FL animals have intrinsic hyperexcitability in the area adjacent to the lesion. The cortex adjacent to the microgyrus, from which the epileptiform activity is most easily evoked is known as the paramicrogyral region or PMR. The cellular changes that cause this increased excitation seen in this model are unknown, however, the knowledge of these mechanisms will help provide special

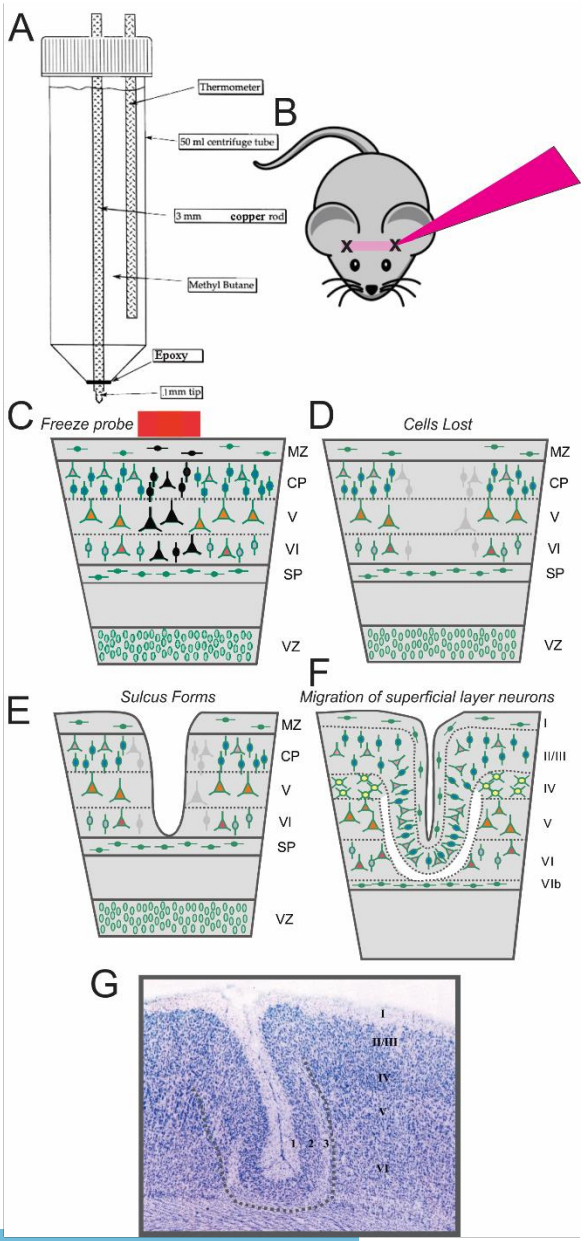

Figure A. *Freeze lesion model of microgyria. A) Freeze probe diagram, modified from Humphreys, Rosen et al. (1991). Probe was placed in dry ice to cool methyl butane. B) Although previous studies in rat utilized a unilateral lesion, for these studies bilateral lesions over somatosensory cortex were made in mice. C) Probe placement results in focal death of cells present in the cortical plate (deep layers). D) Over the next 5-7 days normal processes remove these cells. E) As a result of lost cells a sulcus forms in the normally lissencephalic rodent cortex. F) Normal migration of superficial layer cells into the cortex continues after the lesion. G) Example of a Nissl-stained coronal section through somatosensory cortex containing the induced microgyrus with abnormal lamination and the abrupt transition (gray dashed outline) to normally laminated 6-layered cortex. This example is from a rat. Figures C-G by KM Jacobs.*

targets for therapeutic treatments (Andresen, Hampton et al. 2014). Though the mechanisms are unknown, field epileptiform activity has been evoked in the PMR, laterally adjacent to this malformation. When connections from the PMR region are severed from the microgyrus, the hyperexcitability seen in the PMR region persists (Jacobs and Prince 2005). Afferents avoid the microgyrus and instead, relocate to the PMR (Rosen, Burstein et al. 2000) **(Figure B)**; this is what leads to increased excitatory connectivity in this area (Jacobs, Kharazia et al. 1999, Jacobs and Prince 2005). Increased excitatory afferents must be selective to certain neurons or other components of the circuit in order to overcome the inhibition as with increased excitation among pyramidal neurons (Jacobs, Hwang et al. 1999). Is it just excitatory afferents causing this hyperexcitability?

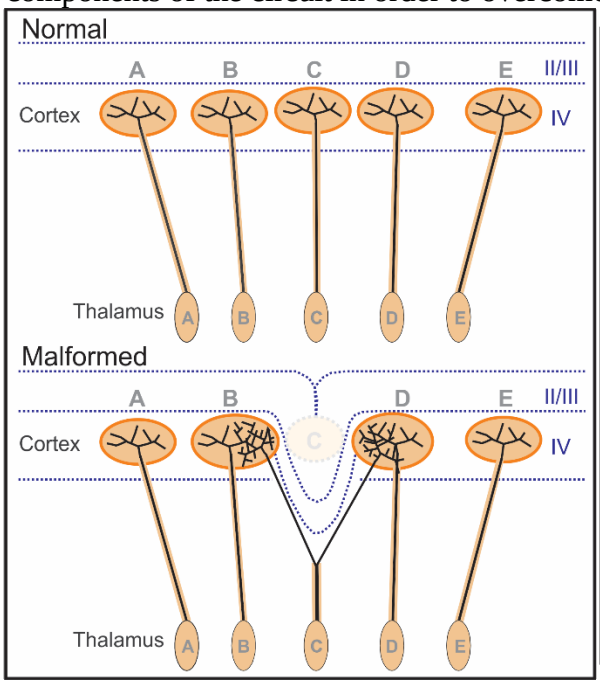

Figure B. *Thalamocortical excitatory afferents avoid the malformed region and project instead to the PMR. In the cartoon at the top, the normal focal projections can been seen. In the lower cartoon depicting the malformed cortex, the thalamic afferents that should have gone to the malformed area instead find the normal layer IV in the adjacent (abnormal) region. This creates additional excitatory afferents in this PMR region. This has been demonstrated both anatomically and functionally. Figure by KM Jacobs.*

Network hyperexcitability was originally identified with field potential recordings in ex vivo slices. Nemes, et al. discussed how pro-epileptic lesions are a predisposing factor for the development of chronic epilepsy if triggered by an event such as ischemia or a seizure. The presence of hyperexcitability increases a patient's

chance of developing epilepsy. This is why stopping the hyperexcitability before seizures occur is key. While the network epileptiform activity begins abruptly and is severe at P12, the functional increase in excitatory activity to layer V pyramidal neurons begins on P9. Thus some other mechanism likely contributes to the initiation of the epileptiform activity. This led us to look at inhibitory interneurons as the cause.

In the PMR region, there are more glutamatergic synapses to layer V pyramidal neurons and interneurons (Jacobs & Prince, 2005). This increased glutamatergic signaling onto certain subtypes of interneurons is important and could affect the excitability of the PMR region (Takano 2011). In the PMR, others from our lab have previously shown that layer V pyramidal neurons receive an increase in these excitatory connections. The increase in excitatory synapses to excitatory neurons occurs at P10, yet we do not see epileptiform field potentials until P12 (**Figure C**). This suggests that there are likely other abnormalities involved in initiating the epileptiform activity.

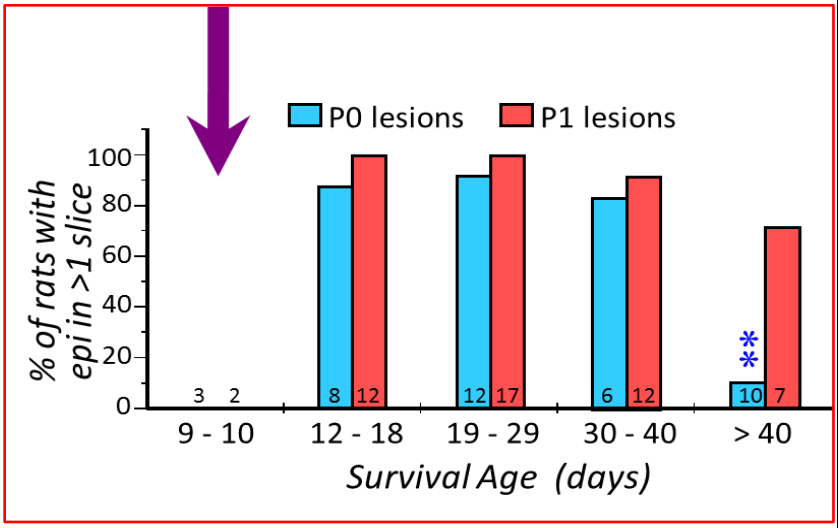

Figure C. *Timing of onset of epileptiform activity. Activity was recorded as field potentials from ex vivo rat slices. Survival age is postnatal day. Note that before P12 (purple arrow) only normal short latency events were evoked. Figure modified (colorized) from Jacobs, Hwang et al. (1999).*

Previous recordings from inhibitory cells showed that inhibitory cells of the PMR receive nearly three times the excitation that controls have. What is the cause of this excitation? The early susceptibility seen that does not coincide with the increased excitation onto pyramidal neurons may be caused by inhibitory neuron changes or changes with their connectivity (George and Jacobs 2006, George and Jacobs 2011, Bell and Jacobs 2014). In addition, since the onset is delayed, this is an important period of latency to study in order to determine the mechanisms of epilepsy, especially since seizures alter many processes such as physiological processes that can worsen hyperexcitability (Bell and Jacobs 2014).

There are a few known mechanisms that can contribute to epileptogenesis and increased hyperexcitability in the FL model. There are thalamocortical afferents that should have projected to malformed region that instead project to the PMR that could contribute but not initiate the hyperexcitability. In addition, there are increased AMPA and NMDA receptors, and decreased GABA receptors. There is increased excitatory input to layer V pyramidal neurons which is an important contributor. Lastly, there are GABAergic neurons decreased in number. This could be an important contributor to network hyperexcitability. After the FL, neurons migrate from the cortical plate and form a bridge-like structure. This structure maintains an early Cl- homeostatic environment which causes GABA to be depolarizing, promoting the formation of this disorganized network and also promoting the abnormal migration of neurons and could affect the hyperexcitability seen in the PMR (Shimizu-Okabe et al., 2007). This depolarizing effect of GABA is essential in order for neurons and interneurons to

migrate (Wang, Kumada et al. 2012). The effect of GABA can affect not only effect excitatory neurons, but can also effect inhibitory neurons.

1.3 Inhibitory interneuron subtypes

In the cortex, 70-80% of the neocortical neurons are excitatory pyramidal cells, the other 20-30% are inhibitory interneurons (White 1989). There are many types of GABAergic interneurons that are distinguishable by their morphology/axonal arborization because certain interneurons are specialized at targeting different domains of neurons, different cortical columns, or even different layers of a column (Markram, Toledo-Rodriguez et al. 2004). Inhibitory neurons have certain characteristics that make them distinguishable from pyramidal neurons such as size, soma shape, action potential firing pattern, and laminar layer location. Interneurons can target other interneurons with inhibitory synapses onto other cells, usually occurring at the dendrites, just as for pyramidal neurons.

Inhibitory interneurons have many functions, they must balance the excitation on different regions of a neuron, and only about 16% of pyramidal neuron synapses are from inhibitory interneurons (Markram, Toledo-Rodriguez et al. 2004). At certain inhibitory synapses there is less depression compared to excitatory synapses, allowing some interneurons to fire at higher frequencies (Galarreta and Hestrin 1998, Wang, Gupta et al. 2002). Inhibitory interneurons must be activated at the right moments, they must constantly be in balance, or else the network can become faulty. Any interruption of the normal balance can lead to disruptions in the normal functioning of the network.

The balance between excitation and inhibition is important when discussing inhibitory cells because in the end, these interneurons control the synchronization at certain frequencies (Konig, Engel et al. 1996, Markram, Toledo-Rodriguez et al. 2004). The diversity seen in inhibitory interneurons is important for the maintenance of the brain's connections. This regulation is important to guarantee the proper processing of stimuli in a given brain region in an unpredictable and ever changing environment (Markram, Toledo-Rodriguez et al. 2004).

Inhibitory interneurons can be differentiated in many different ways, including staining methods and intrinsic firing properties (Connors and Gutnick 1990, Kawaguchi and Kubota 1995, Kawaguchi and Kubota 1996). Among peptides in the neocortex, vasoactive intestinal peptide (VIP) and somatostatin (SS) staining are expressed in different GABAergic cells (Demeulemeester, Vandesande et al. 1988, Rogers 1992, Kubota, Hattori et al. 1994, Kawaguchi and Kubota 1996). Parvalbumin (PV) stained interneurons are immunoreactive for the calcium binding protein PV. These three subtypes are the prominent inhibitory interneurons. SS interneurons have different roles in the cortical circuit (Kawaguchi and Kubota 1996). SS are mostly low-threshold spiking (LTS) neurons and PV interneurons are mostly fast-spiking (FS) interneurons (Markram, Toledo-Rodriguez et al. 2004). FS interneurons have lower input resistances and tend to spread horizontally and control horizontal activity (Gonzales-Burgos, Krimer et al. 2005). SS interneurons are found in the hippocampus, all throughout the neocortex, and tend to be more modulatory. They form synapses on the distal dendrites of pyramidal cells and spread vertically rather than horizontally (Kawaguchi and Kubota 1996).

Rosen et al. (1998) demonstrated that the numbers of PV-expressing neurons are decreased focally in deep layers, but another study that counted all GABAergic neurons showed there was no difference. In normal cortex, PV interneurons are the stronger inhibitory cells (Hu, Gan et al. 2014), however, in the PMR, there are fewer PV interneurons, and they seem to no longer be the main inhibitory cells (George and Jacobs 2011). The Jacobs' lab has used stereology to count numbers of SS, PV and VIP neurons. Within and surrounding the malformation, PV neuronal counts are down while SS and in some cases VIP neuronal counts are increased. We have hypothesized that the PV neurons are more vulnerable to the hypoxic insult while the SS neurons are resilient. It may be that homeostatic attempts to maintain cortical inhibition allow PV neurons and/or synapses to be replaced by SS neurons and/or synapses. This could account for SS interneuron increased strength within the PMR. All of these data together suggest that the interneuron subtypes are differentially affected.

GABAergic interneurons contain many mGluRs that act on then by depolarizing them directly. The result of this is greater inhibition, wherever these synapses are made, which is on both excitatory and inhibitory interneurons (Zhou and Hablitz 1997). In addition, recent studies show that LTS interneurons, due to their electrically coupled networks, are suited to modulate cortical excitability (Gibson, Beierlein et al. 1999). LTS neurons in the PMR have been shown to have an increased maximum frequency when compared to LTS neurons in control tissue (George and Jacobs 2011). Additionally, a decreased firing frequency was seen in FS cells as compared to the LTS interneurons (George and Jacobs 2011). Since the interneuron subtypes are maintained in the PMR, this suggests that differentiation normally

proceeds once the lesion is induced. It could also mean that the mechanisms

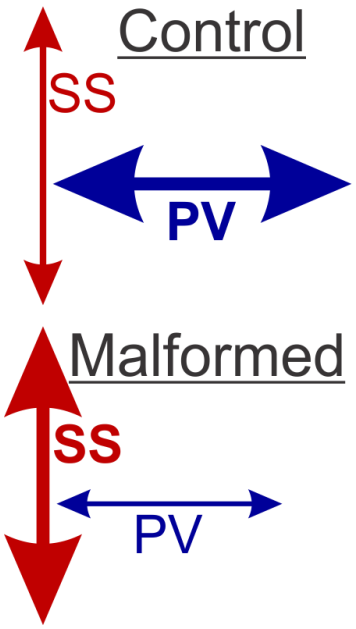

Figure D. *Changes in cortical inhibitory subtypes after malformation. In normal (control) cortex, the PV interneurons project horizontally and provide a powerful suppressant of horizontally (intercolumnar) excitatory activity. In contrast, the SS interneurons normally project intracolumnarly and provide weak or modulatory inhibition onto the dendrites of both excitatory and inhibitory neurons. In malformed cortex, specifically within the PMR, we hypothesize SS neurons are strengthened in function. This is supported by the increased excitatory synaptic activity they receive, the increased maximal firing and increased output (described below) determined optogenetically. This increased function of the inhibition may serve to both synchronize excitatory activity within a column and produce disinhibition due to the contacts onto PV interneurons. Reduced functioning of PV interneurons may then allow the spread of this synchronized excitatory activity across the cortex in the form of epileptiform activity.*

underlying cell type definition are complete in layer V prior to lesion time (George and Jacobs 2011). The interneuronal subtypes can be affected in different ways in both animal models and in human tissue so these interneurons are useful to study (DeFelipe, Garcia et al. 1993, Buckmaster and Dudek 1997, Rosen, Jacobs et al. 1998, Powell, Campbell et al. 2003, Trotter, Kapur et al. 2006). We are interested in these interneurons because we believe that these inhibitory cells are the cause of the epileptiform activity seen in our model. We have previously shown via whole cell patch clamp recordings that the SS interneurons within the PMR receive more excitatory synaptic input and fire action potentials at higher maximal rates compared to control SS interneurons. This increased strength of these intracolumnar interneurons may synchronize excitatory activity within a column. In addition, increased strength from SS to other interneurons may produce network disinhibition. Since these

interneurons project vertically, rather than horizontally like the PV interneurons, they synapse in multiple layers simultaneously. Since these vertical SS synapses are usually modulatory, they normally do not create robust synchrony. It has also been shown that in the presence of mGluRs, SS interneurons fire in an oscillatory manner and that activity can cause an increase in synchrony among the nearby pyramidal neurons (Connors and Gutnick 1990). It is the combination of these two normal processes (intracolumnar projection and synapses onto other inhibitory interneurons) with the strengthening of their function in the PMR that has led us to hypothesize that the PMR has an increase in intracolumnar synchrony. Overall, we hypothesize that PV interneurons that are normally strong become weakened in the PMR, while SS interneurons that are normally weak or modulatory become strengthened in the PMR **(Figure D).**

There are two ways SS interneurons may be producing network excitation with their inhibitory synapses. They synapse onto the PV interneurons, and since they are inhibitory cells, we hypothesize more strongly in the PMR, they inhibit these inhibitory cells, which could lead to excitation. Secondly, since SS interneurons project vertically, they cause the synchronous inhibition and firing of pyramidal neurons. Normally, the SS interneurons are not powerful, but if they become very powerful, they can act to synchronize all activity, providing a jump start to epileptiform activity. It is important to localize the cause of this increased excitation to be able to better target neuronal and receptor subtypes for drug development (Jacobs, Hwang et al. 1999). What about these SS interneurons makes them more powerful in

the PMR? While previous studies have looked at inputs to these neurons, this project looks at the output from these specific subtypes utilizing optogenetics.

1.4 Controlling inhibitory interneuron subtypes selectively

Optogenetics is optical methods combined with genetic methods in order to achieve gain or loss of function of certain events on specific cells in living tissue (Deisseroth 2011). Optogenetics allows for targetable control tools that do three things: deliver effector function, respond to light, and enable technology for: 1) targeting the control tools in cells of interest; 2) electrical recording of evoked activity or other analysis; and 3) delivering light into the tissue that is under investigation (Deisseroth 2011). Neurons can be specifically controlled when they express proteins that are sensitive to light (LaLumiere 2011). Fenno et al. (2011) mentions that with this technology, we have been able to selectively mark SS interneurons with Channelrhodopsin (ChR), enabling us to activate these specific interneurons with blue light. Type I rhodopsin combines light sensation and ion flux into one protein encoded by one gene (Fenno, Yizhar, & Deisseroth, 2011). Fenno et al. (2011) states that with light stimulation, the channel changes conformation and opens. In the absence of further light stimulation, it changes back to the closed conformation. Transgenic mice, as opposed to viral transfection of ChR, allow for greater control over transgene expression because of the use of large promoter fragments (Fenno, Yizhar, & Deisseroth, 2011). The optogenetic system dependent on Cre allows for a direct look at the neural activity of specific neuronal populations and their relationship with animal behavior (Fenno, Yizhar et al. 2011). In order to achieve the ChR expression on SS interneurons, we crossed Floxed stop codon on a ChR-2 expressing

gene in YFP reporter female mice with Cre recombinase in the 3' UTR of the somatostatin locus males. This allowed for the selective activation of the SS interneurons with blue light. We have recently shown with optogenetics that SS interneurons of the PMR produce more output than the same interneuron subtype in control (**Figure E**). We have also shown that epileptiform fields can be generated with light activation of SS interneurons alone (**Figure F**). This suggests that reducing the activity of SS interneurons may be an effective way to prevent epileptiform activity associated with microgyria. We can achieve this via control of metabotropic glutamate receptors. This is beneficial because the effectiveness of therapy is limited in about 30% of all epilepsy cases, and these mGluRs are very good targets (Loscher, Dekundy et al. 2006).

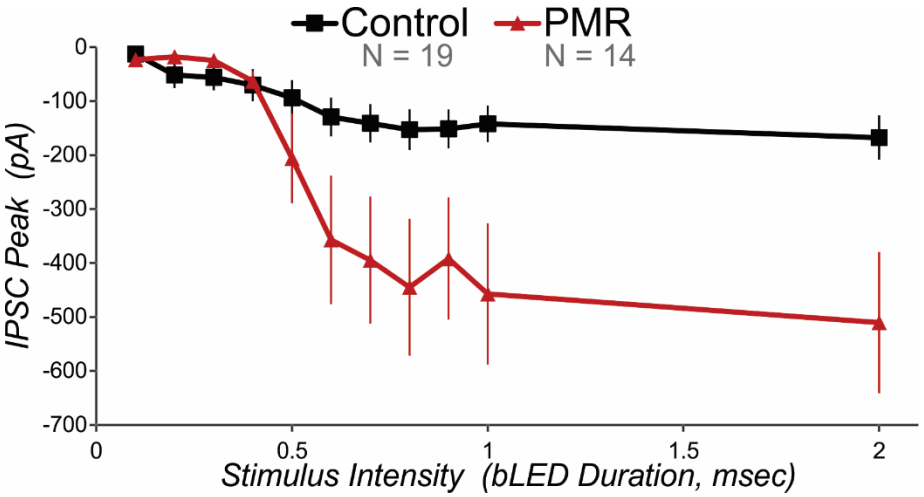

Figure E. *IPSC produced by selective, optogenetic activation of SS interneurons. After mating Cre-SS mice with floxed-ChR mice, ChR is present selectively in SS interneurons (although there may be ~10% error (Hu, Cavendish et al. 2013)). Whole cell patch clamp recordings were made in layer V pyramidal neurons. Blue light (bLED) was applied through the 60X objective above the recorded neuron to activate the ChR in SS interneurons. Data shown is from the work of Nicole Ekanem in the Jacobs lab. A preliminary form of this data was published in the Masters' thesis of Ekanem (2015). This data will serve as the untreated form to which the studies presented in the current Masters' thesis will be compared.*

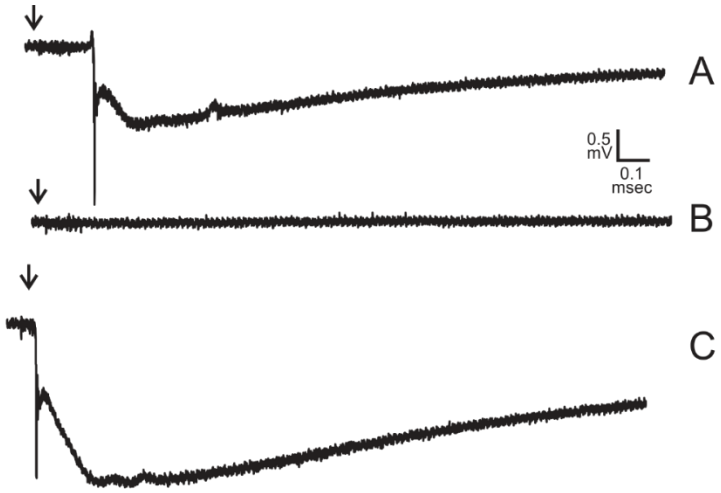

Figure F. *ChR-SS evoked epileptiform field potentials from ex vivo slices containing an induced microgyrus. At the arrow a 2 msec long pulse of blue light was applied through the 60X objective centered on layer V within the PMR (~0.25 mm adjacent to the sulcus) in order to activate the ChR selectively genetically inserted in SS interneurons. Field potential recordings were made within layer V in the center of the applied light. The aCSF bath contained a low level (0.02 mM) of the $GABA_A$ receptor antagonist, Gabazine, in order to increase network excitability. This epileptiform field was not obtained from control slices under the same conditions, suggesting that only within the malformed cortex can activation of inhibitory interneurons produce this network hyperexcitation. These data collected by Weston, Ekanem and Jacobs and originally published as part of Nicole Ekanem's Masters' thesis (2015).*

The mGluRs are members of the G-protein coupled receptor super family (GPCRs) (Jong, Sergin et al. 2014). GPCRs are the largest members of membrane proteins; they also mediate a variety of cellular processes (Rosenbaum, Rasmussen et al. 2009). They are characterized by having seven helical membrane spanning regions (Rosenbaum, Rasmussen et al. 2009) and can interact with many second messengers. They can also be hindered by many different proteins. On the cell surface, mGluRs have GPCR independent signaling through β-arrestin and GPCR dependent signaling (Jong et al., 2014). β-arrestin blocks the receptor/G-protein interaction. This is an adapter protein that targets GPCRs for clathrin mediated endocytosis (Luttrell and

Lefkowitz 2002). The three main functions of β-arrestin are to aid in GPCR coupling efficiency, to sequester GPCRs, and to downregulate/re-sensitize GPCRs (Luttrell and Lefkowitz 2002). It can also recruit signaling proteins to GPCRs that are agonist occupied (Luttrell and Lefkowitz 2002). The mGluRs can act through phospholipase C (PLC) or adenylate cyclase (AC) by coupling with GPCRs directly to ion channels or to second messenger cascades (Szydlowska, Kaminska et al. 2007).

There are 8 different groups of mGluRs that are classified into three groups based on homology, pharmacological profile, and coupling to intracellular pathways (Lujan, Shigemoto et al. 2005). The three 3 different subgroups of mGluRs are named after their agonists.

Group I mGluR modulates the inhibitory interneurons. Group I is made of homomeric receptors containing either subunit 1 or subunit 5 and are thus referred to as mGluR5 and mGluR1. Most agonists are not selective for mGluR1 or mGluR5, but there are very good antagonists that are selective. My thesis focuses on these Group I mGluRs, which are excitatory postsynaptic receptors (Jong, Sergin et al. 2014). Group I mGluRs are proconvulsive by increasing membrane excitability and are excitatory receptors that enhance neurotransmitter release, regulate inhibitory glutamate receptors (iGluR) responses, and control many depolarizing currents (Alexander & Godwin, 2006). Group I mGluRs are modulatory and act slowly via their G-protein and second messenger involvement (Alexander & Godwin, 2006). In addition, they are positioned in many areas that are only active under high neuronal activity conditions. The mGluR1 and mGluR5 null mice show no seizure behaviors (Alexander and Godwin 2006).

The mGluR5 are prominent in areas involved with emotion, motivation, learning, and memory, and also play a large role in many disorders and diseases, including epilepsy (Jong, Sergin et al. 2014). This receptor is a great target because it has been shown to be necessary for induction of epileptiform activity (Wong, Bianchi et al. 2005). As mentioned in Jong et al. (2014), it acts through Gq/11 and regulates cell function via transcriptional profile changes and modulating translation of dendritic mRNAs. It also has an orthosteric binding site on its cytosine rich domain and has allosteric binding sites that when bound by drug, decrease the activity of the main site or can have a neutral effect. Unlike mGluR1, mGluR5 does not experience ligand bias. Ligand bias refers to when a ligand keeps a unique conformation that triggers either a G-protein dependent or independent pathway. Intracellular signaling is present and is thought to be through the actions of β-arrestin. Intracellular signaling has been shown to be an evolutionarily conserved feature and has been shown in *C. elegans* and plants, suggesting that it must play some important role. Intracellular GPCRs can regulate many functions such as inflammatory responses, proliferation, and survival. For intracellular GPCRs, there are two uptake systems: through Na+ dependent excitatory AA (amino acid) transporters or via a cystine/glutamate exchanger (Jong, Sergin et al. 2014).

The mGluRs that are present in the postsynaptic membrane tend to mediate membrane properties via second messenger interactions, and when they are present on the presynaptic terminal, they aid in synaptic vesicle release (Alexander & Godwin, 2006). Targeting this mGluR5 receptor could control glutamatergic signaling due to its modulatory functions for ongoing activity without interfering with the functioning of

ionotropic glutamate receptors (iGluRs) (Alexander & Godwin, 2006). It is thought that Group I mGluRs are unattractive targets because mice with a null mutation for mGluR1 or mGluR5 showed a disruption in cerebellar motor function and long term potentiation (LTP) (Alexander and Godwin 2006). These observations, however, were observed in normal, non FL animals. Previous work in our lab has shown the mGluR5 is enhanced in its expression and in its activity in the PMR region in our FL model relative to its expression and function in control cortex (Figure 1.43A).

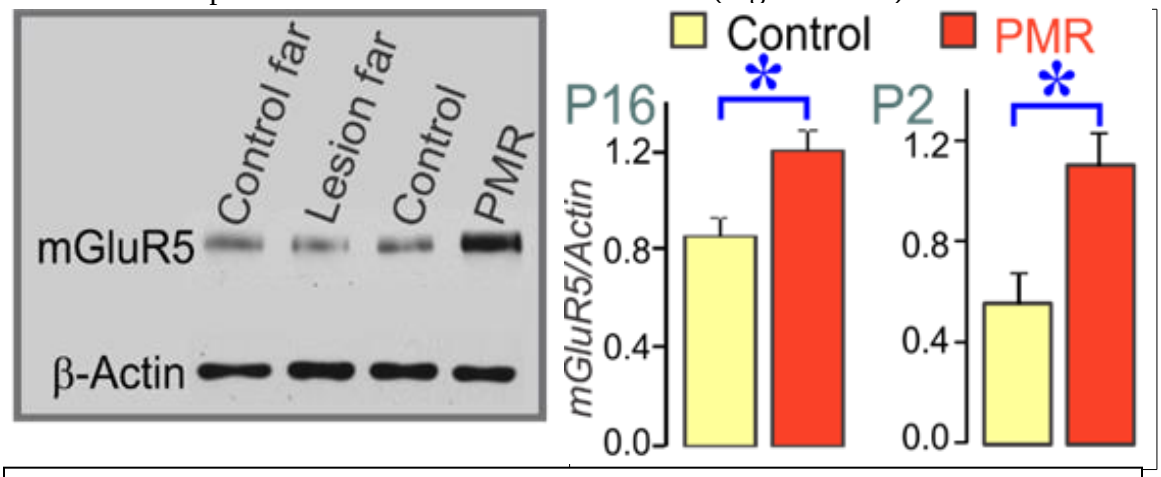

Figure G. *Expression of mGluR5 is increased within PMR compared to control cortex at both P16 and P2. Left panel: Example Western Blot data for mGluR5 (subsequently normalized to b-Actin) for the PMR (1 mm circle of tissue through cortex taken ~0.5 to 1.5 mm lateral to the sulcus, in homologous control cortex, at ~ 2.5 to 3.5 mm lateral to the sulcus (Lesion far) and in homologous control cortex. Right panel: Expression was quantified by digital measurement of the intensity from film exposed to the radioactive blots (NIH's Image program).* * = *t-test, p< 0.05.*

In previous lab studies, antagonists targeting this receptor have been shown to affect LTS but not FS cells when recordings were taken in ex vivo slices in normal cortex. In the PMR, LTS neurons respond more to DHPG, a Group I mGluR agonist, than control neurons did (**Figure H**). In addition, the response to DHPG is only via the mGluR1 receptor in control, but the response in PMR is both mGluR1 and mGluR5. Targeting of the mGluR5 receptor will therefore be less likely to interfere with the

normal functioning tissue and hopefully only target the abnormal areas. We believe that the enhancement of this receptor on SS interneurons is the cause of the epileptiform activity seen in the FL model. We hypothesize that blocking these mGluR5 receptors will lead to a decrease in the excitation seen in the PMR region of our FL animals by decreasing the output of these SS interneurons. Not only do we hypothesize that this will inhibit the epileptiform activity, but we hypothesize that it will aid in other developmental disorders that have epilepsy as a co-morbidity. Since it has been shown that mGluR5 is increased in expression as early as P2 (Figure G), early treatment is suggested. To test this hypothesis, Gabapentin (GBP), a current antiepileptic drug (AED), and MTEP, an mGluR5 antagonist, will be used on PMR and control animals.

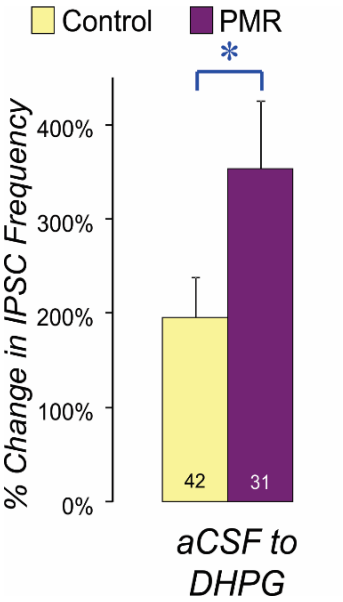

Figure H. *Effect of local application of mGluR1/mGluR5 agonist DHPG (0.01 mM) on IPSCs recorded in layer V pyramidal neurons of control (yellow) and PMR (purple) cortex. In control neurons, DHPG cause nearly a doubling in the frequency of IPSCs, while in the PMR it caused more than a tripling of the IPSC frequency. Numbers of recorded neurons shown lower part of the bars. * = t-test, p< 0.05. In additional experiments it was demonstrated that bath application of an mGluR1 antagonist prevented the increased IPSC frequency associated with local DHPG in controls but not in the PMR. Under these conditions, bath application of an mGluR5 antagonist eliminated the increase in the PMR. Work from George and Jacobs.*

1.5 Potential treatments for malformation-associated epileptiform activity

GBP will be used to test whether it can block the early development of epileptiform activity, as well as whether it changes the increased output from SS neurons. It is a current anticonvulsant that has structural analogy to GABA (Kim, Chang et al. 2009). It can be administered at therapeutic doses, unlike other AEDs that have to be slowly introduced into the system (R. Fisher & Saul, 1997). GBP has a lack of drug interaction, is cleared by the kidney, and exhibits minor side effects compared to most anticonvulsants (R. Fisher & Saul, 1997). In addition, GBP has not been shown to have any long term effects during development (Martin, McClelland et al. 2002). On the other hand, it does have a short-half life and is mainly used as an add-on medication to other anti-seizure medications (Fisher and Saul 1997). GBP blocks the influx of calcium into neurons (Traa, Mulholland et al. 2008) by blocking the interaction of TSP and α2δ-1, a calcium channel subunit (**Figure I**). This is the receptor for TSP mediated synaptogenesis (Eroglu, Allen et al. 2009, Andresen, Hampton et al. 2014). TSP 1/2 is expressed during the postnatal period when many excitatory synapses are forming. TSP is not present in the adult brain when excitatory synapses are greatly reduced (Eroglu, Allen et al. 2009). α2δ-1 is an accessory subunit of voltage gated calcium channels and aids in membrane trafficking (Andresen, Hampton et al. 2014), affects the voltage dependence of activation, and also affects the increase in current amplitude activation and inactivation kinetics (Arikkath and Campbell 2003). These channels can also influence other channels (Andresen, Hampton et al. 2014). GBP inhibits voltage gated calcium channel (VGCC) trafficking and directly inhibits calcium currents, this causes GBP to exert inhibitory effects on intracellular α2δ subunits (Hendrich, Tran Van Minh et al. 2008). All in all, α2δ-1

mediates important functions physiologically and the loss of this subunit can have severe consequences on functions relying on calcium channel trafficking and calcium currents (Arikkath and Campbell 2003). As mentioned in Andresen et al. (2014), this subunit's role in synaptogenesis, however, is independent of the calcium channel. Treatment with GBP for inflammation such as in multiple sclerosis prevents injury-induced excitation as well as a decrease in the amount of reactive astrocytes. It also decreased excitatory input onto layer V pyramidal neurons when used as a treatment in the FL model. GBP seems to eradicate most of the pathologies that are associated with the FL model, such as hyperexcitability, both in vivo and in vitro (Andresen, Hampton et al. 2014). Even though GBP prevents formation of excitatory synapses in vitro and

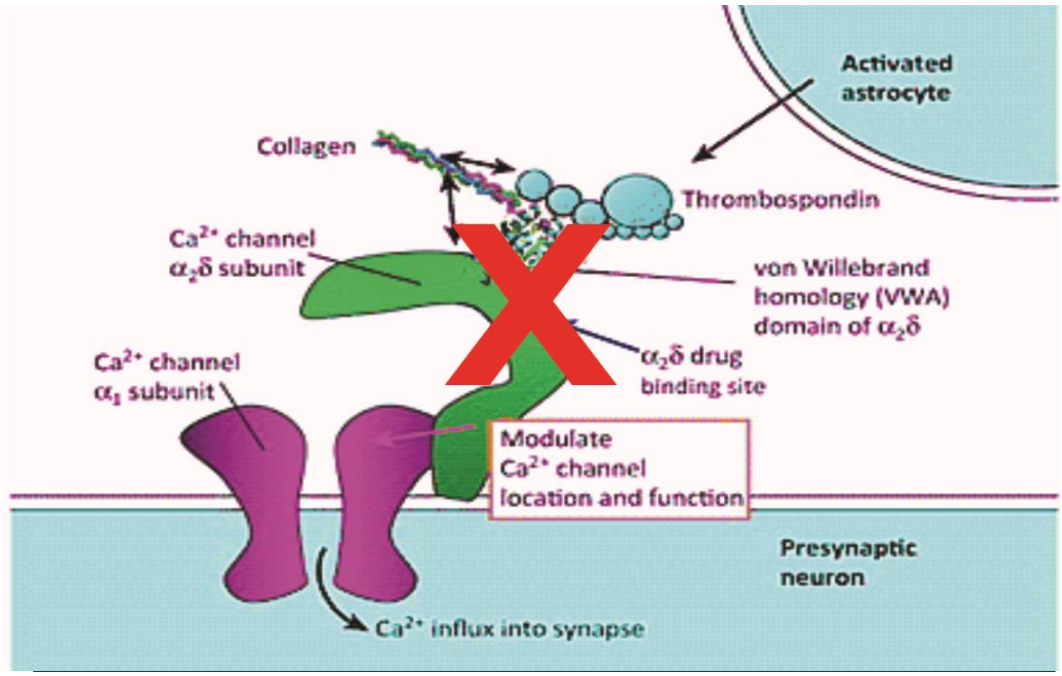

Figure I. *Gabapentin blocks the interaction of thrombospondin and a2d-1 calcium channel subunit, thereby preventing excitatory synapse formation. Figure from Stahl, S. et al. (2013).*

in vivo, it does not affect already formed synapses; since there is a treatment window of 1 week that it is effective (Eroglu, Allen et al. 2009). It is unknown at this point whether GBP affects interneuron function.

When mGluR5 is expression is enhanced, an antagonist could be used (Wong, Bianchi et al. 2005) to reduce activity to normal levels. This is why we chose to test MTEP, an mGluR5 antagonist as a potential treatment for the epileptiform activity occurring in the PMR. The mGluR5 produce effects through a number of intracellular signaling pathways (**Figure J**). The mGlu5 receptors play a role in proper development, because if it is knocked out, barrels do not form due to interruption of the mGlu5 signaling through PLC-β1 (Hannan, Blakemore et al. 2001). A barrel is a specific anatomical unit in layer IV that represents an individual whisker (Woolsey and van der Loos 1970). These barrels make up a somatotopic map in the primary somatosensory cortex (Petersen 2007). Barrels allow for the delineation of functional organization, plasticity, and development (Petersen 2007). When sensory information is received, it is processed within the barrels depending on the whisker-related behavior (Petersen 2007). As previously stated, our model shows an excess of the mGluR5 receptor in PMR tissue as early as P2. There are other mGluR5 antagonists, such as MPEP that have been previously used. We chose MTEP over MPEP because MTEP has been shown to have a greater selectivity for the mGluR5 receptor and is also more highly selective for mGluR5 without having effects on other mGluR subtypes as compared to MPEP (Colmers, Lukowiak et al. 1987, Lea, Movsesyan et al. 2005, Domin, Kajta et al. 2006, Lea and Faden 2006). Additionally, MTEP has shown to be more potent in vitro and in vivo (Szydlowska, Kaminska et al. 2007). MPEP has been shown in rodents and in *Xenopus laevis* oocytes expressing rat AMPA receptors to affect both AMPA and NMDA receptors (Gasparini, Lingenhohl et al. 1999, Olive, McGeehan et al. 2005, Lea and Faden 2006). MPEP has also been known

to have electrophysiological effects on subtypes of NMDA receptors and kainate receptors (Lea and Faden 2006). MTEP has been shown to be effective at low doses and also does not have an effect on NMDA receptors, AMPA receptors, or kainate receptors (Cosford, Tehrani et al. 2003, Slassi, Isaac et al. 2005, Lea and Faden 2006, Loscher, Dekundy et al. 2006, Nagal, Greco et al. 2015). In addition, as discussed in Nagal et al., MTEP has a higher potency in human cloned receptors as compared to MPEP. It has also been shown to penetrate the blood brain barrier well. At behaviorally active doses, MTEP produces complete occupancy of the mGluR5

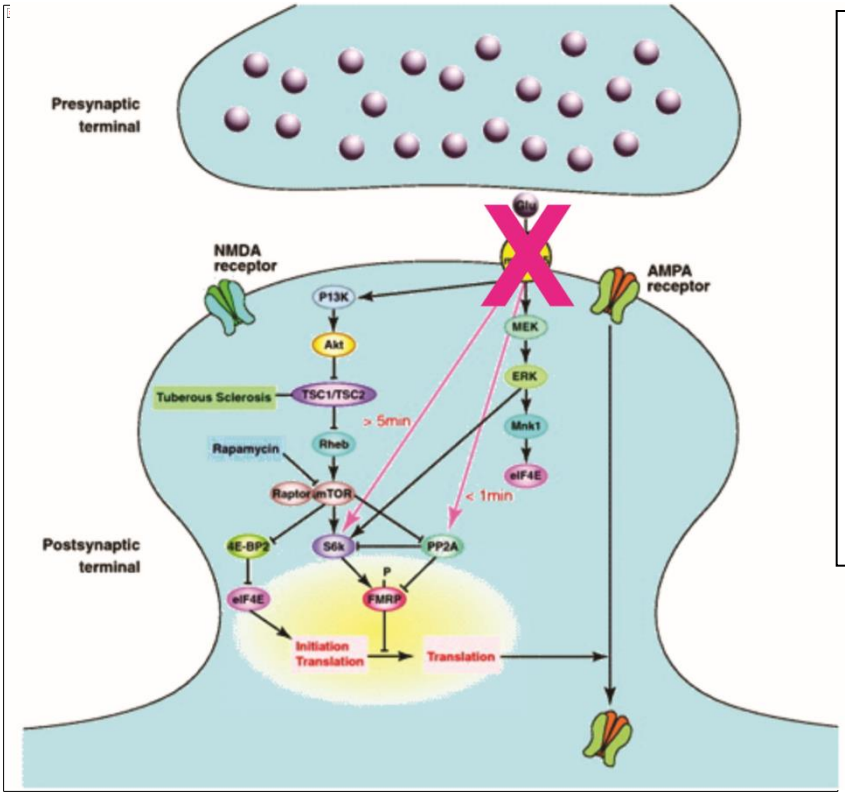

Figure J. *Intracellular signaling pathways activated by mGlu5 receptors. Pink X shows the expected effect of blocking with the MTEP antagonist. Some molecules will be increased while others will be decreased. Figure from Levenga, de Vrij et al. (2010)*

receptor, and based on in vitro affinity, also produces brain free concentrations high enough to occupy the receptor (Nagal, Greco et al. 2015). Together these data suggest that blockade of mGluR5 receptors may be an effective means to reducing epileptiform activity associated with microgyria. We expect that chronic blockade will

have no effect on interneurons in controls, because these receptors are not active on controls. Therefore treatment should be selective on extra function in PMR.

Chapter 2

Potential Treatments for Malformation Associated Epilepsy

2.1: Hypothesis and Objectives

Polymicrogyria, a developmental cortical malformation, can cause intractable epileptic seizures in affected individuals. This disorder has a lack of therapeutic treatments and drives a need to determine the underlying cellular mechanisms of cortical malformations also causing intractable seizures.

These studies utilize a freeze-lesion model for polymicrogyria in transgenic mice that selectively express ChR channels on a particular interneuron cell type. These cells, within an epileptogenic area adjacent to the malformation known as the paramicrogyral region, are thought to be functionally altered as compared to control cortex, and have been shown to contribute to the epileptiform activity seen in the PMR/FL mice. Past studies have implicated the mGluR5 receptor as the cause of the over activation of these SS interneurons. This mGluR5 receptor is enhanced in its expression on SS interneurons in the PMR region, but not in control tissue.

We hypothesize that blockade of the mGluRs will decrease the activity of SS interneurons and thereby prevent the generation of epileptiform activity and increased SS output in malformed cortex. With the following series of experiments, we assessed this by:

1. Evaluating if the drug-treated mice (GBP or MTEP) showed suppressed epileptiform fields in drug-treated PMR vs drug-treated sham-lesioned control animals.

2. Evaluating the output of the SS interneurons in our 4 treatment groups (control-GBP, PMR-GBP, control-MTEP, PMR-MTEP) via whole cell patch clamping of pyramidal neurons with the use of optogenetics.

All recordings were taken from pyramidal neurons in the designated PMR region or homologous control cortex.

2.2: Materials and Methods

Mice

In order to achieve the ChR expression on SS interneurons, we crossed Floxed stop codon on a ChR-2 expressing gene in YFP reporter female mice with Cre recombinase in the 3' UTR of the somatostatin locus males. This allowed for the selective genetic insertion of ChR into SS interneurons, subsequently allowing activation of the SS interneurons with blue light application. Mice are housed in IACUC approved housing and all procedures and protocols are IACUC approved.

FL surgery

On postnatal day 1, aseptic surgery techniques were followed to induce the transcranial freeze lesions. SS-ChR2-EYFP mice were anesthetized by being placed in ice for 3 minutes to induce hypothermia. A coronal incision was made across the skull to expose it. A frozen probe consisting of a copper bar with a 0.1mm pointed tip cooled with dry ice to -55˚C was placed on the surface of the skull for 5 seconds on each hemisphere (bilateral lesion) approximately 0.5 mm from the midline. After surgery, the incision was sutured on the center and Vetbond glue was applied to the rest of the incision. Antibiotic ointment was then applied to the whole suture and the mice were then placed in a heating blanket and allowed to re-warm to normal body temperature. Pups were then returned to

their mother. The pups were weighed for five days following the surgery to ensure they gained weight and therefore had a proper recovery.

SHAM surgery

This surgery comprised of the same series of events as the FL surgery, however, instead of the freezing probe cooled to -55˚C, the probe was room temperature.

Drug administration

Freeze-lesioned and control (sham-lesioned) mice were all given one of two drugs, MTEP or Gabapentin. From postnatal day 1 through postnatal day 7 (P1-P7), the MTEP groups were given daily i.p. injections (at the same time each day) at a concentration of 10 mg/kg MTEP. For the GBP groups, the same protocol was followed (daily i.p. injections from P1-P7) at a concentration of 200 mg/kg. Because only a small volume can be injected into mice pups, for drug injections, the volume was held constant (within a small range) while the mg/ml of the drugs were varied according to animal weight. For MTEP a volume of 0.02 mls was used. Due to the lower solubility of GBP, it was necessary to vary this from 0.02 ml for P1-3, 0.03 for P3-5; and 0.04 for P5-7.

Brain extraction and slice preparations

Mice were anesthetized with isoflurane in a small chamber. Once overdosed, they were decapitated. After decapitation, the brain was excised and quickly removed and placed into cold (-18˚C) sucrose slicing solution containing (in mM): (2.5 KCl, 1.25 NaH_2PO_4, 10 $MgCl_2$, 0.5 $CaCl_2$, 26 $NaHCO_3$, 234 sucrose, 11 glucose). The brain was then transferred to a flat surface and sliced mid-sagittally. One side of the brain was frozen

and stored in a -80˚C freezer to save for later determination of mGluR5 protein levels in the brain tissue using Western blot techniques. The other half was placed on the vibratome stage and 300 µm coronal slices from the somatosensory cortex were taken using a 1000 plus vibratome. Slicing occurred while the brain was in the sucrose slicing solution. Somatosensory cortex was confirmed by using hippocampal morphology

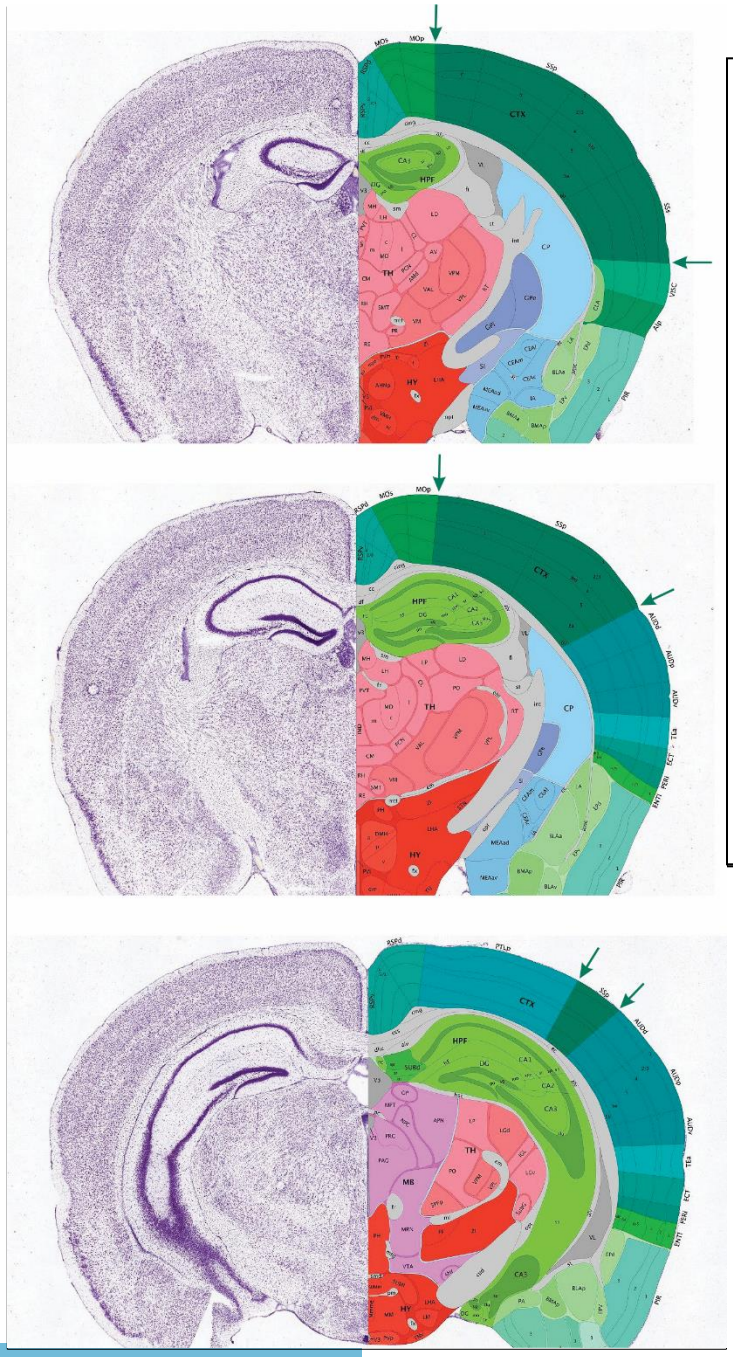

Figure K. *Images from the Allen Brain atlas showing the location of primary somatosensory cortex (dark green, arrows). Website: © 2015 Allen Institute for Brain Science. Allen Mouse Brain Atlas [Internet]. Available from: http://mouse.brain-map.org. Because there is far less somatosensory cortex present in sections with ventral hippocampus, only slices anterior to this level (equivalent to top and middle image) were used for both field potential and patch clamp recordings.*

(**Figure K**). After slicing, the slices were transferred to a warmed holding chamber filled with artificial cerebral spinal fluid (aCSF) infused with 95% O2/5% CO2 to maintain pH. ACSF is comprised of (mM): 126 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 1.0 $MgSO_4$, 1.2 $CaCl_2$,10 glucose, and 26 $NaHCO_3$. Slices remained in the heated chamber of 34˚C for 25 minutes, after which the heater was turned off and the slices cool to room temperature over 20-30 minutes. Slices were kept at room temperature until used for the patch and field recordings.

Patching and Field recordings

Before recording, the slices were transferred from the room temperature bath into the recording chamber with continuously flowing aCSF (~300 mOsm) infused with 95% O_2/5% CO_2 that was heated to 32˚C. In all instances, the aCSF contained 50 μM 2-amino-5-phosphonopen- tanoic acid (APV), an NMDA antagonist, and 20 μM 6,7-Dinitroquinoxaline-2,3-dione (DNQX), an AMPA and kainate antagonist. A high chloride intracellular solution was used (in mM: 70 K-gluconate, 10 Hepes, 4.0 EGTA, 70 KCl, 4.0 Na- ATP, and 0.2 Na-GTP) in the glass pipette for the pyramidal recordings (~3-5 mOhms). Osmolarities and pH of both intracellular solutions were adjusted to 280-290 mOsm and pH 7.3. Biocytin (0.5%) was included in recording pipettes to confirm neuronal morphology post-experiment via subsequent staining (25mg of biocytin was put into 5mL of high Cl- solution).

In malformed cortex, the sulcus could be easily visualized under standard DIC optics. In these slices recording locations were chosen within the PMR, 0.25 – 0.5 mm adjacent to the sulcus or for controls, in homologous cortex (see Figure 1). In all cases the recording location was within somatosensory cortex.

In the patch clamping experiments, an electrical stimulus was applied 100-150 µm lateral to the patched cell using a glass pipette filled with 1 M NaCl. Optical stimulation of the pyramidal cells was achieved using X-cite and XLED1 software (Lumen Dynamics) with the light applied through the 60X objective. To activate ChR expressing SS interneurons, a wavelength of 460 nm was used at an intensity of 100% (**Figure L**).

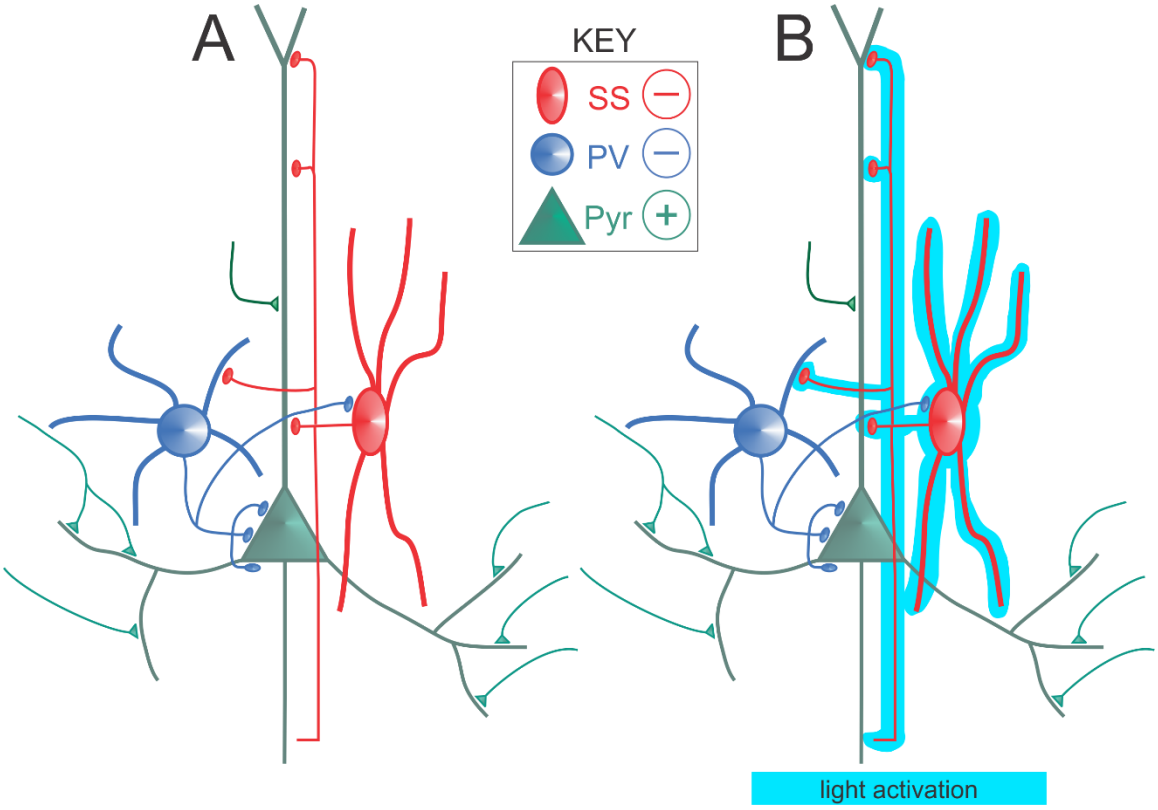

Figure L. *Cartoon illustrating the orientation of and interactions between three cortical neuronal subtypes discussed in this manuscript. SS are the normally weak or modulatory, dendrite-targeting inhibitory interneurons that have ChR genetically inserted to produce depolarization when blue light is applied B). PV are powerful inhibitors synapse on somata and preventing horizontal propagation of excitatory activity. Pyr = pyramidal neurons that are the main excitatory elements within the cortex. They have long apical dendrites. The layer V pyramidal neurons have axons that project not only subcortically, but also intracortically over long horizontal distances, particularly within layer V. The other green synapses (onto pyr) indicate thalamocortical and callosal excitatory afferents. Cartoon developed and modified by KM Jacobs. Here shown modified from that presented in Nicole Ekanem's Masters' thesis.*

These wavelengths were applied through a 60X objective either above the patched cell or 100-150 μm lateral to the patched cell above the stimulating electrode, which was a second condition recorded. MultiClamp 700B Amplifier (Molecular devices) was used and the signal was digitized with pClamp software and a Digidata1440A (Axon CNS Molecular devices).

For field potentials not requiring optical stimulation, the recording electrode was filled with either aCSF or 1 M NaCl. In these experiments, a stimulating electrode was placed in layer V and a recording electrode was placed in layer II/III. An ER1 amplifier (Cygnus Technologies) was used and the signal was digitized with pClamp software and a Digidata 1322A (Molecular Devices).

Protocols

Utilized protocols were as follows: (1) A series of increasing light durations in milliseconds (0.1, 0.2, 0.3, 0.4, 0.5, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2) was applied to determine the effectiveness of SS interneurons in producing IPSCs within the recorded pyramidal neuron with blue light alone. The series was repeated for a total of 3 presentations. The responses were averaged across the 3 presentations prior to measuring amplitude, duration, and area of the light-evoked inhibitory postsynaptic current (IPSC). Finally in cells, (2) the recording was switched from voltage clamp to current clamp mode and a series of hyperpolarizing and depolarizing steps applied (400 msec duration, beginning at -200 pA and stepping at 10 pA for a total of 70 steps), in order to measure intrinsic and cellular properties, and confirm the electrophysiological cell type.

Field potential recordings were made in layers II/III, directly above an electrical stimulating electrode located in deep layers. Care was taken to make sure that recording

and stimulating electrodes were vertically aligned and perpendicular to a tangent at the pia above the recording site. Threshold current level in these experiments was that evoking a short latency negative field of 0.2 mV peak amplitude with an electrical stimulus of 0.02 msec duration. (3) An Epitest at half-threshold (10 sec between stimulus presentations) was then run to test the incidence of epileptiform activity, 10 stimulus presentations at half-threshold current were given. (4) Then, an Epitest exactly as that described above, except at threshold intensity was run. (5) An intensity series was applied by maintaining the current while varying the duration of the electrical stimulus (0.02, 0.04, 0.08, 0.16, 0.32 msec), with the series repeated three times. (6) Lastly, a paired pulse stimulation was applied with paired electrical stimulations at varying durations of electrical stimulation (0.02, 0.04, 0.08, 0.16, 0.32 msec). Because slice health varies, for field potential recordings in order to be included for analysis, the slice had to meet three criteria: a) A threshold current of 10 mA or less; b) an increasing peak amplitude of the short latency field negativity with increasing stimulus intensity; and c) at maximum stimulus intensity, the short latency field negativity must have a peak amplitude of at least 0.6 mV. Over many years the Jacobs' lab has found these to be reliable criteria for detecting slice health. For patch clamp experiments, mostly the visual appearance of the neurons was used as an indicator of health, where unhealthy slices had many cells with swollen soma, nuclei positioned to the side of the slice and unclear membrane borders. It is not possible to obtain patch clamp recordings from this type of unhealthy neurons, thus other criteria thus far have been unnecessary.

Immunohistochemistry

Field potential and patch clamp slices were immediately placed in 4% paraformaldehyde for 24 hours after recording. After this 24 hour period, slices were placed in phosphate buffer saline (PBS) until staining. Whole-cell patch clamp slices with biocytin-filled cells were stained with Avidin (1:500 Texas Red conjugate, Life Technologies) or with Avidin and NeuN (mouse anti-NeuN conjugated with Alexa Fluor 488, Chemicon MAB377X). Images of the stained pyramidal cells were taken with the Zeiss LSM 710 confocal laser scanning microscope. Microscopy was performed at the VCU Microscopy Facility, supported, in part, by funding from NIH-NCI Cancer Center Support Grant P30 CA016059. Images of the slices stained with Avidin and NeuN were obtained with the Scope A1 microscope (Zeiss) and Image Pro Premiere 9.1 (Media Cybernetics).

Data analysis

Data was analyzed using Clampfit (Axon Instruments) and home-written macros in Microsoft Excel. Data are presented as mean ±SEM. Statistical analysis was performed with two-way repeated measures ANOVAs SPSS software (IBM), for measures across intensity series and with z-tests for measures of proportion. For the 2-way ANOVAs, stimulus intensity was the repeated measure, and subject group was the second measure. In all cases, significance was set to $p<0.05$. Throughout the results the current data is compared to that from untreated animals (both naïve controls and PMR). In all cases all data from untreated animals was collected by Nicole Ekanem and Laura Reed and was presented in a preliminary form in the Masters' thesis of Nicole Ekanem (2015).

Chapter 3

Results

3.1 Freeze-lesion histopathology not changed by drug administration

To determine whether the application of drugs altered the cortical lamination of either control or the histopathology of the PMR, slices were immunohistochemically stained for NeuN. Slices were chosen that contained anterior to approximately mid-way through the dorsal hippocampus, as these slices contained the most primary somatosensory cortex (top two pictures in Figure K). After treatment with either MTEP or GBP, the cortical lamination was similar to that in sections from untreated mice, as shown in **Figure 1**. In control, sham-lesioned mice, the normal six

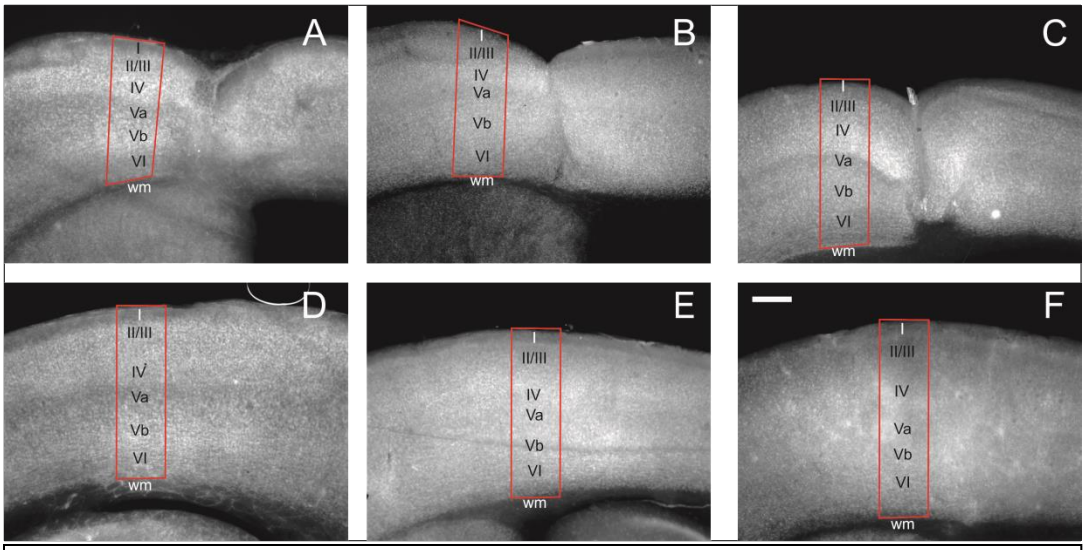

Figure 1. *Lamination within somatosensory cortex for both malformed (A-C) and control brains (D-F), identified with NeuN staining. In each case the red outlined box shows approximate recording location, with layers indicated. For malformed brains, location is within the PMR, 0.25 – 0.5 mm adjacent to the sulcus.* ***A)*** *Non-drug FL;* ***B)*** *MTEP FL;* ***C)*** *GBP FL;* ***D)*** *non-drug naïve control;* ***E)*** *MTEP sham;* ***F)*** *GBP sham. Scale bar in F for A-F = 0.2 mm.*

layers of neocortex were visible in sections through somatosensory cortex (Figure 1 D-F). In freeze-lesioned mice, sections showed the abnormally-laminated microgyrus

and adjacent six-layered PMR region within somatosensory cortex. Treatment with either MTEP or GBP did not qualitatively change the histopathology nor the cytoarchitecture of the adjacent, six-layered PMR region from which recordings were made (Figure 1 A-C).

3.2 Network activity measured with field potential recordings

To determine if the treatments with either MTEP or GBP affected the network excitability, field potential recordings were made from layer II/III during stimulation of deep layers directly beneath the recording site within somatosensory cortex and the PMR (~0.5 mm adjacent to the sulcus). Under these conditions threshold level is determined by adjusting the current level applied with a 0.02 msec pulse until a short latency negativity of 0.2 mV peak amplitude is obtained. A test for epileptiform activity was then performed by presenting 10 stimuli (10 sec interval) at half-threshold and subsequently repeating this at threshold. While an objective quantifiable epileptiform detection system is desirable and under development in the Jacobs lab, it was not available to assess these data. Instead, the expertise of the lab PI (KM Jacobs) was used to identify the presence of epileptiform activity, which has the following characteristics: 1) all-or-none behavior (that is, it is not graded with stimulus intensity); 2) variable form; 3) variable latency; 4) typically long latency relative to that of the short latency response which does vary with stimulus intensity. Only polyphasic deflections at least 2 x the baseline noise were identified as epileptiform events (**Figure 2**). Ictal (seizure) –like activity has a large slope and short time to peak, extremely short peak and often repeated instances of these ictal 'spikes'. In contrast, interictal-like activity is typically lower in amplitude with a much more slowly rising peak, a longer duration peak, and greater presence of polyphasic activity. For all

subject groups studied here, in most cases the epileptiform activity observed was interictal-like, although there were a few instances of ictal-like activity (Figure 2 C, I). In the

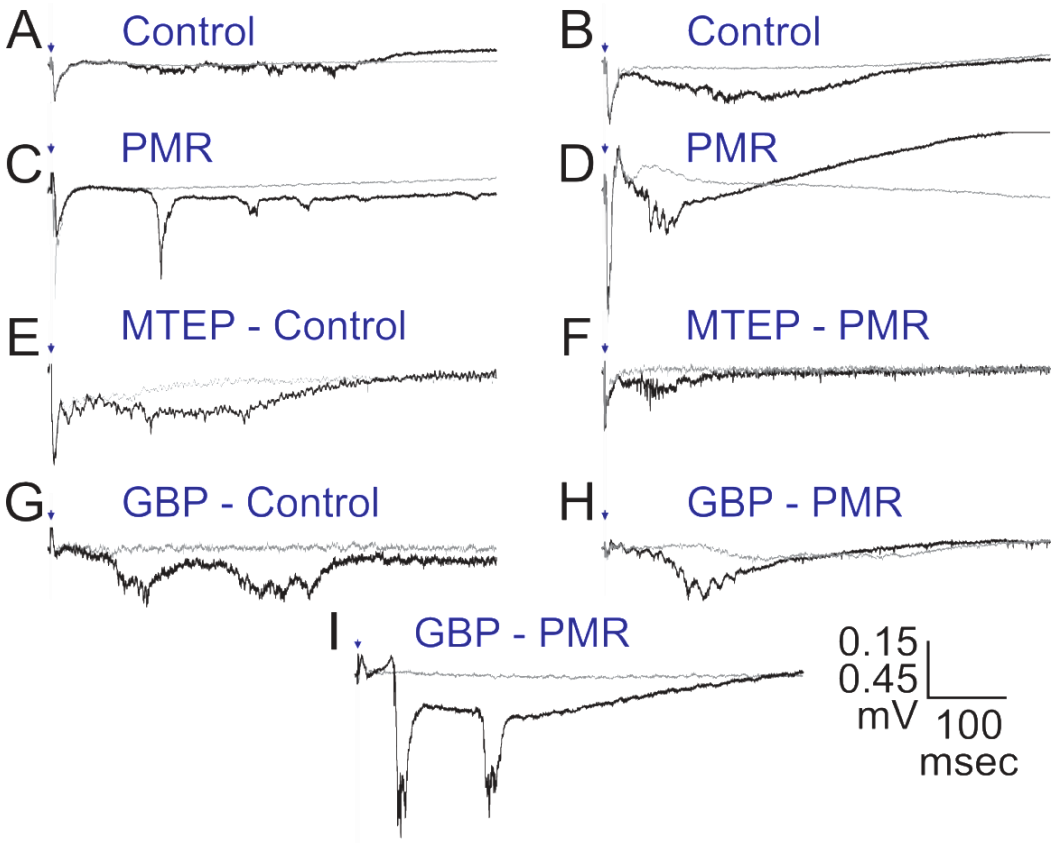

Figure 2. *Examples of epileptiform activity evoked with low stimulus intensity for different subject groups. Small blue arrows indicate time of stimulation. In all cases the black trace is an example of epileptiform activity; and the thinner gray trace shows a non-epileptiform response to the same stimulus presentation (from the same file). This demonstrates the all-or-none behavior of the epileptiform activity.* ***A, B)*** *Non-drug-treated controls from two different animals (A and B). Some epileptiform activity in controls is normal for this age group (P12-21 in rodent).* ***C, D)*** *Non-drug-treated PMR responses were typically larger than those observed in controls. C and D from two different animals.* ***E)*** *MTEP-treated sham control.* ***F)*** *MTEP-treated PMR. For all responses from MTEP-treated mice, epileptiform activity was qualitatively similar to that in un-treated controls.* ***G)*** *GBP-treated sham control.* ***H, I)*** *GBP-treated PMR. Ictal-like activity was observed only in non-drug-treated PMR (C) and in GBP-treated PMR (I). Vertical scale bar = 0.15 for A-H; and 0.45 for I.*

counting of instances of epileptiform activity, both ictal-like and interictal-like were counted as epileptiform activity.

In untreated mice, even controls within the age group tested (P21-21) normally have some hyperexcitability (Luhmann and Prince 1990). However in untreated freeze-lesions, the incidence of epileptiform activity is much higher within the PMR than in homotopic regions of control cortex, whether measured per mice or per slice (**Figure 3**). Surprisingly, controls treated with MTEP had a high rate of epileptiform activity incidence, similar to untreated PMR mice. The epileptiform incidence per slice from PMR mice treated with MTEP was significantly lower than that for slices from the control-MTEP-treated mice (Figure 3B, z-test, $p<0.05$). In addition, the epileptiform incidence per slice was significantly lower for PMR-MTEP compared to that in the PMR-untreated group (one-tailed z-test, $p<0.05$; with two tails, $p =0.08$). Here a one-tailed test was applied because of the expectation that the treatment would reduce the epileptiform incidence.

For treatment with GBP, incidence of epileptiform activity per slice was similar to that in slices from untreated mice. That is, control-GBP was similar to untreated controls and PMR-GBP was similar to untreated PMR. Like their untreated counterparts, for GBP-treated mice the epileptiform incidence per slice was significantly higher for PMR compared to control mice (z-test, $p<0.05$).

When epi incidence was examined per mouse, although the bars appear higher for all drug-treated animals, the incidence was not significantly different from untreated controls (z-tests, $p>0.05$), likely due though to low subject numbers. In future experiments, we expect to obtain at least 9 mice per group.

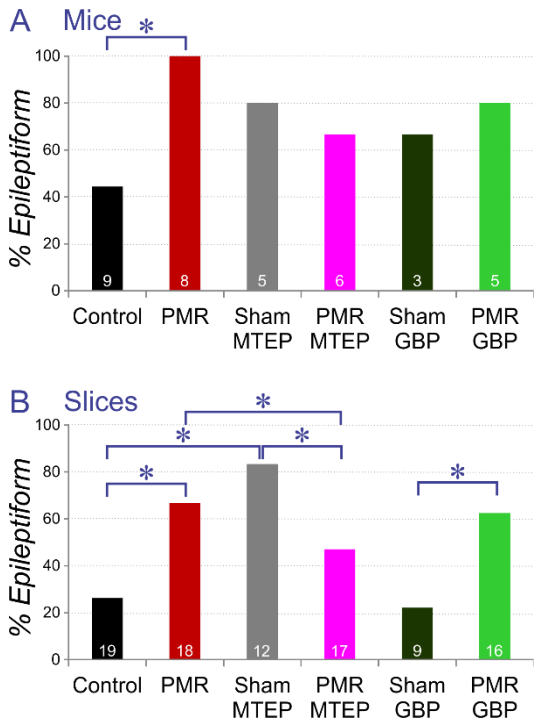

Figure 3. *Incidence of evoked epileptiform activity recorded from field potentials in ex vivo slices and analyzed both per mice* ***(A)*** *and per slice* ***(B)****. Subject group is indicated under each bar. The number of either mice (A) or slices (B) is shown in the bar for each subject group. * = z-test, $p<0.05$. To truly evaluate the per mice results, likely additional subjects must be investigated.*

3.3 Pyramidal neuron identification/differentiation

Neuronal morphology

To choose the neurons from which recordings would be made, the patch electrode was first directed to layer V under low power. It was expected (and subsequently confirmed) that layer V would make up the 3rd quadrant deep to the pia. Under high power and DIC optics, the desired pyramidal neurons were identified morphologically as the ones having large soma; but were most easily distinguished by the large apical dendrite that extended to superficial layers. No other cell types has this apical dendrite. These morphological characteristics were confirmed for some neurons with post-hoc avidin staining of the biocytin that had diffused into the cell via the patch pipette, during the recording (**Figure 4**). It is possible that FL and/or drug-treatment will change detailed characteristics of the pyramidal neuron morphology such as branch length or number. However the main characteristic of the apical dendrite was confirmed for at least some neurons in all subject groups (Figure 4).

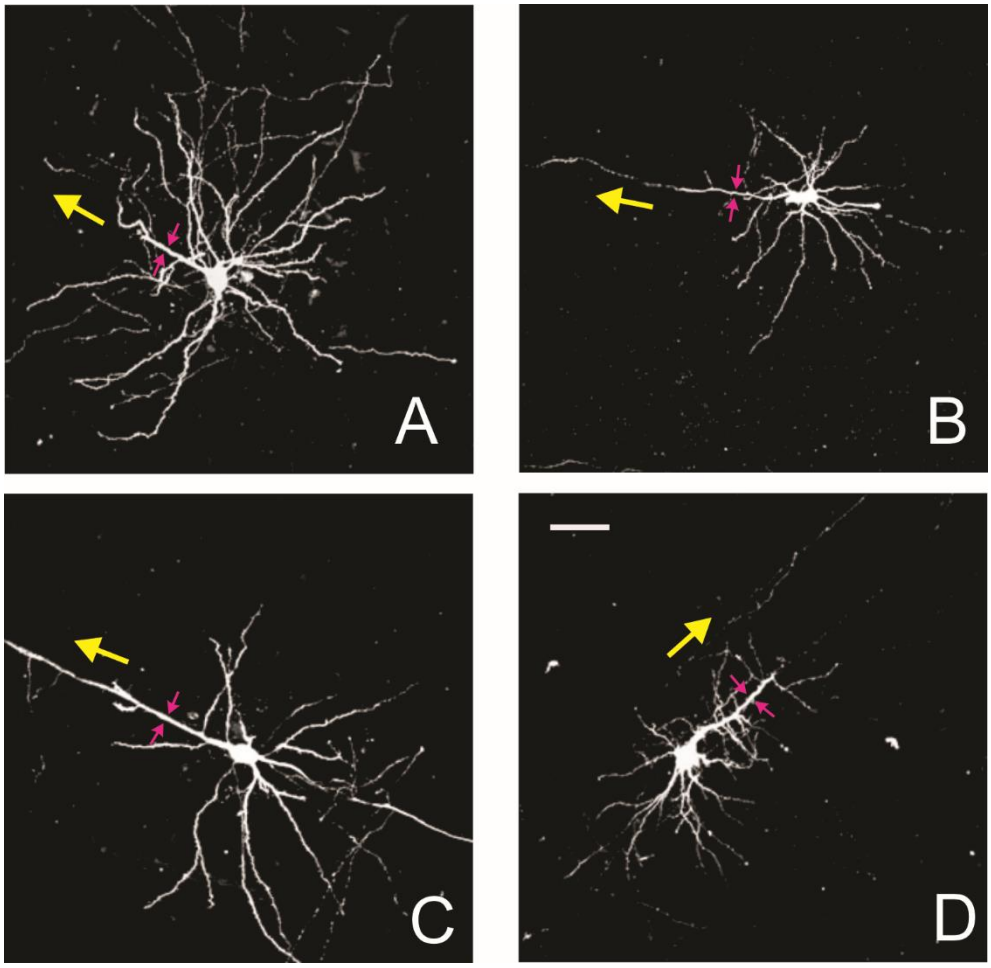

Figure 4. *Confirmation of pyramidal neuronal type for recorded neurons. Biocytin was included in the recording pipette and its presence subsequently identified in the fixed tissue with the application of a fluorescent avidin to which the biocytin binds. Here the large somal size and presence of an apical dendrite (indicated with pink arrows) projecting toward the pia (yellow arrow) indicate that these are pyramidal neurons. Not every recorded neuron can be labeled, but these examples indicate that the neurons targeted in the live slice were correct. Images were taken as maximum projections after a depth profile on a Zeiss confocal microscope.* ***A)*** *Non-drug control;* ***B)*** *GBP-FL;* ***C)*** *Non-drug FL;* ***D)*** *GBP-sham control. Scale bar in D for A-D = 0.02 mm.*

3.4 Optogenetic activation of IPSCs from tissue containing ChR in SS interneurons

When blue light was applied via an LED (bLED) through the 60X objective, in tissue containing ChR in SS interneurons, IPSCs were evoked in pyramidal neurons

(**Figure 5**). Responses were evoked at a relatively short latency and in a graded fashion. That is, increasing the duration of the light to produce a more intense stimulus caused an increase in the peak amplitude of the IPSCs for all subject groups (shown for MTEP- and GBP-treated animals in Figure 5). IPSCs were qualitatively similar between all subject groups.

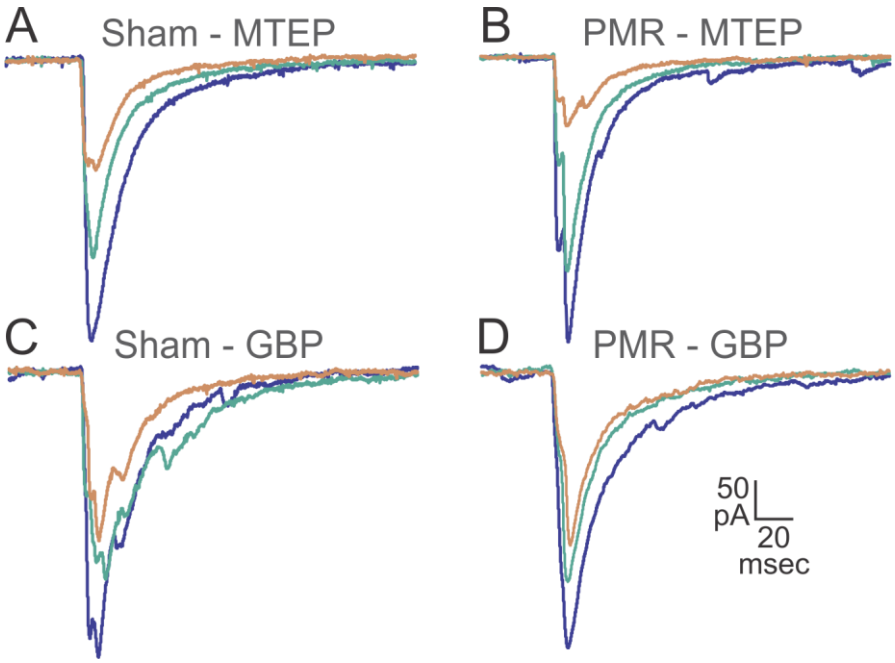

Figure 5. *Examples of IPSCs evoked by the application of blue light (bLED) in tissue with ChR in SS interneurons. Recordings are from pyramidal neurons during activation of SS inhibitory interneurons.* ***A)*** *Sham-injured mouse treated with MTEP;* ***B)*** *FL mouse treated with MTEP, with recordings made within the PMR;* ***C)*** *Sham-injured mouse treated with GBP;* ***D)*** *FL mouse treated with GBP, with recordings made within the PMR. Three intensities are shown: for A & B: 0.3 (brown), 0.4 (green), and 0.8 (blue) msec of light; and for C& D: 0.3 (brown), 0.5 (green), and 0.8 (blue) msec of light.*

3.5 The effect of MTEP treatment on SS-ChR IPSCs in control cortex

Does MTEP treatment change the SS-ChR IPSC in controls?

For all studies on the IPSCs three measures will be presented: peak amplitude; area of the significant response (defined as two standard deviations above the mean of the baseline, which is the region prior to stimulation for each voltage clamp

recording); and duration of the significant response. Each measure is plotted against the 11 stimulus intensities (duration of bLED in msec). The plots for the comparison between untreated controls and MTEP-treated controls are shown in **Figure 6**. To test for significant differences, a 2-way repeated measure ANOVA was used with stimulus intensity as the repeated measure. For this comparison, recordings were made from 19 untreated control and 11 control – MTEP treated neurons. There was no significant effect of subject group or stimulus intensity, however there was a significant interaction of these two (p=0.01, see **Table 1**, where all p values are reported for comparisons shown in Figs. 6-9). For post hoc analyses of the interaction between subject group and stimulus intensity, in all cases a 1-way ANOVA was performed at

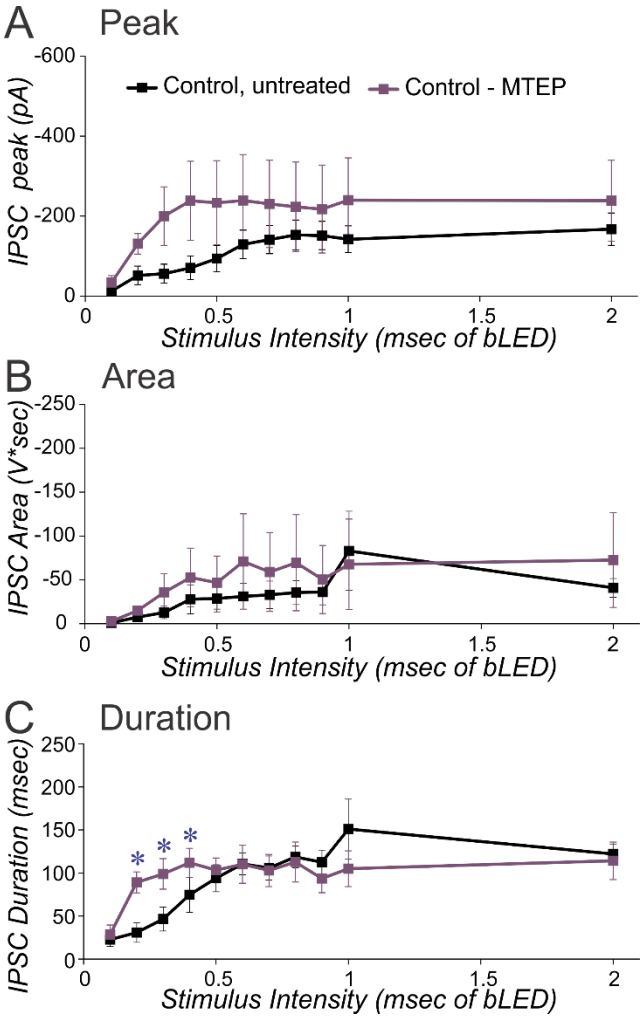

Figure 6. *Comparison of the SS-ChR evoked IPSC between untreated (naïve) controls (black, N = 19) and MTEP-treated sham-injured controls (purple, N = 11). Does MTEP alone have an effect? IPSCs were recorded in layer V pyramidal neurons in tissue with ChR selectively in SS interneurons. Stimulus intensity was generated via increasing durations of bLED. The IPSC peak amplitude* ***(A)****, area of significant response* ***(B)****, and duration of significant response* ***(C)*** *are shown here. Significance was tested with a 2-way repeated measures ANOVA. For peak and area, there was no significant difference between subject groups and no significant interaction between subject groups and stimulus intensity. * = significant difference assessed with post hoc analysis. See text and Table 1 for all p values.*

each stimulus intensity, with the p value adjusted with a Bonferroni correction (0.05/number of comparison tests). In this case there were 11 stimulus intensities, so in order to reach significance at any one level, the p value had to be less than 0.045 (0.05/11). After applying these criteria, there was no significant difference in the peak IPSC between control-untreated and control-MTEP at any stimulus level.

The area of the IPSC was not significantly different for MTEP-treated compared to untreated controls, there was also no effect of stimulus intensity and the interaction was also not significant (see table 1). For IPSC duration, there was an effect of group, an effect of stimulus intensity, and an interaction ($p<0.05$). To further examine the interaction, 1-way ANOVAs were performed at each level, with a Bonferroni adjustment to the p value needed for significance. After applying this correction, there was a significant difference between control-untreated and control-MTEP only at 0.2, 0.3 and 0.4 msec stimulus intensity levels, as shown by asterisks in Fig. 6.

3.6 Does MTEP prevent the PMR-associated increase in SS-ChR IPSC peak?

To determine if MTEP could prevent the PMR-associated increase in the peak of the IPSC evoked with optogenetic activation of SS interneurons, we first compared the results between control-MTEP and PMR-MTEP groups. Should MTEP be effective, it was expected that there would no longer be a significant difference between control and PMR when both were treated with MTEP. That was in fact the case, based on a 2-way ANOVA for just these groups (subject groups N.S. different, $p>0.05$). However it is possible that even without a significant difference that MTEP was not returning the SS interneuron output to the normal (untreated control) levels.

To examine this further, the results for four groups: control-untreated (19 neurons), PMR-untreated (14 neurons), control-MTEP (11 neurons) and PMR-MTEP (11 neurons, see **Figure 7**) were compared. For IPSC peak, although there was no effect of subject group with the 2-way repeated measures ANOVA, there was a significant effect for stimulus intensity and a significant interaction between stimulus intensity and subject group ($p<0.05$). To determine which subject groups were significantly different for which intensities, 1-way ANOVAs with Bonferroni correction to the p-value were used (as described above). This analysis showed a significant group effect for three intensities: 0.2, 0.3, and 0.4 msec. Bonferroni post

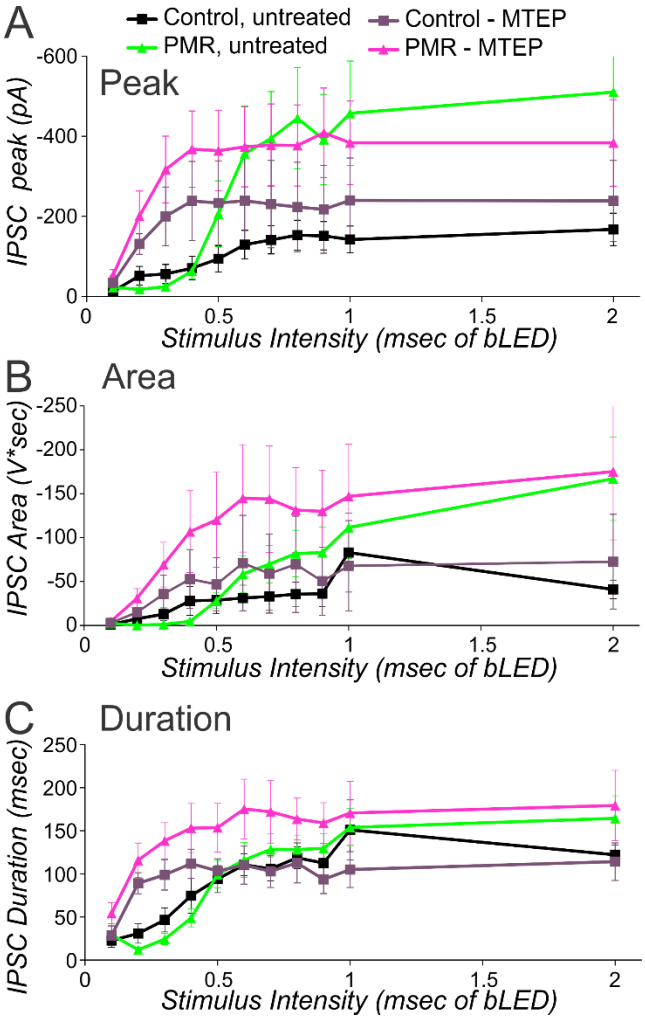

***Figure 7**. Does MTEP prevent the enhanced SS-ChR IPSC associated with the PMR? All features are the same as for figure 6, with 14 PMR-untreated neurons and 11 PMR-MTEP neurons. There was a significant interaction between subject group and stimulus intensity on all measures (two-way repeated measures ANOVA, $p<0.05$). MTEP treated groups were larger than untreated groups at low intensities. See text and Table 1 for further explanation of significant effects.*

hoc analysis of this result showed that the PMR-MTEP group was significantly larger than the control-untreated and PMR-untreated groups at all three intensities.

It is however clear that to draw firm conclusions, additional data will be necessary, given the large error bars and low power (see power in Table 1) associated with the current data. Results for IPSC area were similar to those for IPSC peak with significant effects of stimulus intensity and the interaction between subject group and stimulus intensity. The post hoc analysis showed that only at 0.2 msec was there a significant difference, where PMR-MTEP was once again greater than both control-untreated and PMR-untreated. For the IPSC duration the results were similar to that for peak. The 2-way repeated measures ANOVA showed a significant effect of stimulus intensity and a significant interaction between subject groups and stimulus intensity. Post hoc analyses at each intensity showed a significant group effect only for the 0.2 and 0.3 msec stimuli. At those levels, the PMR-MTEP group was greater than both the control-untreated and the PMR-untreated groups. In addition, the control-MTEP group was also greater than the PMR-untreated group at both intensities and greater than the control-untreated group at 0.2 msec.

3.7 The effect of GBP treatment on SS-ChR IPSCs in control cortex To determine if the second potential treatment, that with GBP had a direct effect on the SS-ChR IPSC, we compared the untreated controls to the GBP-treated controls (**Figure 8**). We again used a 2-way repeated measures ANOVA to test for significant differences (N = 19 and 7 neurons for untreated and GBP). There was no significant effect of subject group and no significant interaction, but there was a significant effect of stimulus intensity ($p<0.05$). The same was true for measures of IPSC area and duration, where there was no significant difference between control-untreated and control-GBP and no significant interaction between stimulus intensity and subject group, but there was a significant effect of stimulus intensity.

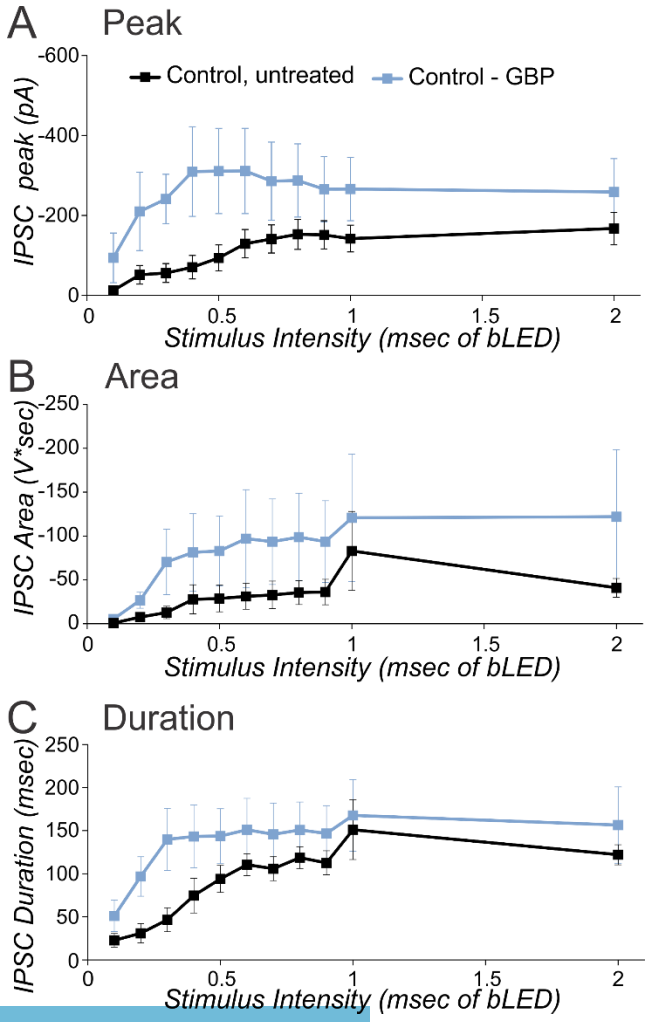

Figure 8. *Does GBP affect the SS-ChR IPSC in control cortex? Untreated controls in black (N = 19) and GBP-treated controls in blue (N = 7). Measures of peak* ***(A)****; area* ***(B)****; and duration* ***(C)*** *of the IPSC are shown. A 2-way repeated measures ANOVA was used to test for significance. There was a significant effect of stimulus intensity only, for all three measures.*

3.8 Does GBP prevent the PMR-associated increase in SS-ChR IPSC peak?

To determine if GBP could prevent the PMR-associated increase in the peak of the IPSC evoked with optogenetic activation of SS interneurons, we first compared the results between control-GBP and PMR-GBP groups. Should MTEP be effective, it was expected that there would no longer be a significant difference between control and PMR when both were treated with GBP. That was in fact the case, based on a 2-way ANOVA for just these groups (subject groups N.S. different, $p>0.05$). However it is possible that even without a significant difference that GBP was not returning the SS interneuron output to the normal (untreated control) levels.

To examine this further, the results for four groups: untreated controls (19 neurons), untreated PMR (14 neurons), control-GBP (7 neurons) and PMR-GBP (11 neurons, see **Figure 9**) were compared. For IPSC peak, although there was a significant effect of subject group with the 2-way repeated measures ANOVA, there was a significant effect for stimulus intensity and also a significant interaction between stimulus intensity and subject group ($p<0.05$). Once there is an interaction, we have investigated this statistically, rather than examining the differences between subject groups. Post hoc analyses of the interaction were performed as described above, with 1-way ANOVAs at each intensity, with a Bonferroni correction applied to the p value. For peak IPSC, there was a significant group effect at stimulus intensities 0.2, 0.3 and 0.4 msec. At these levels the PMR-GBP group was significantly larger than both control-untreated and PMR-untreated groups. In addition, the control-GBP group was significantly larger than the PMR-untreated group at the 0.3 msec stimulus intensity.

For the area of the IPSC, there was an effect of stimulus intensity and an interaction between subject group and stimulus intensity. Post hoc analysis using 1-way ANOVAs with Bonferroni correction applied to the p-value showed a significant effect of group for intensities 0..1, 0.2 and 0.3 msec. At these intensities, the PMR-GBP group was significantly larger than control-untreated and PMR-untreated groups. In addition at the 0.2 msec level, the control-GBP group was significantly larger than PMR-untreated group.

For the duration of the IPSC, the 2-way repeated measures ANOVA showed an effect of subject group, stimulus intensity and a significant interaction between these two. We again focused on the interaction to further understand these results. Post hoc analyses of 1-way ANOVAs at each stimulus intensity with Bonferroni correction applied to the p value, showed significant group effects at the 0.2, 0.3, and 0.4 msec levels. At these levels, the PMR-GBP group was significantly larger than both the control-untreated and the PMR-untreated groups. In addition, at the 0.2 and 0.3 levels, the control-GBP was also significantly larger than both the control-untreated and PMR-untreated groups. It is however still clear that to draw firm conclusions, additional data will be necessary, given the large error bars and low power (see Table 1) associated with the current data.

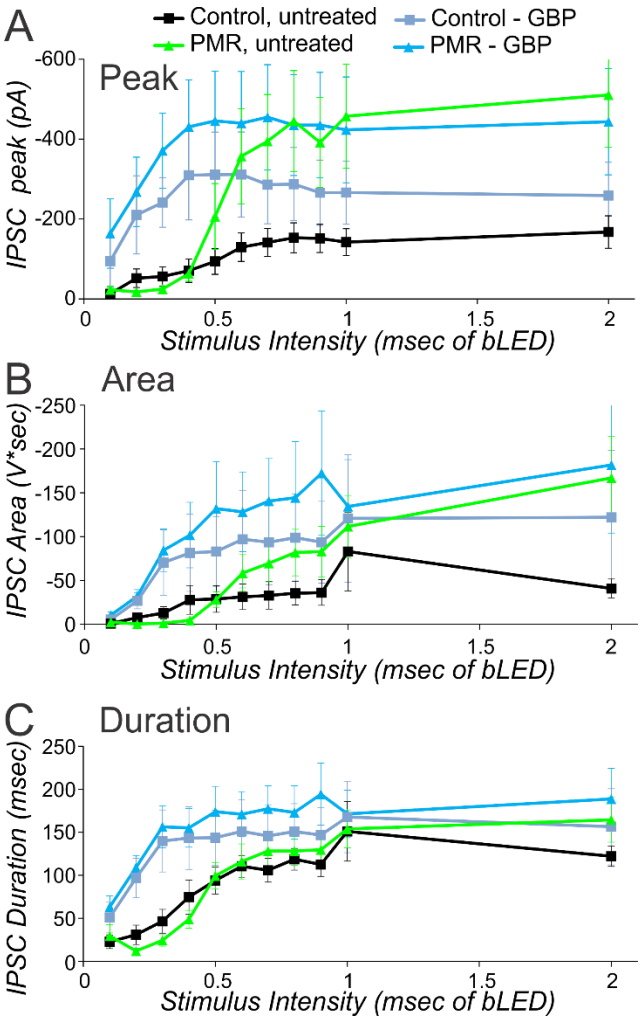

Figure 9. *Does GBP prevent the enhanced SS-ChR IPSC associated with the PMR? All features are the same as for figure 8, with the addition of 14 PMR-untreated neurons and 11 PMR-GBP neurons. There was a significant interaction between subject group and stimulus intensity on all measures (two-way repeated measures ANOVA, $p<0.05$. See text and Table 1 for further effects.*

3.9 Is GBP more effective than MTEP in reducing the SS-ChR IPSC?

While it is clear that for all four drug-treated groups additional neurons are needed to complete this project, here we attempted to compare the GBP and MTEP effects for this preliminary form of the data. To make direct comparisons of the effect of the drug on the PMR-associated changes, all PMR data was normalized to the mean of its respective control. For instance, each untreated PMR peak IPSC value was divided by the mean of the untreated control peak IPSC. This was repeated for MTEP and GBP groups. Thus for any given value if the PMR IPSC value was equal to that of the control, this procedure would yield a normalized value of 1. The results for the three measures of peak, area, and duration of the IPSC are shown in **Figure 10**. For

the untreated group, the peak and the area of the IPSC was 3-4 times that of control on intensities of 0.5 msec and greater. In contrast, for both MTEP and GBP, the values were between 1 and 2 on the normalization scale, further demonstrating the lack of difference between control and PMR in the drug-treated groups. This figure shows that MTEP and GBP were not significantly different in their effectiveness. To test for significance, 2-way repeated measures ANOVAs were again used. The p values and observed power for this data is reported in **Table 2**.

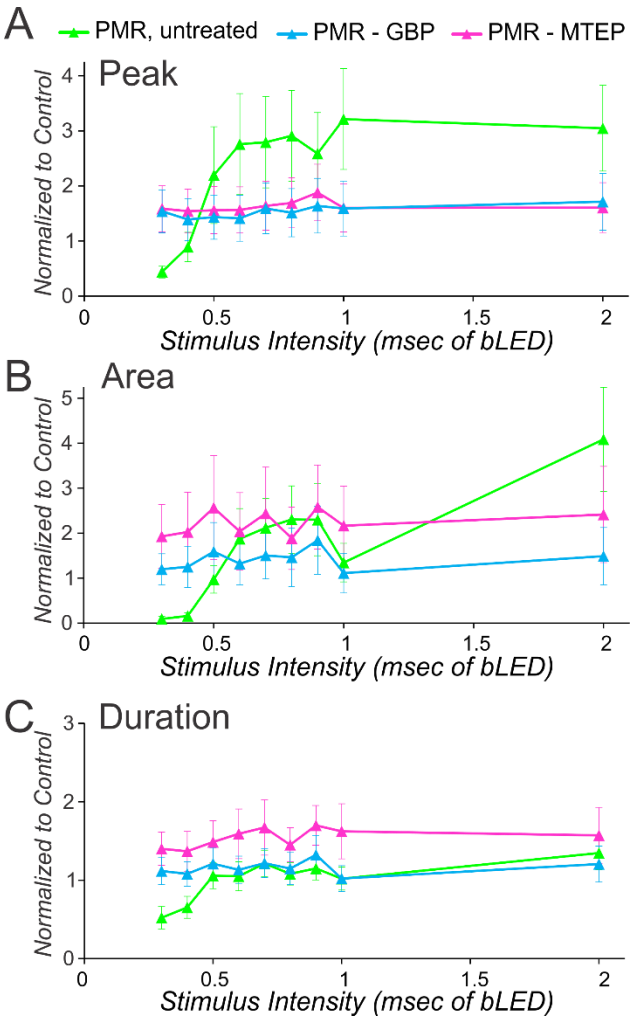

Figure 10. *All PMR data normalized to their respective controls. MTEP and GBP are equally effective in reducing the enhanced SS-ChR IPSC in malformed brain. Measures of peak* ***(A)****, area* ***(B)*** *and duration* ***(C)*** *shown. Untreated in green (N = 14); MTEP in pink (N = 11); and GBP in blue (N=11). See Table 2 for 2-way repeated measures ANOVA p values and observed power for the data shown here.*

p-values	IPSC Peak Amplitude Control-untreated vs Control-MTEP	Control-MTEP vs PMR-MTEP	Control-untreated vs PMR-untreated vs Control-MTEP vs PMR-MTEP	Control-untreated vs Control-GBP	Control-GBP vs PMR-GBP	Control-untreated vs PMR-untreated vs Control-GBP vs PMR-GBP
subject group	0.21 (0.24)	0.31 (0.16)	0.08 (0.56)	0.08 (0.65)	0.41 (0.13)	**0.03 (0.70)**
stimulus intensity	0.05 (0.78)	0.24 (0.43)	**6E-8 (0.99)**	**0.03 (0.84)**	0.09 (0.58)	**0.0001 (1.0)**
interaction	**0.01 (0.91)**	0.99 (0.08)	**0.002 (0.98)**	0.07 (0.71)	0.26 (0.35)	**0.003 (1.0)**
p-values	IPSC Area Control-untreated vs Control-MTEP	Control-MTEP vs PMR-MTEP	untreated vs PMR-untreated vs Control-MTEP vs PMR-MTEP	Control-untreated vs Control-GBP	Control-GBP vs PMR-GBP	untreated vs PMR-untreated vs Control-GBP vs PMR-GBP
subject group	0.55 (0.09)	0.30 (0.18)	0.18 (0.41)	0.23 (0.47)	0.61 (0.82)	0.11 (0.51)
stimulus intensity	0.09 (0.69)	**0.02 (0.69)**	**0.001 (0.99)**	**0.04 (0.77)**	0.14 (0.47)	**0.00004 (1.0)**
interaction	0.31 (0.46)	0.38 (0.17)	**0.001 (0.99)**	0.13 (0.60)	0.67 (0.17)	**0.001 (1.0)**
p-values	IPSC Duration Control-untreated vs Control-MTEP	Control-MTEP vs PMR-MTEP	untreated vs PMR-untreated vs Control-MTEP vs PMR-MTEP	Control-untreated vs Control-GBP	Control-GBP vs PMR-GBP	untreated vs PMR-untreated vs Control-GBP vs PMR-GBP
subject group	**0.03 (0.78)**	0.11 (0.36)	0.06 (0.60)	0.11 (0.36)	0.59 (0.08)	**0.02 (0.73)**
stimulus intensity	**3E-26 (1.0)**	**0.003 (0.98)**	**1E-8 (1.0)**	**0.002 (0.99)**	**0.02 (0.88)**	**2E-7 (1.0)**
interaction	**0.002 (0.99)**	0.19 (0.48)	**0.0002 (1.0)**	0.13 (0.60)	0.49 (0.23)	**0.02 (0.99)**

Table 1. p values and observed power in parentheses for all comparisons tested with a 2-way repeated measures ANOVA shown in figures 6-9. Comparison groups are listed from left to right. IPSC measures of peak, area and duration are listed from top to bottom, respectively. Significant differences ($p<0.05$) shown in red. For these comparisons, N = 19 untreated control, 14 untreated PMR, 11 control-MTEP, 11 PMR-MTEP, 7 control-GBP, and 11 PMR-GBP

p-values	IPSC Peak Amplitude Untreated vs MTEP vs GBP
subject group	0.54 (0.15)
stimulus intensity	**0.004 (0.96)**
interaction	**0.02 (0.94)**
p-values	IPSC Area Untreated Control vs Sham-MTEP
subject group	**0.69 (0.10)**
stimulus intensity	**0.01 (0.89)**
interaction	0.06 (0.87)
p-values	IPSC Duration Untreated Control vs Sham-MTEP
subject group	**0.17 (0.36)**
stimulus intensity	**0.0002 (0.99)**
interaction	**0.03 (0.91)**

values reported as p (observed power)

Table 2. p values and observed power in parentheses for the comparisons shown in Fig. 10 and tested with 2-way repeated measures ANOVA. These data were normalized to the mean of their respective controls. IPSC measures of peak, area and duration are listed from top to bottom, respectively. Significant differences ($p<0.05$) shown in red. For these comparisons, N = 14 PMR-untreated, 11 PMR-MTEP, and 11PMR-GBP neurons.

Chapter 4

Discussion

Epilepsy has been previously attributed to increased excitation or decreased inhibition. With this frame of mind, modern medicine has been unable to develop an effective permanent treatment against the mechanisms of epilepsy for some patients. In order to treat patients with intractable seizures, especially those caused by developmental malformations, it is essential to understand the entirety of mechanisms that could possibly play a role in the abnormal cortical function. Excitatory afferents are increased in the PMR due to the presence of the microgyrus (Jacobs and Prince 2005)**.** This suggests that the hyperexcitability is caused by these extra glutamatergic synapses; however, the early susceptibility to excitation does not coincide with the increased excitation onto pyramidal neurons and may actually be caused by changes in inhibitory interneurons (George and Jacobs 2006, George and Jacobs 2011, Bell and Jacobs 2014). This suggests a role for GABAergic interneurons in epileptogenesis. Previous studies have shown that excitation persists even with enhanced inhibitory function suggesting that the inhibition is not decreasing excitation in the network; blocking of the inhibition causes a decrease in epileptiform activity in some epilepsies or conditions (Mann and Mody 2008). It was shown that in the PMR region, SS interneurons have an increased output compared to PV interneurons (George and Jacobs 2011). In addition, it has been shown that the mGluR5 receptor is enhanced in its presence in the PMR region in FL animals as opposed to control on these SS interneurons.

The overall goal of this study was to determine if blockade of the mGluR5 receptor would inhibit the output from SS interneurons and therefore decrease the overall

excitation seen in the FL model. With the use of field potential recordings and optogenetics with patch clamping the following observations were made:

Patch clamping was used to measure the output of the SS interneurons by selectively activating them with blue light using optogenetics. Field potential recordings were used in order to determine the presence of epileptiform activity in our experimental groups. MTEP was used on PMR and SHAM mice in order to reduce the output from the SS interneurons by blocking the mGluR5 receptor, and therefore, the overall hyperexcitability seen in the FL model. GBP was used as a drug control and was also used to treat PMR and SHAM mice. GBP blocks the interaction of TSP and the α2δ-1 receptor and therefore inhibits excitatory synapse formation.

There was epileptiform activity seen in all groups, even in untreated mice. It is normal, however, for untreated mice to have a certain amount of epileptiform activity due to high concentrations of NMDA receptors and not fully developed GABAergic systems (Luhmann and Prince 1990).

In untreated PMR mice, the incidence of epileptiform activity is higher within the PMR than in homotopic control cortex. This is similar to what's seen in the rat FL model. This increased amount of epileptiform activity in the PMR mice compared to controls suggests a developmental change.

Results also showed a high rate of epileptiform activity in MTEP SHAM mice compared to untreated PMR mice. The incidence of epilepsy per slice in MTEP PMR mice was lower than MTEP SHAM mice. This suggests that MTEP decreases epileptiform activity, however, it also suggests that MTEP affects another aspect of the brain due to the high rate of epileptiform activity in the MTEP SHAM mice compared to

untreated PMR mice. The fact that epi incidence in slices from MTEP PMR mice was decreased– especially as compared to untreated PMR mice – suggests that MTEP may in fact be effective in reducing the malformation-associated causes of hyperexcitability.

MTEP-treated controls did not differ from the untreated controls in peak, area, or duration of IPSCs at the longer light durations. The only difference was seen in the initial intensities (0.2, 0.3, 0.4 msec) for the IPSC duration. For MTEP PMR and control vs untreated PMR and control, there was a significant interaction between the groups at intensities 0.2, 0.3, and 0.4 msec light duration for the peak, at 0.2 msec in the IPSC area for MTEP PMR vs untreated groups and 0.2 and 0.3 msec for IPSC duration for MTEP PMR vs untreated groups. We currently do not know why there is increased SS interneuron output at these low intensities, however, future studies will look into this aspect. Some potential causes of the increased output could be technical error, possible biological differences in current mice, or a biological change due to MTEP in the cortex or elsewhere.

An explanation for the amount of epileptiform activity is possibly due to MTEPs role in excitatory or inhibitory neuron formation. When mGluR5 is knocked out, cortical excitatory neurons receive reduced inhibitory inputs into layer IV, suggesting a role for mGlu5 in the functional development of GABAergic circuits (Ballester-Rosado, Albright et al. 2010). Additionally, mGluR5 plays an important role in radial-glial-mediated neuronal guidance which is important for normal neocortical function (Louhivuori, Jansson et al. 2014) so the blocking of this receptor could have detrimental effects. They showed that the interruption of the mGlu5 receptor hinders the activity of the canonical transient receptor potential (TRPC) channel family which has been shown to mediate the

responses of growth cones to guidance cues through their control of calcium currents. This in turn effects radial glial mediated neuronal guidance and may have an impact on specific neurons that are in the layers of the neocortex (Louhivuori, Jansson et al. 2014). This could affect the formation of the PMR because of the formation of the microgyrus and subsequent redirected afferents.

The mGluR5 receptor on SS interneurons was specifically targeted due to previous work demonstrating its enhanced presence in the PMR, but not in control. Previous studies in our lab have shown that these SS interneurons are more active in the PMR region and could be the cause of the hyperexcitability caused by the synchronous firing of pyramidal neurons due to SS interneuron synchronous inhibition.

The fact that GBP-treated SHAM were similar to untreated controls in their epi incidence suggests that GBP has no effect on normal network excitability. GBP also appeared to have no effect on FL-induced hyperexcitability, since GBP PMR was similar to untreated PMR mice. This, however, is opposite of what was found in Andersen (2014). What could explain the difference in results could be the age that lesions were done as well as the severity of the lesion. We do a bilateral lesion as compared to a unilateral lesion and our mice had the FL at age P1 instead of P0. Andersen (2014) found no difference in the amount of epileptiform activity in GBP treated FL animals compared to SHAM injured animals (Andresen, Hampton et al. 2014), concluding that GBP was able to reduce in vitro cortical hyperexcitability after an induced FL.

The effects of GBP treatment showed significant effects at low intensities of IPSC peak, duration, and area in the GBP PMR group compared to the untreated groups. Again, it is not known why there is increased SS interneuron output at these low

intensities, however, future studies will look into this aspect. Additionally, there was no difference seen in the SS interneuron output in GBP PMR or GBP SHAM groups when compared.

Translation and relevance of project:

Current AEDs cause either a decrease in excitation, like GBP, or cause an increase in inhibition. Most AEDs work fairly well, but not all seizures are treatable with these AEDs. The alternative when AEDs do not work is invasive surgery. AEDs also generally have problems such as problematic drug interaction and other aversive side effects and do not work well in patients with PMG. Targeting the mGluR5 receptor is attractive due to the fact that this receptor is not normally expressed in high amounts in normal control tissue. Because of this, MTEP, an mGluR5 receptor antagonist, may not cause the extensive side effects as other AEDs. MTEP blockade of the mGlu5 receptor shows promise in its ability to decrease SS interneuron output, however, further experimentation is needed. This type of mechanism, if translatable to humans, could result in a successful treatment for previously intractable epilepsies associated with developmental malformations.

These experiments showed that there is a possible role of the mGluR5 in the decrease in epileptiform activity caused by developmental malformations, however, additional drug experiments need to be done to determine MTEP's efficiency in aiding in this process.

Further directions:

Further investigation into the mechanisms of SS interneuron maturation will help further narrow down the areas to which treatments can be applied. One possibility is different maturation times. Knowing the mechanisms of SS interneuron maturation would be a helpful study in order to determine why they have increased expression of mGluR5.

Further studies involving the drug, rapamycin, an mTOR pathway inhibitor, would be a promising next step. The mTOR pathway is one of many pathways activated by the mGlu5 receptor. MTOR is a rapamycin-sensitive serine-threonine kinase that plays a role in mRNA translation initiation, consequently affecting cell growth, dendritic arborization, neuronal morphology, proliferation, and cortical development (Meyuhas 2000, Chen, Atkins et al. 2007, Nguyen, Brewster et al. 2015). Due to MCDs being linked to problems in genes encoding known regulators of the mTOR pathway, blocking this pathway seems to be a logical step in potential treatments (Nguyen, Brewster et al. 2015). Because mGluR5 has been shown to be important in development, narrowing down treatment to a specific pathway activated by this receptor might be the key to a potential therapeutic treatment for intractable epilepsies in order for the partial function of this receptor to remain intact. Additionally, mTOR is a promising next step because some studies have shown that in seizures that are difficult to treat, MTEP's use to block the mGlu5 receptor is ineffective (Witkin, Baez et al. 2008).

There are many future directions to be taken in order to elucidate the role of mGluR5 in the increased output from SS interneurons. Due to the complexity of the cascading pathways activated by this receptor, there needs to be further experimentation in order to determine a specific mechanism to control these SS interneurons.

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