2007

The Role of Inhibitory Interneurons in a Model od Developmental Epilepsy

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THE ROLE OF INHIBITORY INTERNEURONS IN A MODEL OF DEVELOPMENTAL EPILEPSY

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

by

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August, 2007

Acknowledgement

First, I would like to thank my parents for their support over the last 25 years. I would also like to thank Dr. Jacobs for her support and guidance during the year. I would also like to extend a sincere thanks to Amanda Lynn George (AKA Mandy 5 or the MD/PhDbot) for keeping me sane as the year progressed. I expect to see your name quite a bit in future publications. I would also like to thank everyone else who has supported me over the years: Michael, Katie, Matthew, Matt, Rob, Hatcher, Tommy, The other Tommy, Patrick, Lauren, Teo, Cara, Shep, Alex, Jamie, Malcolm, Kelly, Shep, Moo, Markowitz, Matt, Jordan, Tina, Kim, Anya, Melissa, Lynn, Sue, all of my teachers, Bam Bam, Grace, the whole Mendez family, the Longos, the Estes, Ms. Fourness, Jeff, Mantini, Farr, Faquin, the Canfields, the Wolfgangs, Reed, Sylvia and, last but not least, Mary. I am sure I forgot someone but chances are this document will never see the light of day again. Don't take it personally. Also, I would like to thank the cart guys, there were days when you guys were the only thing that got me to school.

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List of Abbreviations

aCSF ..... Artificial Cerebral Spinal Fluid

CB .....Calbindin

ChC .....Chandelier Cells

CT.....Corticothalamic

DBC .....Double Bouquet Cells

FL .....Freeze Lesion

FS ..... Fast Spiking

LBC ..... Large Basket Cell

LTS..... Low Threshold Spiking

MC ..... Martinotti Cell

MCD..... Malformation of Cortical Development

PMG ..... Polymicrogyria

PMZ ..... Paramicrogyral Zone

PV.....Parvalbumin

RS..... Regular Spiking

SEM ..... Standard Error of the Mean

SOM ..... Somatostatin

SS ..... Somatostatin

TC..... Thalamocortical

Abstract

THE ROLE OF INHIBITORY INTERNEURONS IN A MODEL OF DEVELOPMENTAL EPILEPSY

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Virginia Commonwealth University, 2007

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Epilepsy, defined by recurrent seizures, is the one of the most prevalent neurological disorders worldwide (World Health Organization 2007). While many forms of epilepsy are well-controlled by anti-epileptogenic medications, a significant portion of patients have intractable, i.e. untreatable, seizures. The etiology of these seizures is varied, but a significant cause, particularly for patients with intractable epilepsy is developmental malformation. In these cases, an error or interruption during the development of the neocortex produces a structural alteration. Such patients may have other neurological problems, but seizures are the most common symptom. The neuronal mechanisms that link malformation and cortical hyperexcitability are not well understood. Here we have

sought to examine potential mechanisms that result from microgyria, a malformation characterized by excessive numbers of small gyri.

The presence of epileptiform activity indicates that the normal balance of excitation and inhibition has shifted . Two functions of inhibition within neocortex are to prevent spread of excitation, and to modulate the timing of surrounding excitation. Although seemingly contradictory, increasing some forms of inhibition can result in an increase in synchronous excitatory activity. We hypothesize that for certain malformation epilepsies, the inhibitory processes that control timing are increased, creating a hyper-synchronous cortex, while the inhibitory processes that control horizontal spread are decreased, allowing the propagation of such activity.

Here we have examined the network effect of selectively modulating the inhibitory cells that control vertical or columnar cortical synchrony. This modulation is performed via activation of metabotropic glutamate receptors found on the vertically-projecting interneurons but not on those inhibitory cells that control horizontal spread of activity. Our results suggest that the network effect of activating these interneurons is altered in malformed, epileptogenic cortex.

Introduction

Epilepsy and Polymicrogyria

With the accumulation of scientific data concerning brain development with relevance to epilepsy, it has become clear that alterations in brain development may be related to, or causal of, epilepsy (Luders and Schuele, 2006; Crino, 2004; Mischel et al., 1995; Cendes et al., 1995).

The sequential development of the layers of the cortex has been shown to occur in a highly organized manner. It has long been established that layers form in an "inside-out" fashion with neurons destined for the deep layers of cortex migrating first while more superficial layers are established progressively by later migrating neurons that travel through the established layers (Jessell and Sanes, 2000; Chenn et al., 1997).

The development of the cerebral cortex can be seen as the coordinated interaction of three distinct activities or processes. The three processes are neuronal and glial proliferation or neurogenesis, neuronal migration, and neuronal differentiation or organization. While these events occur in a chronological order, there is extensive overlap between the three actions. Alteration of any of these events either alone or in combination can lead to a malformation of cortical development. The timing of the alteration regulates the type of consequent malformation seen in development. Disorders that have an impact on neuronal proliferation tend to result in abnormal cell size and morphology. Alternately, disorders affecting neuronal migration and organization tend to have an effect on neuronal positioning. Four-layered polymicrogyria (PMG), the malformation of interest in our study, is unique in the mechanism underlying its development. While other

malformations of cortical development are the result of an atypical developmental program, four-layered polymicrogyria is the result of cell death occurring within a specific time window during gestation, particularly, during migration (Andermann, 2000; Campos-Castello et al., 1999).

Polymicrogyria is a condition that is identified by the presence of excessive numbers of small but prominent gyri that result in an irregular cortical surface . However, in many instances the outer, molecular layer of the cortex fuses, giving the appearance of an abnormally smooth cortical surface in an MRI. The malformation specifically occurs following the death of cells destined for layers IV-VI. The result of the cell death is the loss of the deep lamina of the cortex while the more superficial layers remain intact (Rosen et al., 1998; Horikoshi et al., 1997; Dvorak et al., 1978). The neuronal cells that form after the time of insult migrate through the area of necrosis left by the cell death and ultimately find the appropriate destination. The final result of such a disturbance is a microgyrus in the cortex of the adult rat which contains layers I-III and the deep portion of layer VI (see fig. i). The malformed areas of cortex associated with this disorder cover a range of both histological varieties and areas of distribution. The spectrum of distribution ranges from unilateral to bilateral and, in some cases, syndromes associated with unique representations of polymicrogyria have been identified.

A variety of animal models of epileptogenic cortical malformations have been developed over recent years that accurately mimic the pathological characteristics of human developmental epilepsy (Chevassus-Au-Louis et al., 1999; Jacobs et al., 1999a). The models are similar to human epilepsies in both their histopathology and their tendency toward cortical hyperexcitability.

The induction of the 4-layered microgyria via cell death represents a unique mechanism of inducing a malformation not only in human pathology but in animal models as well (see Fig. A). The work of Dvorak and Feit showed that a cortical freeze lesion administered to a rat pup on P1 (postnatal day 1) will cause the death of most of the neurons present at that time, under the region of the probe. The neurons killed by the freeze lesion are neurons destined for layers IV, V, and VI. Consequently, in both human 4-layered microgyria and the animal models, the cortex develops lacking these particular layers (see Fig. i). The final result of the freeze lesion is a microsulcus exhibiting dyslamination, i.e. lacking layers IV, V, and the superficial portion of layer VI. The area of dyslamination is surrounded by normal-appearing, six-layered cortex (Dvorak and Feit, 1977).

The exact mechanism underlying the epileptogenesis associated with polymicrogyria remains unclear at this time. It has been shown through experimental induction of microgyri that the disruption of function associated with polymicrogyria extends beyond the visible area of insult. (Jacobs et al., 1999a). The existence of the extended area of disruption can be further supported by the rare success of surgery involving the removal of area of visible malformation (Sisodiya, 2000). Members of our lab have shown that, in the rat model of PMG, epileptiform activity is easiest to provoke in the areas of undisturbed cortex surrounding the malformation, the paramicrogyral zone (PMZ), and persists even with the removal of the microsulcus (Jacobs et al., 1999a). As will be discussed, subtypes of inhibitory interneurons have been shown to be differentially affected in models of PMG (Schwarz et al., 2000; Rosen et al., 1998). Furthermore, It has

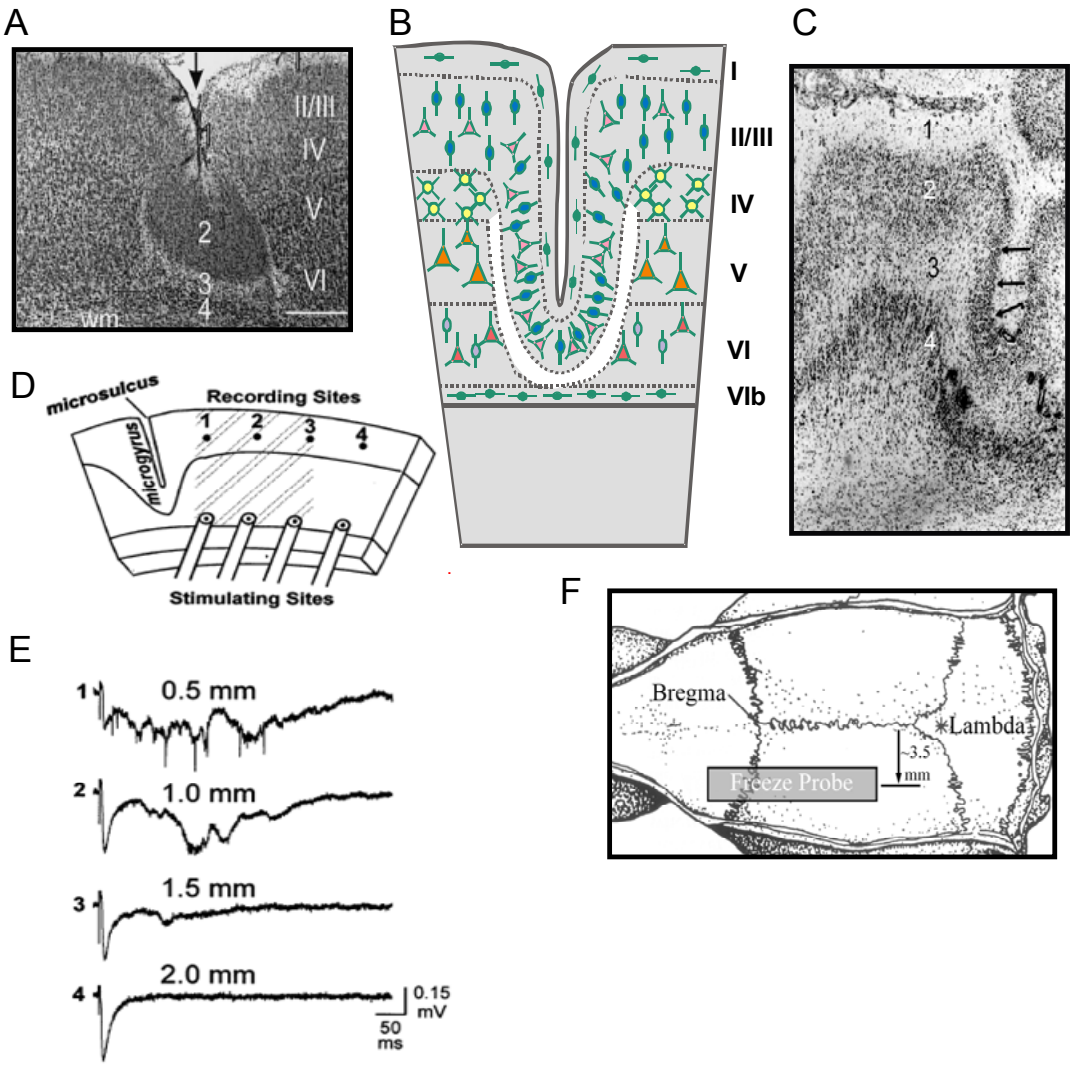


Fig. i The malformation and associated electrical activity. **A:** The induced microsulcus in the rat model labeled to illustrate the abnormal lamination in the microgyrus, as well as, the normal laminatation adjacent to the microsulcus (K.M. Jacobs *et al,* 1999, Epilsepsy Research, 36:165). **B:** An illustration depicting the abnormal arrangement of cortical lamina associated with the microsulcus. Note the existence of layers I-III, a cell sparse layer (white), and deep layer VI **C:** A micrograph from *Dysplasias of the Cerebral Cortex and Epilepsy* of the microsulcus in human cortex (Robain, O., 1996). **D-E:** Example of typical **(D)** electrode placement and (**E**) the corresponding recordings taken in cortex adjacent to the microsulcus (K.M. Jacobs *et al,* 1999, Epilsepsy Research, 36:165). **F:** Placement of probe for induction of freeze lesion modified from Paxinos & Watson, 1998, The Rat Brain in Stereotaxic Coordinates.

been demonstrated that activation of mGluR type I selectively activates one of the altered interneuron subtypes (Beierlein et al., 2000). The efforts described in this thesis are an attempt to further understand the mechanisms of epileptogenesis in the PMZ. Specifically, the changes in inhibition in the PMZ and the role of group I metabotropic glutamate receptors in relation to cortical synchrony is the focus of the study.

Interneurons: A General Overview

When broadly describing the coordinated interactions of neurons in the cortex, it is commonly recognized that glutamate and GABA are the two main neurotransmitters involved in cortical function. The GABAergic interneurons present within the cortex represent 20-30% of the neuronal population (Amaral, 2000). This population of inhibitory interneurons modulates cortical output in a variety of ways, but ultimately exhibits a major influence over the output functions of local networks and the cortex as a whole (Xu et al., 2004; Cherubini and Conti, 2001; Pouille and Scanziani, 2001; Krimer and Goldman-Rakic, 2001; McBain and Fisahn, 2001). Furthermore, interneurons have been shown to play a role in the determination of connectivity that occurs during the critical period of development (Hensch et al., 1998). Following development, one of the main roles of interneurons observed within the cortex is the formation of circuits that limit the spatial spread of excitation and create what is referred to as an *inhibitory surround* (Westbrook, 2000). Furthermore, the network of inhibitory interneurons is important in setting the overall tone of excitability in the cortex (Kobayashi and Buckmaster, 2003; McBain and Fisahn, 2001). Interneurons influence pyramidal cells through feedforward and feedback

inhibition (Pouille and Scanziani, 2001) and play a role in the development of network oscillations (Whittington et al., 1995).

A simple description of the functions of the inhibitory interneurons belies the complexity of the inhibitory network formed by these cells. The interneurons found within the cortex can be categorized into a vast array of subtypes by a number of different means. Different functional subtypes of interneurons can be distinguished based on: chemical markers (Kawaguchi, 1995), morphology (Markram et al., 2004; Kawaguchi, 1995; Lund and Lewis, 1993); electrophysiologically-identified, intrinsic and synaptic properties (Goldberg et al., 2004; Beierlein et al., 2003; Kawaguchi and Kubota, 1993), and connectivity of input sources (Beierlein et al., 2003). In most cases, these characteristics are best used together to confidently identify the interneuron subtype (Markram et al., 2004).

The characteristics elucidated through the identification and investigation of the different interneuron subtypes suggest that many of these interneurons fill a particular functional niche in the signaling pathways of the brain. Despite the complications, two interneuron subtypes have repeatedly been easily distinguished in a number of recent studies: (1) fast-spiking (FS) and (2) low threshold-spiking (LTS) interneurons. Studies suggest that these interneuron subtypes are differentially affected in our model of developmental epilepsy as such an alteration would potentially promote the production of epileptiform activity. An increase in the number of one subtype combined with a decrease in the second subtype may explain why global measures of inhibition show no effect in this

model (Hagemann et al., 2000) yet there is clearly an imbalance between excitation and inhibition.

Low Threshold Spiking Cells and Fast Spiking Cells: A Comparison

Not only do the different characteristics of FS and LTS interneurons allow for their identification, but these very characteristics also offer some insight into their unique roles. For the purposes of this thesis, a description of the differential characteristics of importance between FS and LTS interneurons shall include the 4 distinguishing attributes listed in the previous section: chemical markers, morphology, electrophysiology, and connectivity (see table 1).

Chemical Markers:

One of the first features identified to distinguish interneuron subtypes was the intracellular contents or chemical markers (Kawaguchi and Kubota, 1997). Calcium binding proteins (CBPs) and neuropeptides can be used to identify interneuron subtypes. As figure ii illustrates, the populations of cells that immunohistochemically stain for the neuropeptide somatostatin (SS) are distinct from those staining for the CBP Parvalbumin (PV) throughout the layers of the cortex (see fig. ii, Kawaguchi and Kubota, 1997). A third identifying marker, the CBP Calbindin (CB) has also been used to categorized interneuron subtypes, however some overlap exists between interneurons that stain for CB and those that stain for SS or PV(Kubota and Kawaguchi, 1997; Kawaguchi and Kubota, 1996). When the immunohistochemical findings are considered together, two effectively independent populations of interneurons can be identified as SS/CB interneurons and PV interneurons (Kawaguchi and Kondo, 2002; Kawaguchi and Kubota, 1997; Burkhalter and

	Staining	Typical Morphologies	Orientation	Inputs	Response
FS	PV (decreased in PMG)	DBC MC	Horizontal	TC Cortical	Strong Reliable Depressing
LTS	CB (Increased in PMG) SS	LBC ChC	Vertical	Cortical	Less Reliable Facilitating

Table 1- Summary of Differences Between FS and LTS Cells

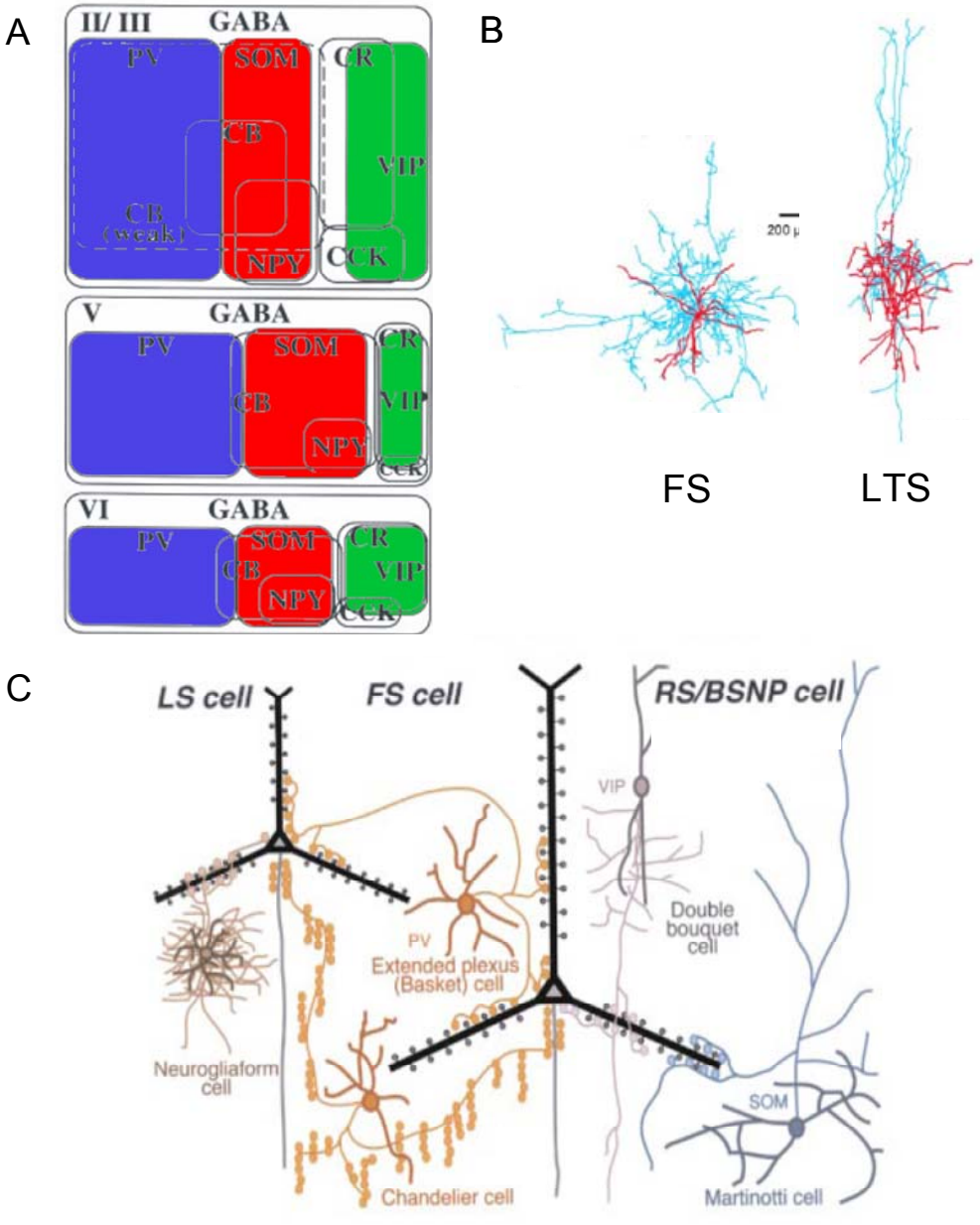


Fig. ii- Interneuron subtypres: distribution and morphology A: Venn diagram illustrating the arrangment of immunohistochemically distinct cell populations wand their distribution throughout the layers of the cortex (Kawaguchi, 1997). B: Typical FS and LTS cells filled with biocytin (Bacci, 2003). C: PV positive FS cells and SS positive LTS Cell (Kawaguchi, 1997)

White, 1997). These immunohistochemically distinct populations represent the two interneuron subtypes of interest.

Interestingly, CB and PV subtypes have previously been shown to be differentially affected in a number of epilepsy models. For instance, a study conducted by Garbelli *et al*, showed a significant reduction in the number of PV-immunoreactive interneurons in a cohort of surgical patients treated for intractable epilepsy (Garbelli et al., 2006). In the same study CB-immunoreactive interneurons showed no significant difference from normal cortex (Garbelli et al., 2006). A study based on the freeze-lesion model of epilepsy showed a 20% reduction in SS-immunoreactive interneurons (Patrick et al., 2006). However, this reduction did not develop until well after the onset of epileptiform activity, suggesting the death of these cells plays little or no role in development of epileptiform activity (Patrick et al., 2006).

As can be seen in figure iii, two separate studies conducted on freeze-lesion epilepsy models of epileptogenesis further suggest the differential alteration of immunohistochemically distinct populations of interneurons. In the first study, the number of PV immunoreactive cells decreased in layers IV and V of the PMG, as well as throughout the layers of the microgyral region (see Fig. iii, Rosen et al., 1998). In another study, the number of GABAergic, CB-positive cells increased (Patrick et al., 2006; see Fig. iii, Schwarz et al., 2000; Rosen et al., 1998). While the specific changes in interneuron populations reported in studies of epilepsy models are still being scrutinized, it is clear that these populations are differentially affected. This change suggests a differential alteration

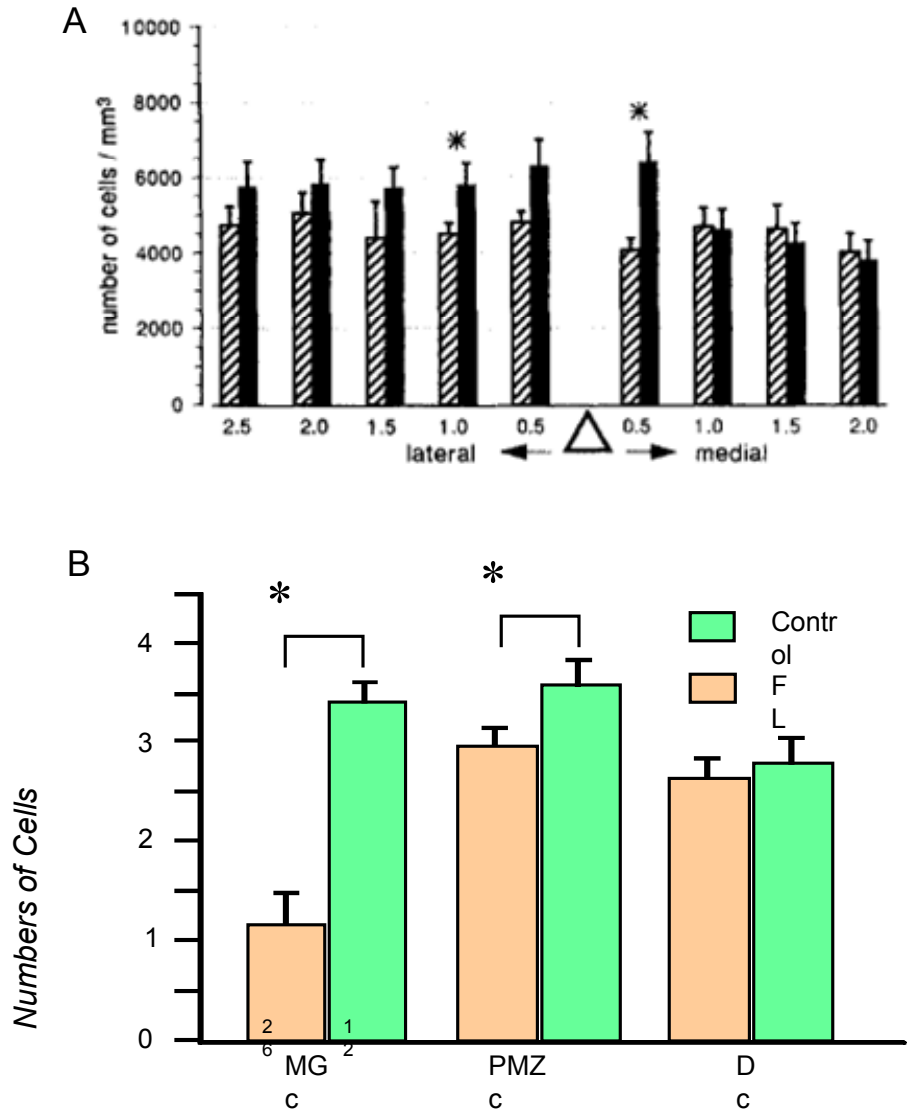


Fig. iii- Figures depicting the differential alteration in cells staining for different CBPs. A: Increased numbers of CB positive cells in the PMG cortex, adjacent to the microsulcus (Schwarz, 2000) B: Decreased numbers of cells staining for parvalbumin in the PMG model in both the microgyrus itself and the adjacent cortex (Rosen, 1998).

of LTS and FS interneurons in epileptogenic cortices and possibly a change in the overall function of the inhibitory system within malformed cortex.

It has been demonstrated that some PV-positive cells immunohistochemically stain for CB as well. Some SS cells also stain for CB (Markram et al., 2004; Kawaguchi and Kubota, 1997; Kawaguchi and Kubota, 1996). In studies of epilepsy models, it has been shown that PV interneurons decrease in number in cortex adjacent to the microsulcus (Rosen et al., 1998). It has also been shown that CB interneurons increase in number in the microgyrus itself and in the paramicrogyral cortex(Schwarz et al., 2000). This suggests that the number of SS/CB interneurons increase in epileptogenic cortex. An increase in SS/CB interneurons suggests an increase in the functional subtype of LTS interneurons while the decrease in PV/CB interneurons indicates a decrease in the functional subtype of FS interneurons.

Morphology and Orientation:

One of the simplest characteristics to distinguish interneuron subtypes from both pyramidal cells and from one another is the identification of the morphological features unique to each. Inhibitory interneurons have many common features, some of which distinguish them from pyramidal neurons. One morphological feature of interneurons that makes them different from pyramidal cells is aspiny dendrites (Douglas and Martin, 2004). A second feature that distinguishes inhibitory interneurons from pyramidal neurons is that their axons project laterally and vertically but these projections are limited and generally do not extend into white matter or distant parts of the brain. This feature of interneurons is

important as it suggests their role is limited to local circuitry within the cortex (Markram et al., 2004; Letinic et al., 2002).

The interneuron subtypes can be distinguished from each other based on the orientation of neuronal appendages (both dendritic and axonal, see Fig. B). While FS interneurons have horizontally-oriented dendrites and axonal arbors, those of LTS interneurons are vertically-oriented (Kawaguchi and Kubota, 1997; Kawaguchi, 1995; see Fig. B, Kawaguchi and Kubota, 1993). The result of this orientation is that LTS interneurons fall within the interlaminar-intracolumnar functional class of interneurons, while FS interneurons tend to be intralaminar-intercolumnar interneurons, (Markram et al., 2004). In addition, FS interneurons tend to synapse on other neurons' somata, or initial segment, while LTS interneurons tend to target neuronal dendrites (Kawaguchi, 1995; Kawaguchi and Kubota, 1993).

Some LTS interneurons have been conclusively identified as Double Bouquet Cells (DBCs) and Martinotti Cells (MCs) (Markram et al., 2004; Kawaguchi and Kubota, 1997) . These cells display a bitufted dendritic morphology and a distinctive, fascicular axonal projection (Somogyi and Cowey, 1981). The axonal collaterals seen on DBCs can extend across all layers and are thicker than the main stem of the axon (Markram et al., 2004). The DBCs are found in layers II-V with the greatest density occurring in the supragranular layers (Markram et al., 2004; Kawaguchi, 1995). DeFelipe and others showed that DBC's appear to be interleaved with pyramidal cells, inhibiting their basal dendrites (DeFelipe et al., 1990). The DBC's also appear to preferentially target dendrites. These cells stain for the CBP, CB (Markram et al., 2004; DeFelipe, 1997).

Another population of interneurons that stain for CB, are the MCs, found throughout layers II-VI of the neocortex. The MCs tend to project to layer I and inhibit the dendrites of the pyramidal cells found there. The axons of MCs have also been noted to spread horizontally in layer I and inhibit neighboring columns as the only source of cross-columnar inhibition in layer I from layers II-VI (Markram et al., 2004; DeFelipe, 2002). Infragranular MCs have also been shown to selectively target layer IV (Wang et al., 2004). Like DBCs, MCs have dendrites with a bitufted morphology, however, these dendritic trees tend to be much more complex than those seen in other interneurons (Markram et al., 2004).

The FS interneurons offer a contrast to the morphologies typical of DBCs and MCs as they project horizontally. Two morphologies typical of these interneurons are large basket cells (LBC's) and chandelier cells (Markram et al., 2004) LBCs are an example of interneurons that express the CBP and PV (Kawaguchi and Kondo, 2002). They have large, aspiny, multipolar dendrites and expansive axonal arborizations that can inhibit neurons in upper and lower layers and in neighboring and distant columns (Wang et al., 2002). ChCs are also interneurons that express PV (Kawaguchi and Kondo, 2002). These are multipolar or bitufted cells that synaptically target axons (Wang et al., 2002). The terminal portions of the axon form short vertical rows of boutons, resembling a chandelier (Markram et al., 2004).

Electrophysiology:

The definitive means for classifying both LTS and FS interneurons is the observation of each cell's response to intracellular injected current. The FS interneurons

are characterized by a fast, non-adapting firing pattern when depolarized. In contrast, LTS interneurons respond to a depolarizing stimulus with either a burst followed by adapting single action potentials or only adapting single action potentials (see fig. iv, Bacci et al., 2003; Kawaguchi, 1993). In addition, LTS interneurons tend to respond to a hyperpolarizing current pulse with a rebound burst of action potentials, occurring after the hyperpolarizing pulse ends. The characteristic firing pattern observed in LTS interneurons is the result of T-type calcium channels that remain inactivated at resting membrane potentials but become available for activation when the cell is hyperpolarized (Goldberg et al., 2004; 2000). These channels are then activated by depolarization from a hyperpolarized state.

Beierlein et al (2003) conducted a comprehensive study to reveal the unique electrophysiological characteristics of LTS interneurons as compared to FS interneurons. The results of this study further illustrate the stark differences in the electrical properties of LTS and FS interneurons. LTS interneurons are distinguished from FS interneurons by longer duration action potentials, larger input resistances, less negative resting potentials, and longer afterhyperpolarizations (Beierlein et al., 2003). Synaptic inputs to and output from LTS interneurons exhibit initially unreliable connections in response to a single stimulation pulse, however they display facilitation with continued stimulation. Over the course of a spike train, excitatory post-synaptic potentials (EPSPs) recorded in the LTS interneurons and inhibitory postsynaptic potentials (IPSPs) recorded in the cells with which they synapse increase in amplitude with a significant dependence on stimulus

frequency. The synaptic input to and output from FS interneurons displays much greater reliability than those of LTS interneurons (see fig iv, Beierlein et al., 2003).

Connectivity of Input Sources:

The FS neocortical neurons receive powerful synaptic inputs from the thalamus, while LTS interneurons receive almost no input from the thalamus (Beierlein et al., 2003; Beierlein and Connors, 2002). This suggests that it is the FS interneurons that determine the inhibitory surround for thalamic input to the neocortex. LTS interneurons are more exclusively dedicated to the neural activity of the local cortical circuit alone (see Fig. iv, Beierlein et al., 2003). The corticocortical connections of the LTS neurons suggest that they play a role in intracortical signal modification (see Fig. iv, Beierlein et al., 2003).

LTS interneurons and Synchronous Inhibition

Findings suggest that inhibitory interneurons play a role in the production of the rhythmic, synchronous activity seen within the cortex (Traub et al., 1999; Whittington et al., 1995). While synchronous activity is normal under certain circumstances, such as slow wave sleep (Steriade, 1997), abnormal, epileptiform activity is characterized by synchronization of large populations of excitatory neurons (2000; Avoli et al., 1999). Previous work has suggested that LTS interneurons can produce synchronous activity in surrounding pyramidal neurons (Beierlein et al., 2000). This synchronization was induced by activating the LTS interneurons with metabotropic glutamate receptor (mGluR) agonists. The mGluR agonists produce depolarization, and ultimately rhythmic firing in the LTS interneurons. Paired recordings of an LTS and pyramidal neuron showed that IPSPs occur in pyramidal neurons coincident with the action potentials in LTS

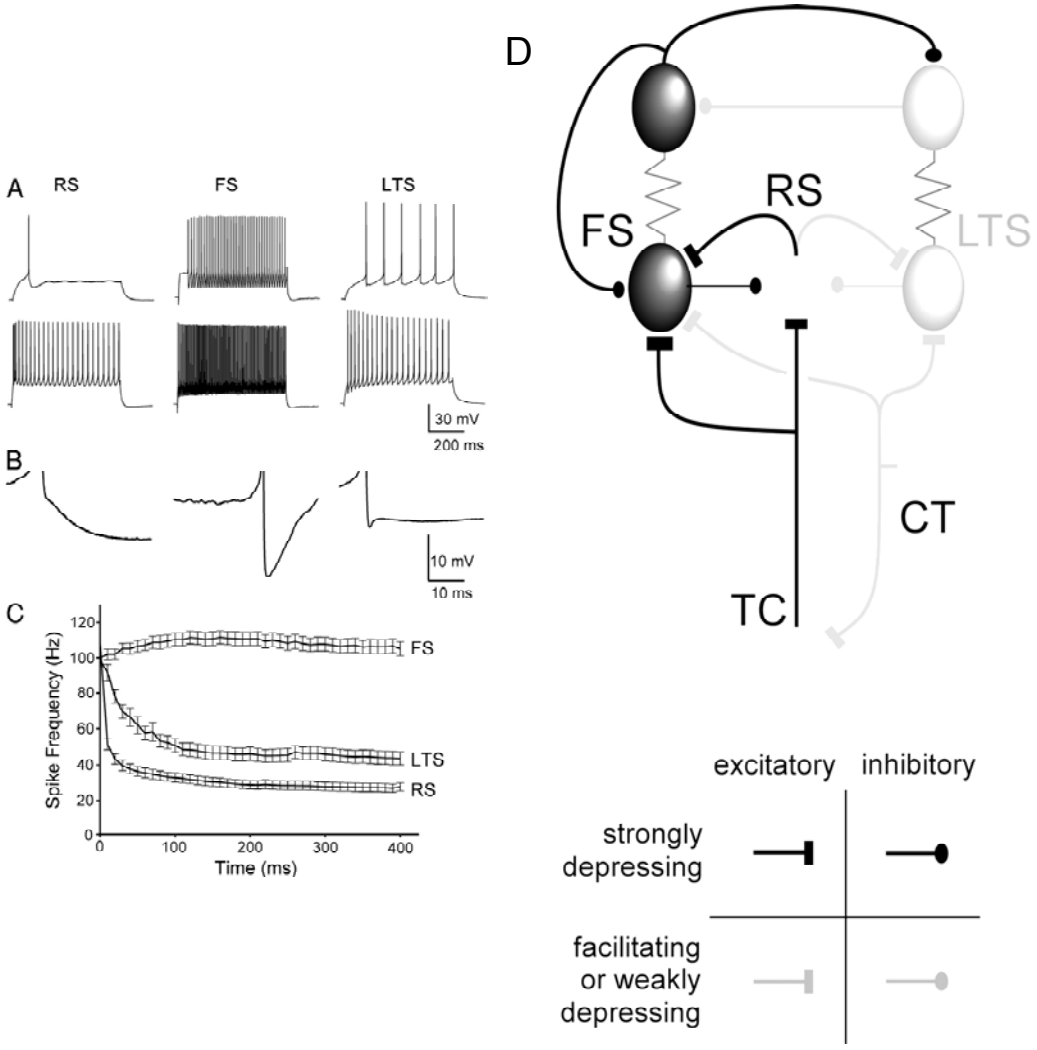


Fig. iv A: The recorded response of different cell types following stimulus input. B: The afterhyperpolarizations of the different cell types. C: Short term adaptation of evoked response in cell types D: The synaptic connections of LTS and FS interneurons and pyramidal cells in the cortex. note the lack of direct thalamic input to the LTS cells and the gap junctions connecting interneurons of the same subtype (Beierlein, 2003).

interneurons (Beierlein et al., 2000). These findings suggest that LTS interneurons play a role in local synchronization of surrounding pyramidal neurons within a cortical column.

Synchrony among excitatory neurons is a necessary prerequisite for epileptiform activity. Given the vertical orientation of the LTS interneurons and their increase in number suggested by the increase in immunohistochemically-labeled CB cells (Schwarz et al., 2000), in cortical malformations, it seems likely that an increase in LTS interneurons or their output could initiate an interlaminar-intracolumnar synchronous epilepileptiform discharge, that then spreads horizontally, due to a decrease in the number of FS/PV interneurons, observed in this model. Furthermore, some findings suggest that the excitatory input to FS interneurons is decreased while the excitatory input to LTS interneurons is increased in PMG cortex (George and Jacobs, 2006). All of the changes to LTS interneurons seen in epileptogenic cortex support the possibility that LTS interneurons play a role in the hyperexcitability and synchronization associated with epileptiform activity.

Glutamate and Glutamate Receptors in Brief

Glutamate is the major excitatory neurotransmitter in the mammalian CNS; its actions are mediated by ligand-gated ion channels (ionotropic receptors) and G-protein coupled (metabotropic) receptors. Activation of these receptors is responsible for basal excitatory synaptic transmission and many forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD), that are thought to underlie learning and memory. These receptors have also become potential targets for therapies for CNS disorders such as epilepsy.

The ionotropic glutamate receptors are ligand-gated ion channels. The binding of glutamate to ionotropic receptors results in the direct activation of the channels and the depolarization of the cells where they are located. The metabotropic glutamate receptors differ from other glutamate receptors in the nature of events that occur following their activation. Glutamate receptor activation results in the initiation of intracellular, second messenger systems (Horikoshi et al., 1997). These two types of receptors interact in the fine tuning of neuronal responses under different conditions (Lujan et al., 1997).

Metabotropic Glutamate Receptors

The mGluRs are made up of at least seven subtypes that have been subdivided into three groups, based on sequence similarity, pharmacology and intracellular signaling mechanisms. Group I consists of receptors made of (1) mGluR1 or mGluR5 subunits . Group II consists of receptors made of (2) mGluR2 and mGluR3 subunits. Finally, group III consists of receptors made of (3) mGluR4, mGluR6 and mGluR7 subunits. Group I Metabotropic glutamate receptors stimulate phosphatidylinositol (PI) hydrolysis and Ca2+ signal transduction and, in some cell types, Phosholipase C stimulation or the formation of cAMP (Conn and Pin, 1997; Hayashi et al., 1993).

Recently the localization of group I mGluRs has suggested that the subtypes, mGluR1 and mGluR5 activate different cell types (Beierlein et al., 2000). In particular, activation of mGluR1 has been shown to induce synchronous spiking in networks of LTS interneurons. The unique localizations of each group I receptor subtype are the reason for our interest in this particular group.

Lopez-Bendito and colleagues conducted research that illustrated differential localizations seen between the Group I subtypes during development (Lopez-Bendito et al., 2002b). At P0 both mGluR1 and mGluR5 were seen within the cortex in varying, low levels. These levels increased until P21, at which time adult concentrations were reached. During the first week of postnatal development, mGluR1 was detected at very low levels, mainly in layer I. The mGluR1 expression was seen on cells that resemble Cajal-Retzius cells (an inhibitory interneuron subtype). Over the course of development, the layer I levels decreased while levels detected in layers II-VI increased until P21. The mGluR1 detected later in development was found mainly on interneurons expressing bipolar or multipolar morphology, typical of SS or LTS type interneurons (Lopez-Bendito et al., 2002a).

The changing expression of mGluR5 throughout postnatal development differs from that of mGluR1. During the first postnatal week neurons of layers II and III showed no mGluR5 immunoreactivity and only a diffuse distribution was seen in layers IV-VI. However, apical dendrites from pyramidal cells in deeper layers were labeled for mGluR5 (Lopez-Bendito et al., 2002a). By postnatal week three, neuropilar staining of mGluR5 could be seen in layers I-IV with the same intensity of staining seen in all layers following this period. In the adult animal, mGluR5 immunolabeling was seen exclusively in the neuropil surrounding cell bodies of both pyramidal and non-pyramidal cells (Lopez-Bendito et al., 2002b).

The highest concentration of group I mGluRs occurs perisynaptically and along the extrasynaptic membrane. Studies have also shown that both mGluR1 and mGluR5

receptors display the highest receptor density in the perisynaptic annulus around the postsynaptic membrane specialization (Lujan et al., 1996).

In some cases mGluRs have been shown to occur in the same cells that express intracellular markers indicative of LTS interneurons. For example, Stinehelfer et al found that 70-80% of cortical cells that stained positive for mGluR1α were also positive for the peptide somatostatin. These neurons were also found to stain positive for mGluR1. This double-labeling suggests a high abundance of mGluR1 occurring on LTS interneurons within the cortex (Stinehelfer et al., 2000). In contrast, Lopez et al found that mGluR5 appeared mainly in pyramidal cells with some occurrence in interneurons (Lopez, 2002). It is this distribution of mGluR1 that allows for the selective activation of LTS interneurons with little effect on neighboring cells. In other words, Stimulation of mGluR1 would allow for the selective observation of LTS cell activity.

A Rationale for the Project

Developmental cortical malformations are a common trait exhibited by many patients suffering from intractable epilepsy (World Health Organization, 2007). While the correlation between malformations and epilepsy has been established, the specific mechanisms underlying the onset of epilepsy remains unclear.

Within normal cortical circuitry, inhibitory interneurons play a role in the extent of spread of excitation and are responsible for the specificity with which signals are conducted through the cortex. An alteration in the distribution of interneurons within malformed cortex could be responsible for the abnormal electrical activity associated with such malformations. Research has shown that interneurons that stain for PV and those that

stain for SS and CB represent two distinct populations. Horizontally-oriented FS interneurons stain for PV while vertically-oriented LTS interneurons stain for SS/CB (Markram et al., 2004; Kawaguchi and Kubota, 1997; Kawaguchi, 1995; Kawaguchi and Kubota, 1993). Additionally, our preliminary evidence suggests that these immunohistochemically distinct populations change differentially following the induction of an epileptogenic malformation (Schwarz et al., 2000; Rosen et al., 1998). The interneurons that stain for SS/CB increase in malformed cortex (Schwarz et al., 2000), while the PV interneurons decrease in number in malformed cortex (Rosen et al., 1998).

We originally hypothesized that stimulation of mGluR1 receptors would activate LTS/SS specific interneurons and lead to an increase local synchrony in a way that could be measured with field potentials. Complicating this study is the fact that no selective agonists for mGluR1 receptors are commercially available. Thus we had to use an agonist that activates both mGluR1 and mGluR5 receptors and in separate experiments, antagonists that are selective for either mGluR1 or mGluR5 receptors to attempt to tease apart the role of enhancing LTS interneuron activity through mGluR1 receptors. Surprisingly, the results did not suggest that mGluR activation increased local synchrony at the network level, as measured by field potentials. However, our results do suggest that mGluR receptors produce different network effects in PMG relative to control neocortex. We expect that subsequent studies will further illuminate the nature of this difference.

Methods and Materials

Animals and Surgery

All experimental procedures were performed in accordance with protocols approved by Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC). A transcranial freeze lesion was performed on a total of 85 Sprague-Dawley rats on postnatal day (P) 1, using the following procedure. Prior to the surgical procedure, general anesthesia was induced via hypothermia by surrounding the rat pup with ice for a duration of 4-6 minutes until response to tail pinch was absent. The anesthetized rat was then removed and placed in a shallow ice bath for the duration of the procedure. Following the application of betadine to skin over the skull of the rat, an incision was made in the skin in the anterior-posterior direction to expose the left half of the skull overlying the somatosensory cortex. The freeze lesion probe was chilled to -50° C in solid Carbon Dioxide and maintained at this temperature for the duration of the procedure. The probe used consisted of a metal bar that tapered to a flattened, rectangular end (5x2 mm) that was applied to the rat pup. The cold probe was applied for 5 seconds to the exposed skull over the rat pup ~3.5mm lateral to the centerline of the skull with the rostral portion of the probe overlying Bregma (see Fig. i). Following application of the probe, the incision was sutured and the rat pups were placed under a warming lamp until ambulatory and exhibiting proper skin color. Antibiotic ointment was then applied to the

wound and the pup was returned to the dam within 2 hours. Post-operative examination and weighing of the pups was conducted to insure proper recovery from the procedure.

Preparing Cortical Slices

At age P12-P20 the rat was anesthetized using isoflurane until tail pinch yielded no response. Following the administration of anesthesia the rat was decapitated and the brain removed. During the procedure the exposed brain was immediately covered with 0°C sucrose slicing solution containing (in mM) 234.0 Sucrose, 11.0 Glucose, 2.5, KCl, 1.25 $NaH_2PO_4 \cdot H_2O$, 10.0 $MgSO_4 \cdot 7H_2O$. 0.5 $CaCl_2 \cdot 2H_2O$, and 26 $NaHCO_3$. Upon removal the brain was transferred directly to a 0°C sucrose slicing solution bath maintained at a pH of 7.4 when saturated with 95% 0_2- 5% CO_2 gas and consisting of the same composition described previously. The site of the freeze lesion was identifiable as a small depression in the pial surface.

The brain was prepared for slicing by removing the brain portions rostral, caudal, and ventral to the area of interest. The brain was then placed in a Pelco 102 Vibratome Sectioning System™ using Krazy Glue™ as an adherent. During slicing the brain was kept immersed in the ~0°C sucrose slicing solution. Coronal slices of 300-400µm thickness were made through the cortex containing the microsulcus and areas adjacent to it. Slices were made from homologous regions of control cortex. Following sectioning, the slices were placed immediately into an oxygenated, normal aCSF solution containing (in mM) 126.0 NaCl, 3.0 KCl, 1.25 $NaH_2PO_4 \cdot H_2O$, 2.0 $MgCl_2 \cdot H_2O$, 2.0 $CaCl_2 \cdot 2H_2O$, 10.0 Glucose, and 26 $NaHCO_3$. The slices were kept in the solution at 34°C for 45 minutes and room temperature from that point onwards.

Electrophysiology

After slices were allowed to return to room temperature they were transferred via pipette to a Harvard Apparatus interface slice chamber, that maintained a constant temperature of 34°C, a constant flow of 95% 0_2- 5% CO_2 gas over the surface of the slice, and a constant flow (~1 ml/min) of normal aCSF over the slice. Glass pipettes (3-10 MΩ), pulled on a Narishige model PP-830 pipette puller and filled with 1.0 M NaCl were used for recording field potentials. Two recording electrodes were placed in a line orthogonal to the overlying pia, with the first electrode in superficial neocortical layers (0.4-0.6 mm deep to the pia) and the second placed in layer V (1.1-1.4 mm deep to the pia). In slices from freeze lesioned animals, this array of electrodes was placed ~ 1.0 mm from the sulcus, a location that has previous been shown to be epileptogenic. The array was placed in homologous cortex in slices from control (unlesioned) animals. The signal from the first recording electrode was amplified using a Cygnus ER-1 amplifier, with a gain of 1000X, and low pass filtered at 3K. The signal from the second recording electrode was first amplified using AXON Instruments AxoClamp 2B, low pass filtered at 3 K. This provided a gain of 10X, so the signal was then taken to a Cygnus FLA-01, to reach an overall gain of 1000X. A stimulating electrode was placed below the deeper of the two recording electrodes on the layer VI/ white matter border, 'on-column' (in a line orthogonal to the pia) with the recording electrodes. Electrical pulses were applied using a World Precision Instruments (WPI) DS 8000 Digital stimulator in conjunction with a WPI 360 Stimulus Isolator. Signals were digitized with an Axon Instruments Digidata 1322A and recorded with Axon Instruments Clampex software.

A given slice was deemed viable if a 0.6 mV peak field negativity could be elicited at any current less than or equal to 10 mA with a square pulse width of 0.32 sec or less. Furthermore, the response recorded at the superficial recording electrode was required to exhibit a 0.2 mV response at less than or equal to 10 mA with a pulse width of .04 ms or less. The current at which the superficial electrode exhibited the 0.2 mV response was designated as *threshold* current, and current amplitude was maintained at this level for the remainder of recordings from the slice. Stimulus intensity changes were then applied by altering the pulse width. In order to detect the incidence and amplitude of any intrinsic epileptiform activity, for each slice an epileptiform search protocol was administered that consisted of stimulating the slice at threshold (threshold current at 0.02 ms pulse width) 1/30 sec for 5 minutes.

In order to measure the intrinsic excitability of the neuronal network at the site of the recording electrodes, a stimulus intensity protocol was applied. This consisted of pulses at 0.02, 0.04, 0.08, 0.16, and 0.32 ms pulse widths at threshold current level. This series was repeated 3X and the responses averaged at each stimulus intensity level.

In order to test the effect of various mGluR agents, the following drug protocol was utilized. The slice was stimulated at the middle intensity (0.08 ms), at a rate of 0.033 Hz, for 5 min prior to drug application and 25-45 min after switching to aCSF containing the drug of interest. With our flow rate, the drug reached the slice chamber ~5 min after switching the solution. Following the drug file protocol, both the epileptiform search protocol and the stimulus intensity protocol were repeated while the drug-containing aCSF was still being applied to the slice.

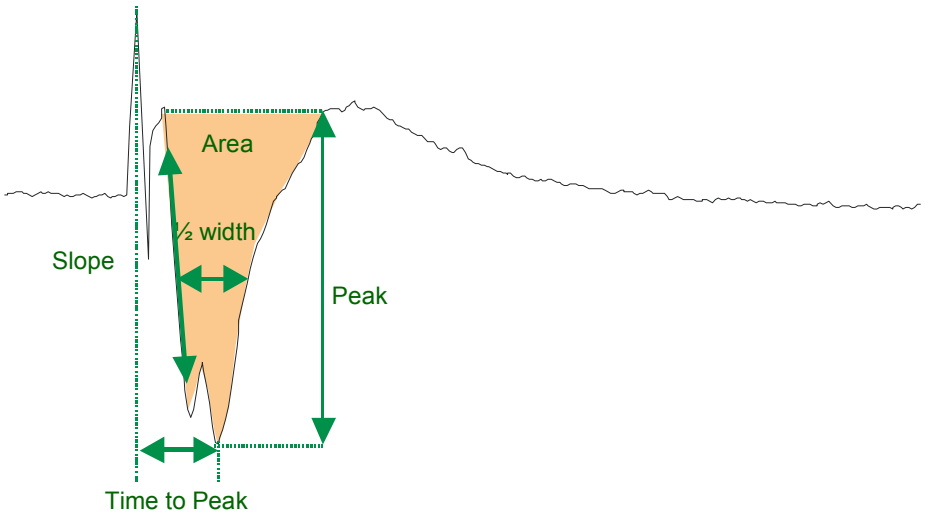
Drug Administration

To examine the effect of mGluR group I receptor agents, the following drugs were applied alone: (1) Group I agonist, (S)-3,5-dihydroxyphenylglycine (DHPG, 10 μM); (2) the mGluR1 antagonist R,S-1-aminoindan-1,5dicarboxylic acid (AIDA, 30 mM) and the mGluR5 antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP, 10μM). These concentrations have previously been shown to be effective at activating or blocking the receptors (George and Jacobs, 2006; Kim et al., 2006; Fazal et al., 2003).

Data Analysis

Field potentials were analyzed using Igor Pro software (Wavemetrics). For the drug profile, every 5 consecutive responses were averaged (a 2.5 min period). The following characteristics were measured for the short latency field potential (35 msec from field onset): peak, area, time-to-peak (TTP), halfwidth, and slope (see Fig. v). Percent change from the first 2.5 min period (pre-drug) was then calculated for individual slices. These values were then averaged for each group (Control superficial layers, Control deep layers, PMG superficial layers, PMG deep layers).

A



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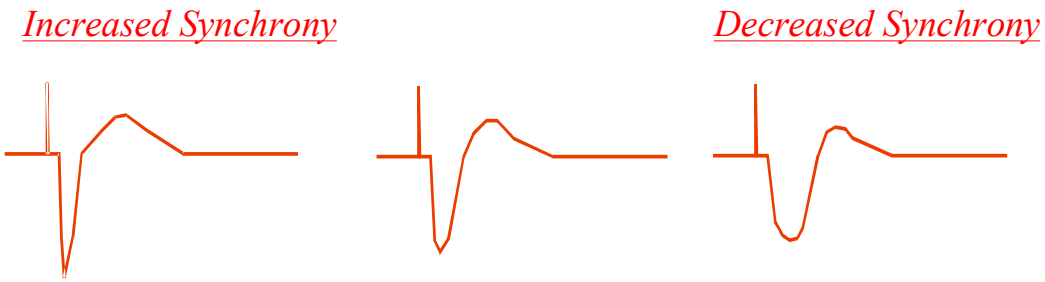

Fig v Typical field potential and measurable characteristics A: An example of a typical excitatory field potential with measureable characteristics indicated. B: An illustration a a field potential and the changes indicating a decrease or increase in local synchrony.

Results

Comparison of Control and PMG field potentials in normal aCSF

Analysis of the stimulus intensity series in normal aCSF consisted of averaging the sweeps for all of the *pre-drug* files at each intensity. Although we attempted to normalize the data obtained across slices by starting with a field potential of the same peak negativity amplitude (0.2 mV) at threshold stimulus intensity (20 μsec, and lowest current), there was still some slight variation in the resulting amplitude. To compensate for this, we compared the excitability of control and PMG slices, by normalizing the values for each slice to the value obtained at threshold stimulating current. 2-way ANOVA tests were performed using the Control and PMG and superficial and deep layers as the fixed fixed factors.

Superficial Layers

The comparison of characteristics of the evoked short latency field potential negativity measured during the stimulus intensity series for superficial layers can be seen in figure 1. In the superficial layers, both area and peak of the field were significantly greater in PMG slices than in control slices at all measured intensities at or above 2x the threshold intensity (2-way ANOVA, $p=0.002$ for area and 0.001 for peak). Likewise, halfwidth values in the superficial layers of the PMG slices were significantly greater than in control slices at all intensities $\geq$2x (2-way ANOVA, $p=0.001$). The PMG superficial

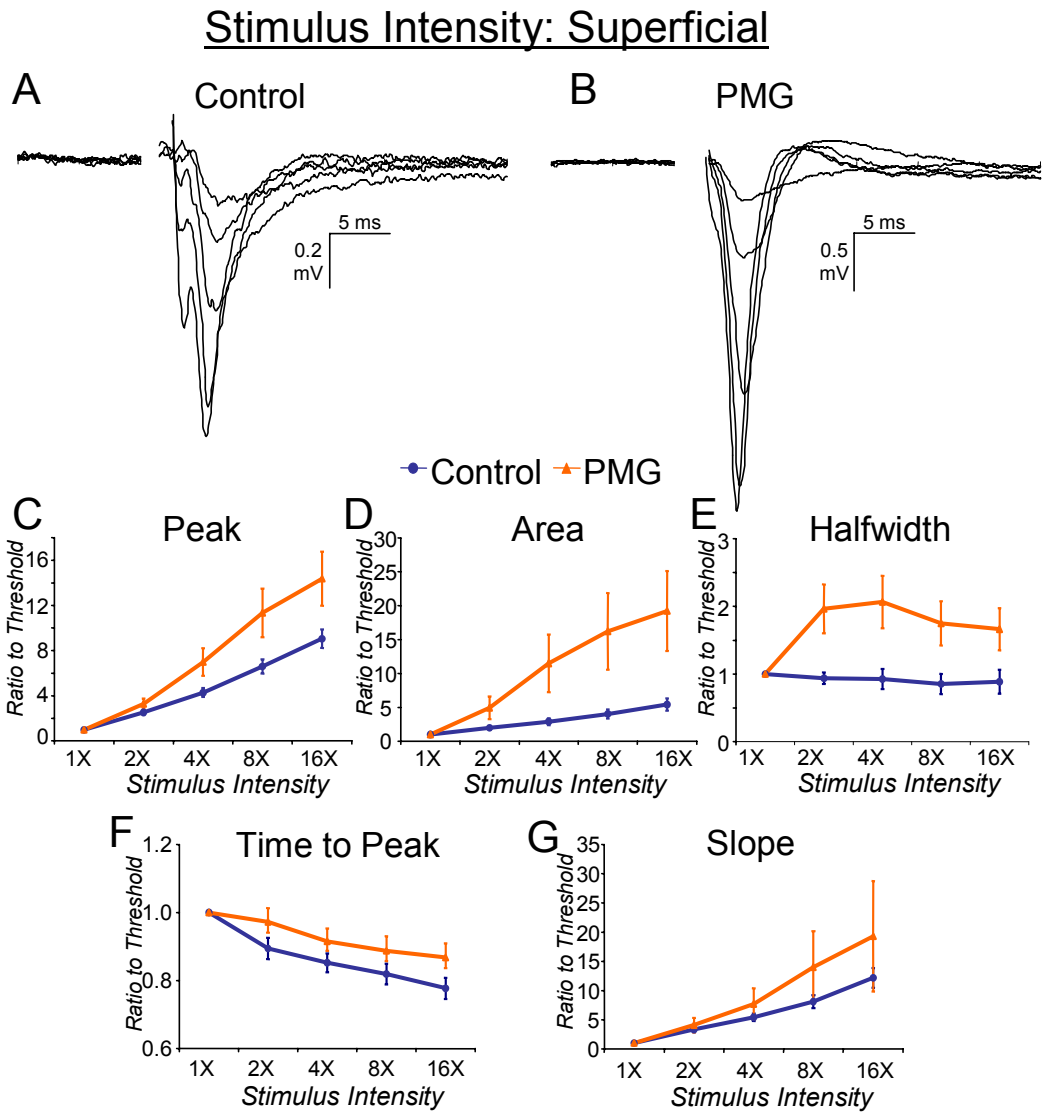

Fig. 1 Comparison of field potential recordings from superficial layers of control and PMG cortex in normal aCSF. **A-B:** Typical examples of evoked field potentials recorded during stimulus intensity series. Traces shown are averages of 3 sweeps at each stimulus intensity, recorded in superficial layers of control slices (A) and PMG slices (B). **C-G:** Changes in characteristics of the evoked field potential presented as a ratio to that recorded at the threshold stimulus intensity. Blue = control (20 slices), orange = PMG (20 slices). The characteristics shown are: (C) peak, (D) area, (E) halfwidth, (F) time-to-peak, and (G) slope.

layers also showed greater TTP values than the same layers in control slices ($p=0.01$). There was no difference in slope for PMG compared to control slices. For both peak and time to peak, there was a significant effect of stimulus intensity, however in all cases, there was no interaction between stimulus intensity and experimental group (control vs PMG).

Deep Layers

The comparison of characteristics of the evoked short latency field potential negativity measured during the stimulus intensity series for the deep layers can be seen in figure 2. As in the superficial layers, there was a significant increase in peak, area, and halfwidth values recorded at all intensities $\geq$2x in the PMG slices relative to the controls (2-way ANOVA, $p=0.002$). There was a significant effect of stimulus intensity on the measures of peak and area, but in no cases was there an interaction between stimulus intensity and experimental group. There was no difference seen between the deep layers of the control and PMG slices in either slope or TTP measurements.

Drug Applications

Analysis of the drug files consisted of averaging of the sweeps for each 2.5 minute period over the course of the recording. All data presented represents measurements made on these averages. Within the following section the term *pre-drug* will refer to the sweeps averaged between 5 to 2.5 minutes, prior to the application of the drug. The term *late-drug* refers to the time period 35-37.5 minutes after beginning the drug application (drug will reach the chamber 5 min after beginning application). All results are reported as the mean ± the standard error of the mean.

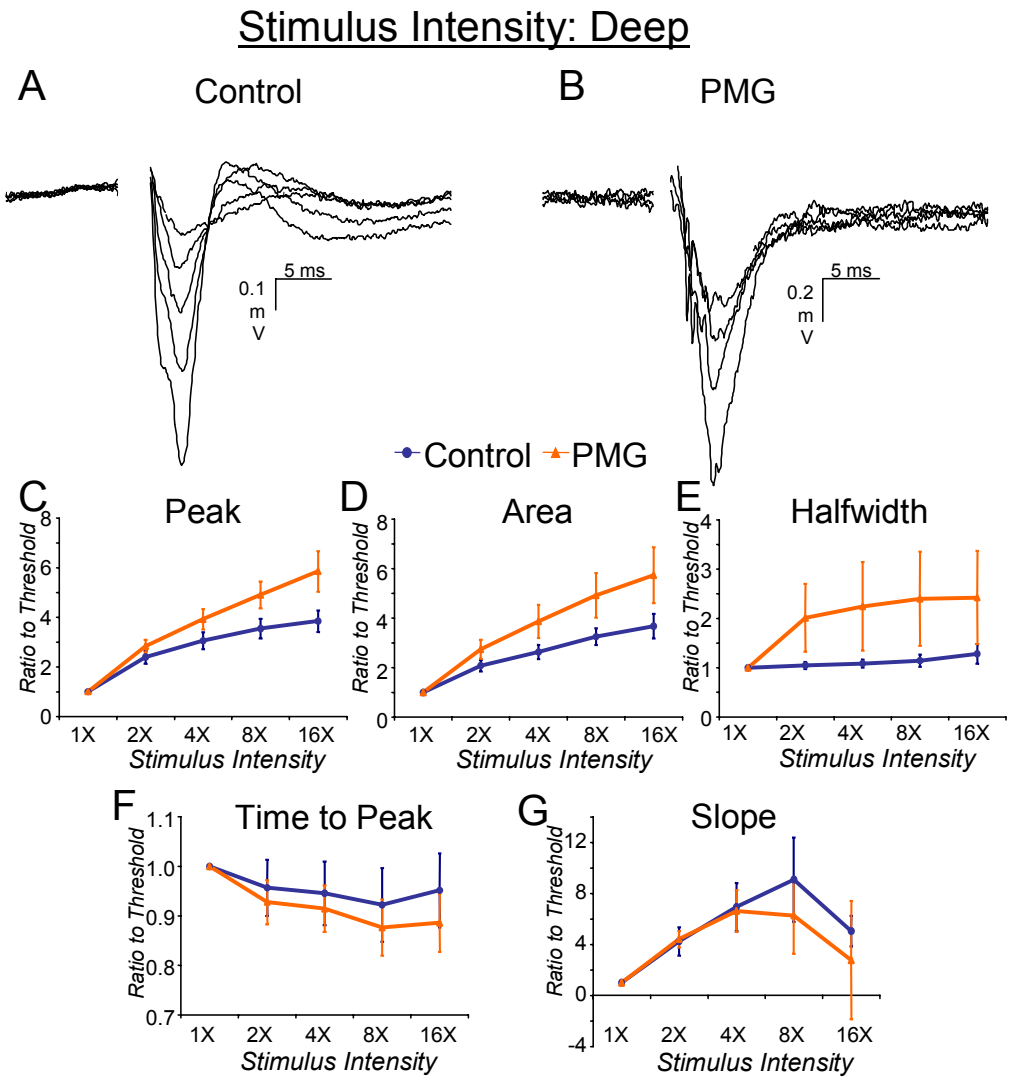

Fig. 2 Comparison of field potential recordings from deep layers of control and PMG cortex in normal aCSF. **A-B:** Typical examples of evoked field potentials recorded during stimulus intensity series. Traces shown are averages of 3 sweeps at each stimulus intensity, recorded in deep layers of control slices (A) and PMG slices (B). C-G: Changes in characteristics of the evoked field potential presented as a ratio to that recorded at the threshold stimulus intensity. Blue = control (20 slices), orange = PMG (20 slices). The characteristics shown are: (C) peak, (D) area, (E) halfwidth, (F) time-to-peak, and (G) slope.

Effect of DHPG (mGluR Group I Agonist) in Control Slices

In the control slices subjected to the solution of 10 μM DHPG, significant changes were observed in several characteristics of the field potentials (see Fig. 3). In both the deep and superficial cortical layers the peak field negativity decreased during application of DHPG (Figs. 3A, C, E). A comparison of pre-drug versus late-drug peak amplitude showed a significant decrease of 17±6% in superficial layers and 26±11% in deep layers (t-test, $p<0.05$). Despite the decrease in peak, within superficial layers there was an increase in the overall area (59±35%, t-test, $p<0.05$) and an increase in the halfwidth (19±11%, t-test, $p<0.05$). These changes reflected an enhancement of the late part of the field, despite the decrease in the peak negativity (Fig. 3A). These changes in the late part of the field were not observed in deep layers, where overall there was decrease in and slowing of the response. The area of deep field negativity was decreased by 24±15% (t-test, $p<0.05$), while the time to peak was increased (12±6%, t-test, $p<0.05$), and the initial slope of the field negativity was decreased by 34±10% (t-test, $p<0.05$, Fig. 3C, E, F).

Effect of DHPG in PMG Slices

Overall, DHPG had less of an effect on recordings from PMG slices compared to the effects in control slices (Fig. 3B, D, E, F). In PMG superficial layers, there was no effect on the peak field negativity. In contrast to the increase in area seen in control slices, for PMG slices, there was a slight but significant *decrease* in the area (7±4%, t-test, $p<0.05$). The only other change in the PMG superficial field potential was a slight but significant decrease in the initial slope (6±4%, t-test, $p<0.05$). At the PMG deep recording site there was significant decrease in the peak of the field potential negativity (14±4%, t-

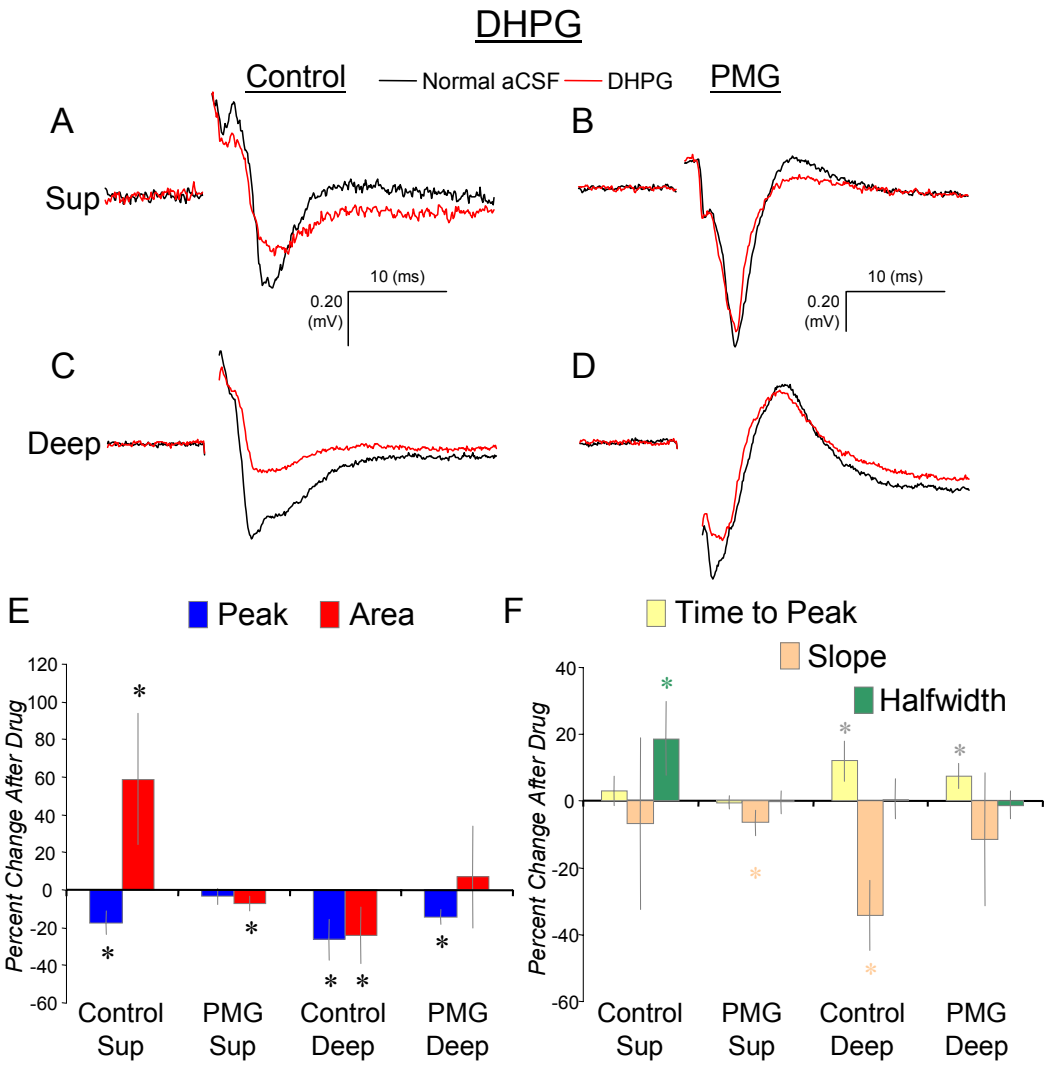

Fig. 3 Effects of DHPG application on characteristics of field potentials. **A-C:** Typical examples of evoked field potentials recorded during drug trial. Traces shown are averages of the first (black) and last (red) 10 recordings taken during the drug trial from: (A) superficial control; (B) superficial PMG; (C) deep control; and (D) deep PMG cortical layers. **D-E:** Average percent change in characteristics of the evoked field potential following application of 10 uM DHPG, for 14 control and 22 PMG slices.

test, $p<0.05$) and an increase in the time to peak (8±4%, t-test, $p<0.05$), similar to the effect observed in the control deep recording. In contrast to control slices, for the PMG deep recording site, DHPG had no effect on area or slope. There was also no effect of DHPG on the halfwidth for the PMG deep field potential.

Effect of AIDA (mGluR1 antagonist) in Control Slices

If the effects observed after DHPG application were mediated by mGluR1 receptors *and* if there is enough glutamate released under normal slicing conditions to activate these receptors, then we would expect that application of AIDA alone would generally show the opposite effect of that for DHPG. For control slices, this was mostly true (Fig. 4). In both superficial and deep layers of control slices, 30 μM AIDA added to the bathing medium caused an increase in the peak field potential negativity (12±5% and 9±2%, respectively, t-tests, $p<0.05$ for both, Fig. 4A, C, E). In general, the effects observed after AIDA application were modest, suggesting that these receptors may not be completely activated with ambient levels of glutamate under slice conditions, or that 30 μM may not completely block these receptors. This concentration of AIDA did not appear to significantly affect the late component of the short latency field negativity in superficial layers. AIDA produced an increase in the area of the superficial control field potential (11±3%, t-test, $p<0.05$) that appeared to be due primarily to the increase in peak amplitude. AIDA did not produce an effect on any of the measures of timing (TTP, halfwidth, slope) for the control superficial field.At the control deep recording site, the increase in peak was the only significant effect, although there was a trend toward an increase in area, but too much variability for significance (Fig. 4E).

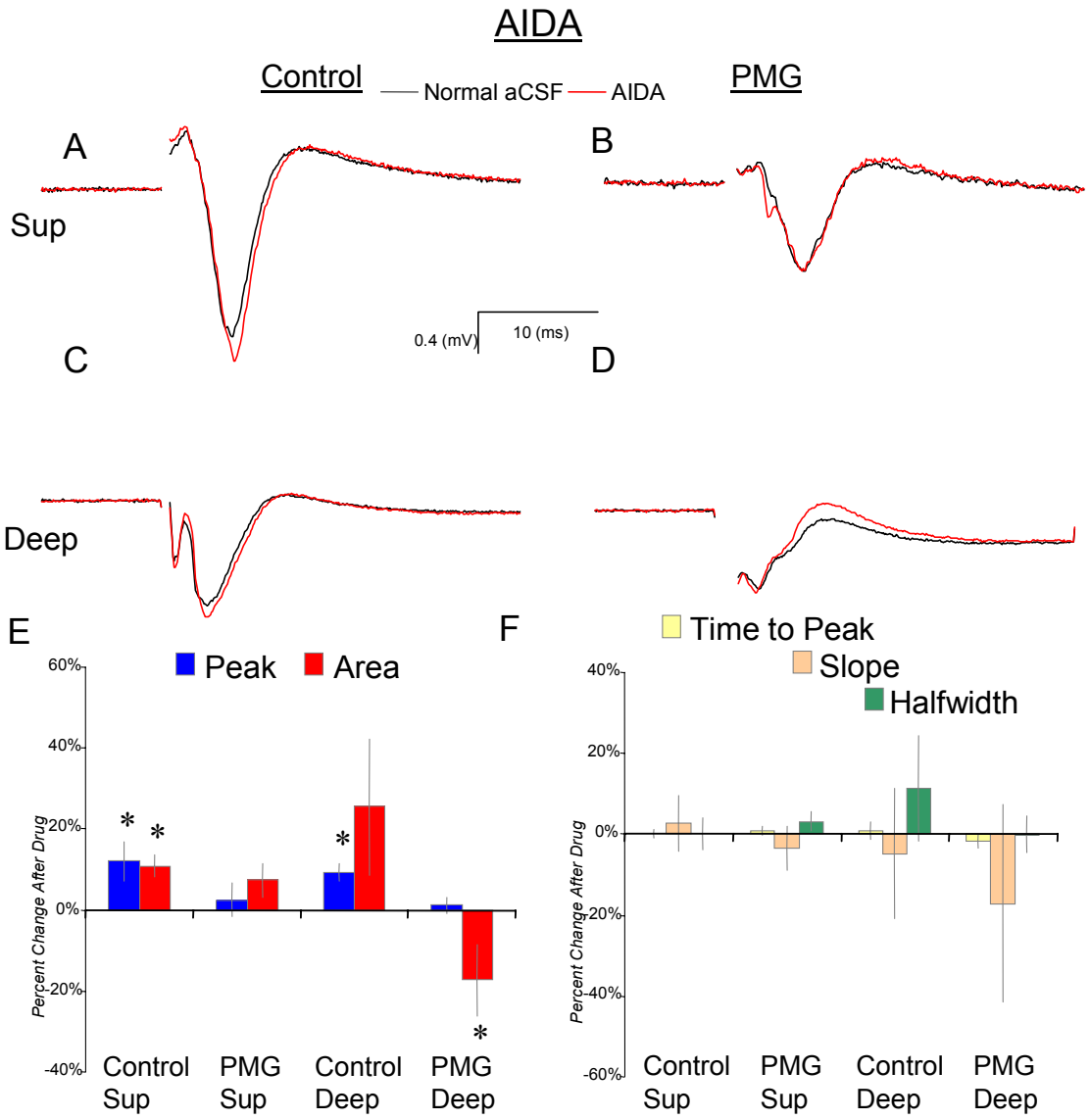

Fig. 4 Effects of AIDA application on characteristics of field potentials. **A-C:** Typical examples of evoked field potentials recorded during drug trial. Traces shown are averages of the first (black) and last (red) 10 recordings taken during the drug trial from: (A) superficial control; (B) superficial PMG; (C) deep control; and (D) deep PMG cortical layers. **D-E:** Average percent change in characteristics of the evoked field potential following application of 30 uM AIDA, for 13 control and 14 PMG slices.

Effect of AIDA in PMG Slices

Following the administration of AIDA to PMG slices, field potentials displayed no significant changes in the characteristics of peak, TTP, slope, or halfwidth regardless of the layers from which they were recorded (Fig. 4B, D, E, F). However, area decreased by 17±9% (t-test, $p \leq 0.05$) in the deep layers of the PMG slices following AIDA administration. In the superficial layers, area showed no significant change in value from the pre-drug time period to the late-drug time period.

Effect of MPEP (mGluR5 antagonist) in Control Slices

Application of 10 μM MPEP did not affect either peak or area of the field potential in control slices at either superficial or deep recording sites (Fig. 5A, C, E). This drug did however affect the timing of the field recorded in superficial layers only (Fig. 5F). The TTP was increased by 12±4%, while the halfwidth was increased by 22±10% (t-tests, $p<0.05$ for both). These effects appeared to be due to an increase in the late component of the field potential, similar to the effect produced by DHPG. The fact that these effects were in the same direction as those produced by DHPG, suggests that DHPG may be having dual effects on the late superficial field, mediated by different receptor subtypes.

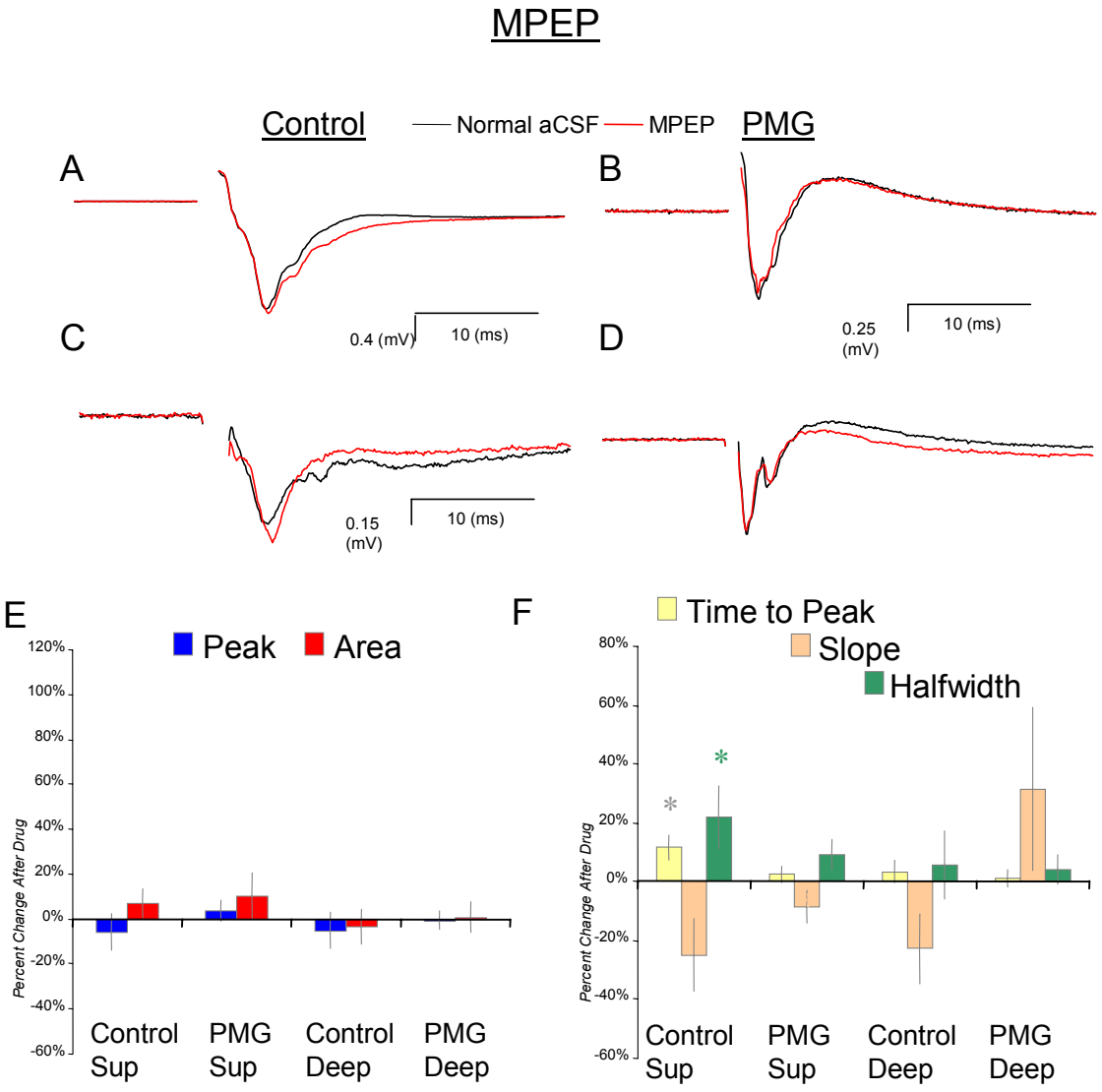

Fig. 5 Effects of MPEP application on characteristics of field potentials. **A-C:** Typical examples of evoked field potentials recorded during drug trial. Traces shown are averages of the first (black) and last (red) 10 recordings taken during the drug trial from: (A) superficial control; (B) superficial PMG; (C) deep control; and (D) deep PMG cortical layers. **D-E:** Average percent change in characteristics of the evoked field potential following application of 10 uM MPEP, for 9 control and 12 PMG slices.

Effect of MPEP in PMG Slices

In both the superficial and deep layers of PMG cortex, no significant changes were observed in any measures of the field potential negativity after application of MPEP (Fig. 5B, D, E, F).

Discussion

Pre-drug Comparison of Control and Lesioned Slices

The data gathered from the pre-drug administration of the stimulus intensity protocol was used to compare differences in excitability and local synchrony between the slices. It is well accepted that the evoked neocortical field potential negativity primarily reflects excitatory synaptic input onto the apical dendrite of pyramidal neurons, and that it is difficult to directly discern inhibitory postsynaptic potentials from this type of recording. The amplitude of the field potential negativity, however will be a reflection of the sum of excitatory and inhibitory synaptic activity (Mitzdorf, 1985; Nicholson and Freeman, 1975). Additionally, the characteristics of the field potential reflect the overall timing of the synaptic activity (see Fig. E). Previous studies utilizing intracellular recordings have demonstrated that the result of stimulation within neocortex produces within pyramidal neurons, first an excitatory postsynaptic potential (EPSP), then an inhibitory postsynaptic potential (IPSP) that overlaps the EPSP (Westbrook, 2000).

The EPSP is due, largely, to α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors activated by glutamate, and by the voltage dependent Ca^{2+} channels (g_{Ca}). This EPSP causes the early depolarization of the neuron. The IPSP occurs only slightly later and is responsible for the hyperpolarization of the neuron. The IPSP is the result of the activation of GABA receptors and calcium-

dependent K^+ channels (gK, Westbrook, 2000). Changes in the timing or amplitude of the EPSP or IPSP will have an impact on the overall postsynaptic potential of the cell and, ultimately, the recorded field potentials (see Fig .vi).

Despite the fact that this freeze-lesion model of microgyria has been recognized as useful for studying epilepsy associated with malformation for more than 15 years, no previous description of the differences in the short latency field negativity in PMG compared to control cortex has been published. Not surprisingly, the overall excitability of PMG cortex appears to be greater than that of control cortex, as indicated by greater percent increase in peak, amplitude, and halfwidth of the field negativity with increasing stimulus intensities (see fig. 2). These changes were significant in both superficial and deep layers. All of these changes indicate a general increase in excitatory activity or a decrease in overall inhibitory tone. The increased excitation indicated by the field potentials could be the result of the changes in the components of the postsynaptic potentials discussed above.

A possible explanation for the alteration of excitability is an change in the numbers or distribution of receptors found on the pyramidal cells. Zilles *et al.* demonstrated a 10-20% decrease in $GABA_A$ receptors in all layers of cortex adjacent to the microsulcus (Zilles et al., 1998). The decreased number of GABA receptors could also explain why the level of excitation was greater in the PMG cortex. Another possible cause of increased excitation is an increased number of excitatory synapses in PMG cortex. Previous work by Zsombok and Jacobs has shown that the excitatory afferent hyperinnervation of pyramidal, *but not inhibitory* neurons is an active and ongoing process prior to onset of

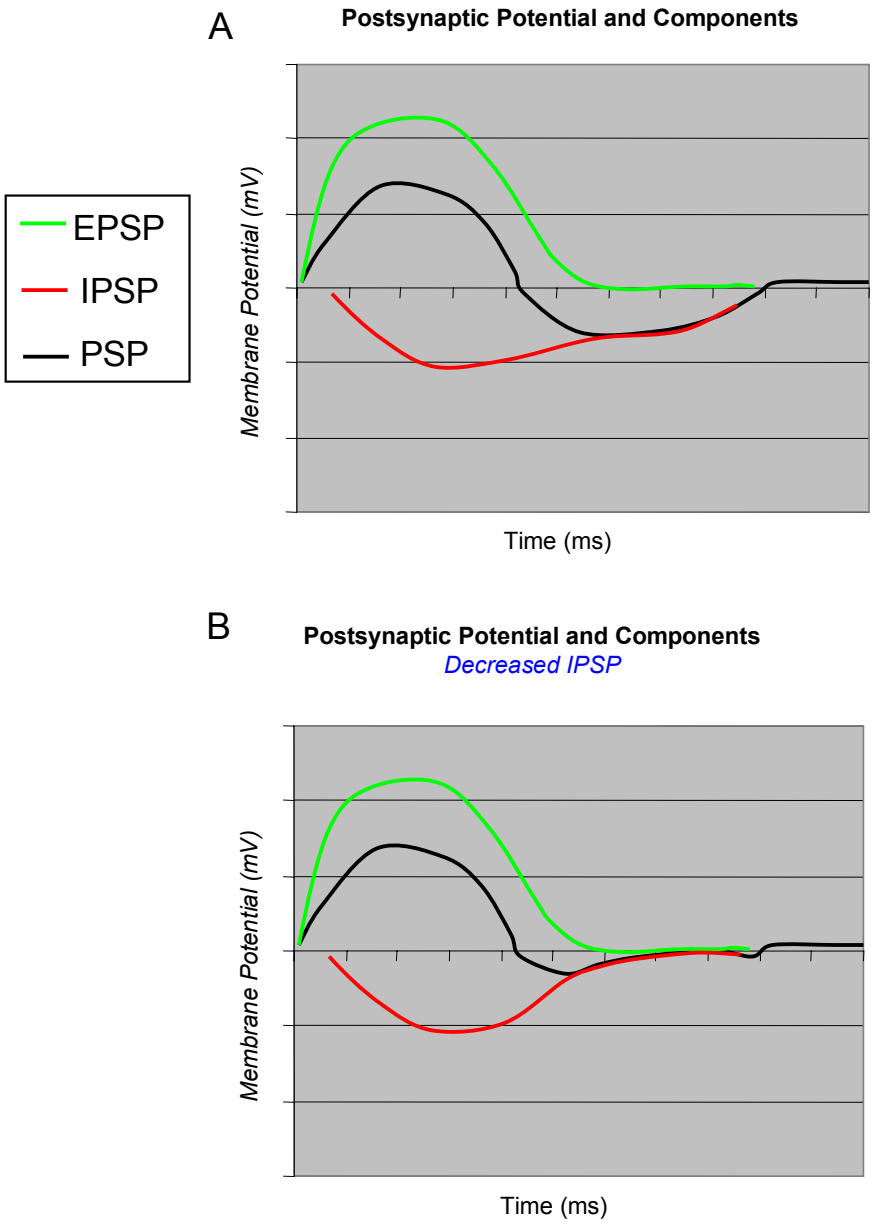

Fig. vi Drawing to illustrate the timing of inhibitory and excitatory postsynaptic currents. A: The green line shows an EPSP in isolation, the red line shows an IPSP in isolation. The black line shows what would be recorded as the sum of these two potentials. B: The same diagram, but with a decrease in the late IPSP component.

epileptogenesis (Zsombok and Jacobs, 2004). This hyperinnervation could be responsible for the more powerful field potentials we recorded. A final cause of the increased excitation seen in the PMG cortex is a potential increase in the number of excitatory, glutamate receptors. It has been shown that the NMDA subtype of receptor is enhanced in the PMG cortex, although the most dramatic increase is seen within the microgyrus itself (McDonald et al., 1990; Bode-Greuel and Singer, 1989; Monaghan et al., 1985). Also, the AMPA subtype of glutamate receptor is increased both within the microgyrus and in the PMG. However, the AMPA increase is not necessarily isolated to pyramidal cells so the effect of this alteration is debatable (Kharazia et al., 1998).

The increase in TTP for the superficial recording site in PMG compared to control slices was surprising, and in contrast to other data collected in the lab simultaneously (Pham & Jacobs, unpublished observations). One possible explanation for these discrepant results is a different alignment of the superficial electrode relative to the stimulating electrode. The measure of TTP will increase with distance from the activation site for neocortical field potentials. The distortion of the lamination due to the malformation, may cause slight errors in aligning the three electrodes in a plane perpendicular to the pia, although recordings were typically made far enough from the site of the malformation that the lamination and pial course was obvious and normal. To further investigate this and determine if the issue is alignment of the electrodes, we have begun experiments in which the superficial layer recording electrode is moved in 100 mm steps lateral and medial away from the 'online' site above the stimulating electrode. Further analysis will be necessary to

determine whether these differences were due to alignment or true changes in the projections to the superficial layer neurons.

The Drug Files

The application of DHPG to control slices caused a significant decrease in the peak field potential negativity for both superficial and deep layers (see Fig. 3). Work in both our lab (George and Jacobs, 2006) and other labs (Beierlein et al., 2000) has shown that group I mGluR agonists, such as DHPG significantly increase the activity of interneurons and the frequency of IPSCs recorded in pyramidal neurons. Thus, the decrease in field potential peak observed with the current experiments is likely due to an increase in inhibitory synaptic input that when summed with excitation, produces a smaller field potential negativity.

While the effect of DHPG on the peak of the field potential negativity was similar for both superficial and deep layers, the effect on timing measurements of the field potential was different for superficial versus deep recordings sites in control slices (see Fig.3). In the superficial layers of the control slices the increase in area and halfwidth of the field negativity, combined with a decrease in peak, may suggest a desynchronization of local activity produced by DHPG. These changes indicate that the excitatory activity may have essentially spread out over time. Another possibility is that the late aspect of the field negativity was selectively increased. The LTS interneurons that are activated by DHPG tend to make chemical synapses with FS interneurons, as well as with pyramidal neurons (Gibson et al., 1999). Gibson et al (1999) used paired cell recordings to determine the

intercellular connections between inhibitory LTS and FS cells. They found that inhibitory interneurons of the same type are often paired electrically. Inhibitory chemical synapses were seen between FS cells, as well as, FS and LTS cells. LTS cells rarely form inhibitory synapses on one another (Gibson et al., 1999). This arrangement supports the possibility that activation of LTS cells could cause inhibition of FS cells (see Fig. iv, Gibson et al., 1999). Thus, inhibiting an inhibitory interneuron could ultimately lead to an increase in expression of excitation (see Fig. vi). It is unclear why the timing would affect only the late part of the field, however it is possible that that the effect also occurs earlier but is masked by the simultaneous more direct increase in inhibition from LTS interneurons.

In the deep layers of the control slices, DHPG produced a less synchronous onset of excitatory activity. This decrease in synchrony is illustrated by the increased TTP and the decrease in slope and peak. An overall decrease in the level of excitatory activity measured in the deep layers of the control slices is indicated by the decreased area and peak of the field potentials. The decrease in the overall response seen in the control, deep layers could be indicative of increased inhibitory tone and, consequently, a decrease in the number of excitatory neurons available for activation. A possible account for the increased inhibitory tone is the fact that DHPG would activate the mGluR1 on the LTS subpopulation of inhibitory interneurons (Stinehelfer et al., 2000). The decrease in synchrony is surprising because we expected the activation of the LTS interneurons to synchronize the excitatory activity of the pyramidal cells. We expected increased inhibition to translate into increased synchrony of excitatory elements, but mediated by LTS interneurons, as shown by the Connors group, and described above. The activation of mGluR5 on pyramidal cells could

account for this unexpected result (Lopez-Bendito et al., 2002a). What we expected was that LTS interneurons would inhibit pyramidal cells in a synchronous manner. We expected this inhibition to be strong enough to limit pyramidal cell activity to the temporal gaps in the inhibition by the LTS network. With mGluR5s activated on pyramidal cells, the inhibitory input from the LTS network may not be strong enough to completely block excitation of pyramidal cells. Effectively, the increased excitability caused by the mGluR5 activation may have offset the increased inhibition produced by the LTS cell activeation. It is also possible that this type of synchrony does not translate to a large enough network to be observed in a field potential type of recording. Another possibility is that an increase in synchrony occurred but was masked by the more dramatic changes in overall increased level of inhibition.

The application of DHPG to PMG slices produced some changes similar to those seen in the deep layers of the control slices. The PMG superficial layers showed a decrease in area and slope similar to the control deep recording site. The decreased area represents a possible decrease in the overall level of excitatory activity. The decreased slope could be an indicator of decreased synchrony of the excitatory elements responsible for the early component of the field potential. The decrease in peak and increase in TTP seen in the PMG deep layers indicates a reduction in excitation or increased inhibitory tone and a possible reduction in synchrony. Changes seen in both layers of both PMG and control slices could be entirely explained by a decrease in excitatory activity, but may also be indicative of a decrease in synchrony of excitation.

The overall reduced effectiveness of DHPG in PMG cortex was surprising, since other members of the lab have simultaneously shown that the percent increase in IPSC frequency after DHPG application is greater in individual pyramidal neurons of PMG compared to control cortex (George and Jacobs, 2007). One possible reason that an increased activation of LTS interneurons might not lead to an overall greater effect on the inhibitory network is the loss of electrical connections between LTS cells. As described in the introduction, Connors group has shown that application of mGluR agonists activates LTS interneurons, subsequently induces oscillations in these cells, during which the cells fire in oscillating bursts. This network activity is likely mediated by the electrical connections between the LTS interneurons (Amitai et al., 2002) .This is in turn is therefore likely necessary to enable the subsequent synchrony of surrounding pyramidal neurons. Work done by other members of my lab has shown that there is a possible reduction in these electrical connections between interneurons in PMG cortex (Pham and Jacobs, 2007). Such a reduction in gap junction-mediated electrical connectivity would hinder the LTS networks' ability to become excited in a synchronous manner. If this is the case, increased activation of individual inhibitory neurons would not have the same overall network effect. Another possible explanation for this altered network effect of DHPG is that the pattern of synaptic connections in PMG cortex may be altered (Jacobs et al., 1999b), a change that could also account for the differential changes in levels of excitation seen in PMG and control cortex.

The effects of AIDA were generally the opposite of those produced by DHPG, while MPEP had little effect. These results suggest that most effects observed after DHPG

were mediated by the mGluR1 receptors, and therefore likely due to changes in activation levels of LTS and similar vertically-oriented interneurons.

The fact that AIDA alone produced a change in the field potential indicates that ambient levels of glutamate in control slices that are high enough to activate mGluR1s. The lack of change seen in the PMG slices, along with other work done in our lab, suggests that PMG slices may be less sensitive to AIDA blockade than controls (George and Jacobs, 2007). The increased peak and area seen in the control, superficial layers could be the result of decreased excitation of the LTS interneurons and the resulting decrease in inhibitory tone. In the deep layers of the PMG slices, the opposite change in area occurred. This effect could be explained partially by the decreased sensitivity to AIDA blockade mentioned above (George and Jacobs, 2007). However, a decrease in sensitivity to AIDA blockade would be expected to yield no change in excitability, not a decrease. The decrease in excitability is unexpected and may be the result of altered distribution of mGluR1 in PMG cortex. The alteration of mGluR1 distribution in PMG cortex has not been shown and this idea remains purely speculative.

Significant changes seen with the application of MPEP were minimal although some common trends were seen between the different recordings. An increase in halfwidth and TTP in the control, superficial layers suggests a change in the timing of the excitatory activity. However, in these recordings there is no indication of changes in the level of excitation, i.e. peak or area. These findings suggest a desynchronizing effect of MPEP in superficial control layers. The mGluR5s are found predominantly on pyramidal cells within the cortex (Lopez-Bendito et al., 2002a). Consequently, the desynchronizing effect

caused by the mGluR5 antagonist could be a result of the decreased excitability of pyramidal cells, since mGluR5 receptors function to excite these cells. The trends seen in PMG superficial layers following MPEP application suggest changes similar to those seen in control superficial slices.

The results as a whole strongly indicate an alteration of network connections in the PMG cortex. Previous work done by my lab and others has shown that mGluR group I agonists cause synchronous activity of LTS interneurons (George and Jacobs, 2006; Beierlein et al., 2000). Application of DHPG failed to produce changes in field potentials suggestive of an increase in synchrony in either control or PMG cortex, however a significant reduction in excitability in control slices suggests that inhibitory interneurons were activated in a fashion that affected the network observed in the field potential recording. The failure of similar effects in PMG cortex could be linked to a failure in proper connectivity and synchronizing mechanisms of mGluR-sensitive interneurons.

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World Health Organization. World Health Organization web site . 2007

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