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Characterization and development of a stroke-induced model of acquired epilepsy in organotypic hippocampal slice cultures: role of the cannabinoid CB1 receptors in modulation of neuronal excitation and inhibition

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CHARACTERIZATION AND DEVELOPMENT OF A STROKE-INDUCED MODEL OF ACQUIRED EPILEPSY IN ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES: ROLE OF THE CANNABINOID CB$_1$ RECEPTORS IN MODULATION OF NEURONAL EXCITATION AND INHIBITION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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LIST OF ABBREVIATIONS

4-AP	4-aminopyridien
AE	Acquired epilepsy
AEA	arachidonylethanolamine ("anandamide")
2-AG	*sn*-2-arachidonylglycerol
AM251	*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carbamoxide
AM404	(all Z)-*N*-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide
AM630	6-iodo-pravadoline
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APV	2-amino-5-phosphonopentanoic acid
ATP	adenosine triphosphate
ºC	degrees Celsius
Ca^{2+}	calcium ion
cAMP	cyclic adenosine 3', 5' –monophosphate
CB_1	cannabinoid receptor subtype 1
CB_2	cannabinoid receptor subtype 2
CNS	central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CP55,940	(-)-*cis*-3-[2-hydroxy-4-(1,1-dimthylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol
CP232871	1-(2-chlorophenyl)-4-cyano-5-(4-methoxyphenyl)-1H-pyrazole-3-carboxylic acid phenylamide
DAG	*sn*-1-acyl-2-arachidonolylglycerol
DG	dentate gyrus of the hippocampus
DSE	depolarization induced suppression of excitation
DSI	depolarization induced suppression of inhibition
EEG	electroencephalogram
EGTA	glycol-bis(2-aminoethylether)-N,N,N2,N 2 tetraacetic acid
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
FAAH	fatty acid amide hydrolase
γ	gamma
GABA	γ-aminobutyric acid
GD	gestational day
GPCR	G-protein coupled receptor
GTP	guanine-5'-triphosphate
$[^{35}S]GTP\gamma S$	guanylyl-5'-[(γ-^{35}S)]thio-]triphosphate
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HNC	hippocampal neuronal culture
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential

JZL184	4-nitrophenyl 4-[bis(1,3-benzodioxol-5-yl)(hydroxy)methyl]piperidine-1-carboxylate
K^+	potassium ion
KA	kainic acid (kainate)
LY320135	4-[[6-Methoxy-2-(4-methoxyphenyl)-3-benzofuranyl]carbonyl]benzonitrile
LTD	long-term depression
LTP	long-term potentiation
μM	micromolar
MAGL	monoacylglycerol lipase
Mg^{2+}	magnesium ion
mGluR	metabotropic glutamate receptor
min	minute
mM	millimolar
mRNA	messenger ribonucleic acid
mV	millivolts
n	sample size
Na^+	sodium ion
NMDA	*N*-methyl-D-aspartate
O-1812	(*R*)-(20-cyano-16,16-dimetyldocosa-cis-5,8,11,14-tetraenoyl)-1'-hydroxy-2'-propylamine
OGD	oxygen glucose deprivation
OHSC	organotypic hippocampal slice culture
pA	picoamps
PF-3845	N-(pyridine-3-yl)-4-(3-(5-(trifluoromethyl)pyridine-2-yloxy)benzyl)piperdine-1-carboxamide
QX-314	N-(2,6-Dimethylphenylcarbamoylmethyl)triethylammonium bromide
RT	room temperature
SE	status epilepticus
S.E.M.	standard error of the mean
sec	seconds (s)
SR141716A	*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride
SREDs	spontaneous, recurrent epileptiform discharges
Δ^9-THC	delta-9-tetrahydrocannabinol
TTX	tetrodotoxin
URB597	cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester
WIN55,212-2	*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-yl](1-napthalen-yl)methanone mesylate

ABSTRACT

CHARACTERIZATION AND DEVELOPMENT OF A STROKE-INDUCED MODEL OF ACQUIRED EPILEPSY IN ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES: ROLE OF THE CANNABINOID CB$_1$ RECEPTORS IN MODULATION OF NEURONAL EXCITATION AND INHIBITION

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Stroke is the most common cause of acquired epilepsy in persons 35 and older. The massive increase in extracellular glutamate during stroke causes a cascade of intracellular events that can lead to cell death or the molecular changes that initiate the development of epilepsy. In addition, many studies point to a modulatory role of the endocannabinoid system in controlling seizures. Animal models of stroke induced-acquired epilepsy have been difficult to develop. Therefore, this dissertation was initiated to develop an organotypic hippocampal slice culture model of acquired epilepsy and examine the changes in distribution and function of the endogenous CB$_1$ receptor system. We utilized 4-aminopyridine and glutamate to induce separate excitotoxic injuries to slice cultures. Both injuries produced significant cell death acutely following the injury. After

a latency period, we observed a significant increase in the number of slice cultures that displayed electrographic seizures in both injury models. Western blot analysis demonstrated that the cannabinoid CB_1 receptor protein was significantly upregulated following injury with glutamate. Immnohistochemical studies demonstrated that this receptor upregulation was likely specific to the glutamatergic terminals. Electrophysiological experiments were performed to study endocannabinoid modulation of inhibitory and excitatory signaling in the CA3 pyramidal cells. We demonstrated that depolarization induced suppression of excitation (DSE) was enhanced in slice cultures that had undergone glutamate injury. This indicated that the upregulation of CB_1 receptors following glutamate injury was physiologically functional, as it enhanced cannabinoid control of the excitatory signaling. These studies support the hypothesis that there is a functional alteration of CB_1 receptors in the epileptic state that acts to suppress seizures. The development of an organotypic hippocampal slice culture model of stroke acquired epilepsy provides a unique tool to study the neuronal plasticity changes associated with epileptogenesis. It also provides a practical model to study pharmacological agents that may be useful in preventing or treating epilepsy.

GENERAL INTRODUCTION

A. Epilepsy

Epilepsy is a debilitating neurological condition that affects persons worldwide. It is one of the oldest conditions known to mankind, characterized by recurrent seizures. The word "epilepsy" is derived from the Greek word "epilambanein" which means "to seize or attack" (Kobau *et al.*, 2005). Normal brain function relies on interconnected neuronal networks in the brain to generate synchronized activities. A transformation of otherwise normal brain rhythms may lead to epileptic seizures, resulting from sudden, usually brief, excessive electrical discharges in neurons in different parts of the brain (Lutz, 2004). The clinical manifestations of seizures will vary and depend on where in the brain the disturbance first starts and how far it spreads. Symptoms such as loss of awareness or consciousness, disturbances of movement, sensation, mood or mental function may occur transiently (Kobau *et al.*, 2005; Lutz, 2004).

It is estimated that epilepsy affects about 1% of the human population, with a lifetime incidence approaching 3% (Lutz, 2004). The incidence is highest in the first year of life and again in elderly persons. It is a disorder that affects an estimated 2.7 million people in the United States and costs about $15.5 billion in medical costs and lost or reduced earnings and productivity each year (Kobau *et al.*, 2005). Each year, about 200,000 new cases of epilepsy are diagnosed in the United States. People of low socioeconomic status, those who live in urban areas, and members of some minority

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populations are at increased risk for epilepsy. In addition, patients with epilepsy tend to rate their quality of life lower than people without epilepsy, reporting approximately 8 more physically and mentally unhealthy days per month than persons with no history of epilepsy (Kobau *et al.*, 2005). It is a truly debilitating condition that limits independence, as most states require 6 seizure-free months before a person can obtain a driver's license. It also limits social interaction, as there is a lack of understanding of the disease, leading to negative attitudes, beliefs and stigma causing discrimination in the workplace and in schools (Kobau *et al.*, 2005).

More than 40 recognized types of epileptic syndromes can be classified into two categories, generalized and partial, based on excitatory activation in the brain (Lutz, 2004). Generalized epilepsies result in seizures occurring throughout the cortex because of a generalized lowering of the seizure threshold, and are often genetically determined. They typically involve both brain hemispheres from onset. Generalized seizures can be further sub-classified into generalized tonic-clonic, absence, tonic, atonic, and clonic and myoclonic seizures, based on clinical presentation (Porter and Meldrum, 2004). Tonic-clonic are the most common generalized seizures, consisting of sustained contractions (tonic) and oscillating contraction and relaxation (clonic) components (Morimoto *et al.*, 2004). Absence seizures represent a distinct type of generalized seizure, generated by thalamocortical loops without a strong motor system recruitment (Elger and Schmidt, 2008; Morimoto *et al.*, 2004). Partial seizures result from the focal point of the excitation beginning in a localized fashion (Elger and Schmidt, 2008). Partial seizures are sub-classified into simple partial and complex partial seizures, based on extent of spread. A partial seizure may also lead to a generalized seizure, termed "partial seizure secondarily

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generalized" (Porter and Meldrum, 2004). Generalized and partial seizures are diagnosed by the clinical presentation as well as EEG findings. The most severe type of seizure is termed status epilepticus (SE). SE is a medical emergency that is associated with a high morbidity and mortality. It is characterized by a seizure lasting longer than 30 minutes or recurrent seizures without regaining consciousness for greater than 30 minutes. Clinically, 25% of SE cases occur in patients who have been diagnosed with epilepsy. At some point in their lives, 16% of patients with epilepsy will experience an episode of SE (DeLorenzo, 2006), often precipitated by a change in medication or lack of adequate blood levels of an anti-epileptic medication. Twelve to thirty percent of patients with a new diagnosis of epilepsy first present in SE, indicating that it is often the precipitating event to epileptogenesis (DeLorenzo *et al.*, 2005).

Though medical science has advanced in the diagnosis and treatment of epilepsy, the cause of the disease still remains unknown for many patients. Known causes include traumatic brain injury, stroke, CNS infections (meningitis, encephalitis, etc.), brain tumors, brain malformations, and febrile seizures in infancy (DeLorenzo *et al.*, 2005). It is still unclear how some of these initial insults can lead to epileptogenesis, the development of epilepsy. Another portion of epilepsies are termed cryptogenic and presumed to be genetic (Elger and Schmidt, 2008; Kobau *et al.*, 2005) though very little is known about the genes that underlie epileptogenesis (Elger and Schmidt, 2008). Mutations of voltage-gated Na^+, K^+, and Cl^- channels, as well as ligand gated $GABA_A$ and acetylcholine channels are known to exist in idiopathic epilepsies (Elger and Schmidt, 2008). Over 70 epilepsy susceptibility genes have been found in humans, yet cannabinoid receptors and other components of the endogenous cannabinoid system have

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not yet been implicated as mutations in epilepsy subjects (Lutz, 2004). Seizures may also occur due to acute and temporary conditions, such as fever, metabolic disturbance, or alcohol withdrawal. These seizures do not lead to the diagnosis of epilepsy unless the seizures recur in the absence of the acute and temporary condition (Elger and Schmidt, 2008; Kobau *et al.*, 2005).The type and cause of seizures is of great importance clinically, as it influences which types of medical interventions are most appropriate.

B. Current treatments of epilepsy

Current medical interventions include anti-epileptic drugs (AEDs), surgery, and vagus nerve stimulation (VNS). Drugs such as phenytoin, carbamazepine, valproate, and the barbiturates are classically used to treat partial seizures and generalized tonic-clonic seizures. Newer drugs, such as lamotrigine, gabapentin, oxcarbazepine, topiramate, vigabatrin, and levetiracetam are becoming more useful in industrialized countries (Porter and Meldrum, 2004). Exact mechanisms of action are not known for all AEDs, but some are known to act by altering Na^+, K^+, and Cl^- conductance, membrane potentials, and the concentrations of amino acids and the neurotransmitters norepinephrine, acetylcholine, and GABA (Porter and Meldrum, 2004). Ethosuximide, valproic acid, and sodium valproate are generally used in the treatment of absence seizures. Ethosuximide reduces the low-threshold T-type Ca^{2+} current, seen as therapeutically relevant in thalamic neurons, while the mechanism of action for valproic acid is poorly understood. Other drugs useful in the management of epilepsy include the benzodiazepines, which act on $GABA_A$ receptor-chloride ion channels, and acetazolamide, a diuretic that may act by inducing mild acidosis in the brain (Porter and Meldrum, 2004). Though one or more

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AEDs effectively control seizures in 60-70% of persons with epilepsy, other means of medical intervention are considered for those individuals who do not respond or cannot tolerate AEDs.

Adjunctive resective surgery is a standard of care for a small group of properly selected patients with drug-resistant partial epilepsy, especially mesial temporal lobe epilepsy (Elger and Schmidt, 2008). The surgical approach chosen depends on many considerations including the localization and the extent of the epileptogenic zone, MRI findings, preoperative monitoring, and the risk–benefit balance of the resective surgery itself. With surgery, 25-30% of patients are seizure free with no AEDs, while another 25-30% can be seizure free with adjunctive AED therapy (Elger and Schmidt, 2008). Vagus nerve stimulation is useful for some of the drug-resistant population with partial seizures if surgery is not an option. Stimulating electrodes are implanted in the left vagus nerve and the pacemaker is implanted in the chest wall or axilla. Use of this device reduces the number of partial seizures by one-third with continued AED therapy (Elger and Schmidt, 2008). Even with these many options, 30% of patients with epilepsy do not obtain satisfactory control of their seizures, with temporal lobe epilepsy (TLE) being the most common and drug resistant type of partial epilepsy (Morimoto *et al.*, 2004). This large population clearly demonstrates the need for further exploration of other pharmacological targets to control neuronal hyperexcitability.

C. Stroke induced acquired epilepsy

Stroke is a catastrophic event with significant morbidity and mortality. It is estimated that there are more than 795,000 strokes per year in the United States and that

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140,000 of them result in death (Klein-Ritter, 2009). Stroke is defined by the World Health Organization as the clinical syndrome of rapid onset of focal (or global, as in subarachnoid hemorrhage) cerebral deficit, lasting more than 24 hours or leading to death, with no apparent cause other than a vascular one (Warlow *et al.*, 2003). There are three types of stroke: ischemic, primary intracerebral hemorrhagic, and subarachnoid hemorrhagic, with ischemic strokes accounting for about 80% of all strokes in white populations (Warlow *et al.*, 2003). Stroke is one of the leading causes of acquired epilepsy (Hauser and Hesdorffer, 1990; Hauser *et al.*, 1991), yet little is known about the underlying basic mechanisms leading to stroke-induced epilepsy. The incidence of developing epilepsy is highest in the first year of life and again in elderly persons. In fact, among persons 35 and older, stroke is the most common known cause of epilepsy (Forsgren *et al.*, 1996). Compared to the population in the community, patients who have had a cerebral infarct have a 17-fold greater risk of experiencing recurrent seizures (Ferro and Pinto, 2004). As a result of the increase in the proportion of older people and the future effects of current smoking patterns in less developed countries, it is estimated that by 2020, stroke mortality will have almost doubled (Warlow *et al.*, 2003). This also provides an indication that the incidence of stroke related epilepsy will continue to rise without continuing research efforts into prevention and treatment of this debilitating disease.

D. Glutamate in stroke

Glutamate plays a central role in neuronal injury during an ischemic brain injury. Focal impairment of cerebral blood flow restricts the delivery of oxygen and glucose to

the neurons. This inhibits oxidative phosphorylation by the mitochondrial electron transport chain and depletes the cellular stores of energy rich ATP and phosphocreatanine (Lipton, 1999). The loss of cellular ATP causes a compensatory switch to anaerobic metabolism, resulting in the accumulation of lactate and hydrogen ions precipitating acidosis (Katsura *et al.*, 1993). In addition, loss of cellular ATP impairs the energetics required to maintain normal ionic gradients. With this energy loss, normal membrane potential is lost and neurons depolarize, activating voltage-dependent Ca^{2+} channels. This, in turn allows for the release of excitatory amino acids (particularly, glutamate) into the extracellular space. In addition, reuptake of glutamate is impeded, as it is an energy dependent process, further increasing the extracellular concentration of glutamate (Dirnagl *et al.*, 1999). The extracellular glutamate concentrations rise from micromolar to millimolar levels from the ischemic core to the surrounding penumbra (area around the core of the stroke). The excessive glutamate continuously acts on post-synaptic glutamate receptors, flooding the post-synaptic neurons with Na+ and Ca^{2+} ions. This also allows for the passive entry of Cl$^-$ ions, creating an osmotic gradient that leads to cell swelling and immediate cell death. In cells that do not die in this immediate time frame, the intracellular Ca^{2+} levels continue to rise with activation of NMDA receptors and metabotropic glutamate receptors. The rise in intracellular Ca^{2+} is thought to initiate a series of cytoplasmic and nuclear events that impact the development of tissue damage (Dirnagl *et al.*, 1999) and cause delayed neuronal cell death (see Figure 1). In cells that survive the insult, much experimental evidence exists to suggest that the rise in intracellular Ca^{2+} plays a role in the development of epilepsy (DeLorenzo *et al.*, 2005).

Calcium is a major signaling molecule in neurons and free neuronal calcium is

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tightly regulated by various mechanisms in the cell. Brief, controlled elevations of Ca^{2+} are essential for neurotransmitter release and long-term potentiation. Following a CNS injury, there is a significant elevation in intracellular Ca^{2+} concentrations (Raza *et al.*, 2004; Raza *et al.*, 2001; Sun *et al.*, 2004). The cells that survive these elevated Ca^{2+} levels continue to maintain elevated intracellular Ca^{2+} levels in several models of epileptogenesis (Raza *et al.*, 2004; Raza *et al.*, 2001; Sun *et al.*, 2004). There are several possible mechanisms that allow this extended elevated Ca^{2+} to lead to epileptogenesis. Increased Ca^{2+} may alter GABA receptor recycling, altering neuronal excitability (Blair *et al.*, 2004). Elevated Ca^{2+} also alters gene transcription, protein expression and turnover, neurogenesis, neuronal sprouting and many other physiological processes (Morris *et al.*, 1999). Indeed, pharmacologically blocking this extended elevation of Ca^{2+} inhibits epileptogenesis in *in vitro* models (Nagarkatti *et al.*, 2010).

Figure 1.

Flowchart of the events underlying neuronal cell death or epileptogenesis following ischemia/stroke. Note that the increase in extracellular glutamate plays a central role in the cascade of molecular events that lead to cell death or epileptogenesis. ATP – adenosine triphosphate; Na^+/K^+-ATPase – energy dependent sodium potassium pump.

Figure 1.

Ischemia

↓ATP (from ↓glucose/O2)

loss of function of Na+/K+-ATPase

↓Glutamate uptake by glia   Depolarization of cell

↑ release of glutamate

↑ **Extracellular glutamate**

NMDA and Non-NMDA receptor activation

Ca2+ influx   Na+ influx

passive Cl- influx

Epileptogenesis   Delayed cell death

Osmotic swelling

Transcriptional changes
Protein expression
Neurogenesis
Neuronal Sprouting, etc.

Immediate cell death

E. Current models of epileptogenesis

Studying acquired epilepsy in the laboratory is a challenging task and the current models do not necessarily reflect all the injury types that lead to epileptogenesis in humans, so the need for more models of epileptogenesis is imperative. In addition, studying acute seizure events *in vivo* and *in vitro* has been easier to accomplish than actually producing epileptogenesis. Current methods of provoking acute seizure events *in vitro* include the addition of agents such as 4-aminopyridine (Albus *et al.*, 2008), or pilocarpine (Poulsen *et al.*, 2002), or by removal of magnesium from the extracellular solution (Albus *et al.*, 2008; Sombati and Delorenzo, 1995). *In vivo* models include the use of pilocarpine (Raza *et al.*, 2001), kainic acid (Cavalheiro *et al.*, 1982), organophosphates (Deshpande *et al.*, 2010), and maximal electric shock (Wallace *et al.*, 2001). These insults produce acute seizures and have allowed for research into anti-epileptic agents such as Levetiracetam (Angehagen *et al.*, 2003) and carisbamate (Deshpande *et al.*, 2008). Models of epileptogenesis are more limited in the literature. *In vitro* models include the low magnesium and glutamate injury models in hippocampal neuronal culture (HNC) (Sombati and Delorenzo, 1995; Sun *et al.*, 2001). The low magnesium model utilizes the removal of magnesium from the extracellular solution for a period of 3 hours. The removal of magnesium leads to excessive activation of the NMDA receptor and subsequent electrical discharges by the cell, similar to the clinical correlate of status epilepticus (Sombati and Delorenzo, 1995) Twenty-four hours after the magnesium is returned to the media, the neurons display electrographic spontaneous recurrent epileptiform discharges (SREDs). This model has provided valuable information about the cellular processes that occur after a status epilepticus-like injury,

resulting in epilepsy. Studies have shown that Ca^{2+} handling in these cells becomes impaired and the intracellular Ca^{2+} concentrations remain elevated for the life of the neuron (Pal *et al.*, 2001). This model has also led to some insights about possible pharmacological intervention to prevent epileptogenesis utilizing dantrolene (Nagarkatti *et al.*, 2010) or carisbamate (Deshpande *et al.*, 2008). Hippocampal neuronal cultures have also been utilized in the glutamate injury model of epileptogenesis (Sun *et al.*, 2001). Treating the cells with 20µm glutamate for 10 minutes causes a significant injury, similar to what is observed during stroke (Sun *et al.*, 2001). Similar to the low magnesium model, the surviving cells have a continuously elevated intracellular Ca^{2+} concentration and develop SREDs (Sun *et al.*, 2001; Sun *et al.*, 2004).

In vivo models of epilepsy have generally focused on SE-like injuries by utilizing various neuro-toxins. Pilocarpine is a muscarinic agonist commonly used to experimentally induce SE. In the rat model of pilocarpine induced AE, rats are administered a single dose of scopolamine (to block peripheral effects) before a single dose of pilocarpine. Pilocarpine administration produces limbic seizures resulting in SE and brain damage when administered to rats (Turski *et al.*, 1983). Muscarinic cholinergic excitation in the CNS is thought to occur as the result of blockade of K^+ conductance, mediated by voltage-dependent Ca^{2+} and Na^+ conductances, resulting in prolonged depolarizations and subsequent cellular damage (Turski *et al.*, 1983). After latency period of several weeks, rats begin to have spontaneous, recurrent seizures that persist for the animal's lifetime. This model has been thoroughly characterized and shows many correlates to human temporal lobe epilepsy (Cavalheiro *et al.*, 1991; Mello *et al.*, 1993). Similar to *in vitro* models, studies have shown a substantial increase in intracellular

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neuronal Ca^{2+} during SE and following the injury. In fact, intracellular hippocampal Ca^{2+} levels remain elevated for the life of the animal (Raza *et al.*, 2001). In contrast to *in vitro* models, epileptogenesis following a stroke-like injury has been much more difficult to produce. A cerebral artery occlusion model in perinatal rats has been successful in producing acquired epilepsy (Kadam *et al.*, 2010). This model is significant to studying epilepsy associated with hypoxic-ischemic induced perinatal encephalopathies. However, it is not an accurate model for the most common group affected by stroke AE; persons over 65. In adult rats, Hartings et al. (Hartings *et al.*, 2003) has shown that middle cerebral artery occlusion does, in fact, produce non-convulsive seizures acutely following the injury, but there are no animal models of stroke induced acquired epilepsy known in the literature.

Stroke induced epilepsy is a major problem in modern neurology. Current models in the laboratory can provide some insights, but they also have limitations. *In vitro* models are ideal for studying individual cellular effects of an injury, but they are limited by lost morphology when cells are cultured. Anatomic factors and the presence of different cell types plays a role in the stroke penumbra, and these factors can not be adequately studied in a hippocampal neuronal culture (HNC) model. *In vivo* models of stroke have had limited success in producing AE, as current models have observed non-convulsive seizures acutely, without monitoring the animals for long term acquired epilepsy (Hartings *et al.*, 2003). Perinatal models of ischemia have produced AE, but the developing brain has many key differences from the adult brain in this model. In addition, animal models are very costly and time consuming and may not be suitable for the first step in testing new antiepileptic agents. These factors have led our laboratory to the

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development of an organotypic hippocampal slice culture model of acquired epilepsy. Organotypic brain slice cultures maintain basal cellular and connective organization and several fundamental *in vivo*-like characteristics that offer a feasible alternative located in complexity between primary cell cultures and intact animals. Following an initial and brief depression caused by explantation, many synaptic components are expressed at steady levels in long-term hippocampal slice cultures, allowing detailed investigations of mechanisms of brain synaptic function (Bahr *et al.*, 1995).

F. Organotypic hippocampal slice cultures

The preparation of slice cultures was first developed in the 1970s (Crain, 1973) by use of the static method in which a slice was incubated in a sealed Maximov chamber with culture medium. This was followed by the roller-tube method in which slices are adhered to a glass coverslip with a collagen or plasma clot and rotated in medium-containing tubes (Gahwiler, 1981). The properties of brain slices maintained in this method have been described well (Gahwiler, 1988), however, most recent studies with organotypic hippocampal slice cultures (OHSCs) utilize the interface method first reported by Stoppini et al. (Stoppini *et al.*, 1991). The interface method maintains slices on a porous membrane at the air-medium interface. Cultures prepared with this method are unique in that they are the first cultured tissues to exhibit long-term potentiation (LTP) (Bahr, 1995). Extensive studies of OHSC development have revealed *in vitro* similarities to normal *in vivo* development. The distinct laminar structure and the morphology of different cell types is present at the time of culture and are well preserved by long term culturing (Bahr *et al.*, 1995). Glial markers develop gradually over the first

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2 weeks of culture, followed by a stable maintenance for at least 30-60 days, closely matching the astrocyte proliferation and myelination profiles during rat brain development *in vivo* (Bahr *et al.*, 1994; Bahr *et al.*, 1995; Bahr, 1995). In addition to maintaining normal morphology and development, long-term hippocampal slice cultures also reach a level of functional maturity. Studies have shown that long term cultures possess the capacity for many synaptic plasticity events including paired-pulse facilitation, post-tetanic potentiation, LTP, and inhibitory post-synaptic potentials (Bahr, 1995; Muller *et al.*, 1993a; Stoppini *et al.*, 1991; Vanderklish *et al.*, 1995).

The maintenance of normal morphology and physiology has increased the popularity of OHSCs in studying many neurological conditions. OHSC models have been validated in many to investigate mechanisms and treatment strategies for neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, epilepsy, and stroke (Cho *et al.*, 2007; Noraberg *et al.*, 2005; Sundstrom *et al.*, 2005). Excitotoxic injury has been induced in OHSCs by exposure to N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainic acid (KA), or domoic acid. Interestingly, CA1 pyramidal cells are more susceptible to cell death upon exposure to NMDA and AMPA (Kristensen *et al.*, 2001; Noraberg *et al.*, 2005), while CA3 is more vulnerable to KA and domoic acid (Holopainen *et al.*, 2004; Noraberg *et al.*, 2005). OHSCs have also been used extensively to study the stroke-like, energy failure conditions in an oxygen-glucose deprivation injury (OGD). When exposed to OGD, CA1 pyramidal cells are the most susceptible, as *in vivo*, displaying NMDA glutamate receptor mediated cell death (Bonde *et al.*, 2005; Noraberg *et al.*, 2005). OGD has been studied extensively to examine various neuroprotective agents (Moroni *et al.*, 2001; Pringle *et al.*,

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1996). However, the physiological consequences of excitotoxic or OGD injuries has not been fully explored in OHSCs. An advantage of OHSCs is the ability to maintain and study cultures long term after manipulation. This distinct advantage provides an ideal system to study the long term physiological changes following a stroke-like injury.

Seizure activity in OHSCs has been studied using various methods. Using the roller drum method of culture (Muller *et al.*, 1993b) bicuculline and picrotoxin induce epileptic activity and a reduction of dendritic spines. Other groups using bicuculline to induce epileptiform activity in slice cultures grown in the interface method have demonstrated increased gap junctional function and expression (Wang and Bausch, 2004). The application of kainic acid for 48 hours induced increased supragranular Timm staining of mossy fiber and CA1 derived axonal sprouting onto dentate granule cells (Routbort *et al.*, 1999). They showed that this axonal sprouting contributed to hyperexcitability in KA treated cultures. These studies support the use of OHSCs in the development of models of acquired epilepsy.

G. History of *Cannabis* use to treat seizures

Ancient medicine has long known that *Cannabis* extract has pharmacological properties useful for the treatment of many ailments. There is evidence from as early as the BC era that *Cannabis* was used to treat pain (Lutz, 2004). Ancient eastern medicine has also exploited *Cannabis* extract for the treatment of many other ailments, including pain, headaches, insomnia, fever, and a variety of GI disorders. Perhaps one of the earliest written testimonies of *Cannabis* use as a means to control seizures dates back to 1464. In a treatise on hashish written by the poet Ali ben Makki, it states that hashish was

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prescribed to the son of the Caliphate Councillor who was suffering from seizures. In effect, "it cured him completely but he could not be without the drug ever after" (Lutz, 2004; Mechoulam, 1986). The use of hashish was widely illicit in medieval Arab society, though it was widely used recreationally among the poor, therefore its use as an anti-epileptic may have been influenced by Indian medical traditions, where *Cannabis* -based medicines were often used to treat convulsions, along with many other neurological disorders (Mechoulam, 1986).

Early medical studies of the therapeutic uses of *Cannabis* were not undertaken by Europeans until the 1840s. The British scientist and physician O'Shaugnessy tested some of the pharmacological uses of *Cannabis* by administering extracts of *Cannabis* resin to patients with rheumatism, tetanus, rabies, infantile convulsions, cholera, and delirium tremens. He reported that the convulsions and spasms associated with rabies and tetanus were well controlled with high doses of the *Cannabis* extract. He also noted positive results in cases of infantile convulsions (Mechoulam, 1986; Robson, 2001). After these observations were published, the medicinal use of *Cannabis* expanded rapidly. By 1854, *Cannabis* was in the United States Dispensatory and commonly used in many home remedies (Robson, 2001). In 1890, a British neurologist named J.R. Reynolds, Queen Victoria's personal physician, reported on the role of *Cannabis* in the treatment of epilepsy, stating that while *Cannabis* "was very useful for attacks of violent epileptic convulsions, it was absolutely useless for attacks of petit mal epilepsy" (Mechoulam, 1986). However, the peak of the use of *Cannabis* as a home remedy had already passed by that time due to variable potency, poor storage stability, unpredictable response to oral administration, increasing availability of synthetic alternatives, and concerns about

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recreational use (Robson, 2001). Therefore, Reynolds' report was largely ignored by the medical community (Lutz, 2004). *Cannabis* was banned in the United States and other countries in the early 20th century, hindering its use as a medical remedy.

Though the various *Cannabis sativa* preparations were the most widely used illicit drugs in the world for centuries, little was known about the chemical composition until the 1960s. The major active component of marijuana, Δ-9-tetrahydrocannabinol (THC), was not isolated in a pure form until 1964 (Lutz, 2004; Mechoulam and Gaoni, 1965). In addition, cannabidiol, another constituent of marijuana was also isolated. These compounds were tested in various seizure models (discussed later), but their mechanisms of action were not really understood until further studies elicited more information about the brain's endogenous cannabinoid system, known as the endocannabinoid system.

H. The endocannabinoid system

The endocannabinoid system consists of the two cannabinoid receptors (CB_1 and CB_2), their endogenous ligands and associated enzymes. The cannabinoid CB_1 and CB_2 receptors are G-protein-coupled receptors (GPCRs) that belong to the rhodopsin GPCR family (Reggio, 2006). The CB_1 receptor is mainly found on neurons in the CNS, while the CB_2 receptor is found predominantly in immune tissues, though it is present at lower levels in the CNS (Munro *et al.*, 1993; Van Sickle *et al.*, 2005). A major advance in cannabinoid research came with the discovery and cloning of these unique receptor proteins that mediated many of the behavioral and physiological effects of cannabinoids, leading to the molecular characterization of the CB_1 (Devane *et al.*, 1988; Matsuda *et al.*, 1990) and CB_2 (Munro *et al.*, 1993) receptors. Further advancing the field was the

discovery of several novel cannabinoid ligands and endogenous cannabinoid (endocannabinoid) compounds, including the two major brain endocannabinoids, N-arachidonyl ethanolamine (anandamide, AEA) (Munro *et al.*, 1993) and 2-arachidonylglycerol (2-AG) (Mechoulam *et al.*, 1995), with regional differences in which one or the other predominates in the brain (Alger, 2004). The CB_1 receptor transduces signals in response to AEA, 2-AG, and noladin ether, as well as Δ-9-THC (Reggio, 2006). It also transduces signals in response to synthetic compounds such as the non-classical cannabinoid CP 55940 and the aminoalkylindoles, typified by WIN55212-2 (Reggio, 2006). Several CB_1 antagonists have been reported, among them SR141716A, AM630, LY320135, O-1184, and CP272871(Reggio, 2006). SR141716A has been shown to exert is effects as a competitive CB_1 antagonist, as well as an inverse agonist (Reggio, 2006).

The CB_1 receptor is one of the most abundant neuromodulatory receptors in the CNS, with levels up to 10 times that of any other GPCR (Herkenham *et al.*, 1990; Sim *et al.*, 1996) and it is expressed at high levels in the hippocampus, cortex, cerebellum, and basal ganglia (Herkenham *et al.*, 1990; Herkenham *et al.*, 1991; Tsou *et al.*, 1998). The endocannabinoid molecules and the endocannabinoid system regulate hippocampal excitability (Ameri *et al.*, 1999; Chemin *et al.*, 2001; Steffens *et al.*, 2003) and modulate neuronal excitation and inhibition in animals and humans (Chen *et al.*, 2003; Steffens *et al.*, 2004). In the hippocampus, it has been proposed that the endogenous cannabinoids, AEA and 2-AG act as retrograde signaling molecules that mediate the suppression of GABA release from presynaptic terminals following depolarization of a CA1 pyramidal neuron. However, CB_1 function has also been observed on glutamatergic terminals and

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transcripts encoding CB_1 receptors were shown to be present in glutamatergic neurons of the hippocampus and amygdala. Thus, the overall effect of CB_1 receptor activation in the hippocampus can be a reduction in both the inhibitory and excitatory transmission. In addition, Hajos and Freund propose that in the hippocampus, CB_1 receptors are preferentially localized to GABAergic terminals and that another, non- CB_1, non-CB_2 cannabinoid receptor mediates the effects of CB_1 receptor agonists on glutamatergic release (Freund and Hajos, 2003; Hajos and Freund, 2002a; Hajos and Freund, 2002b; Smith, 2005).

Discoveries in the last decade have been crucial to discovering the role of endocannabinoid signaling as a regulator of synaptic communication. The molecular and anatomical organization of the endocannabinoid system at most synapses also allows for perturbations of the system to contribute to several neurological diseases. The CB_1 receptor is the most abundant G-protein coupled receptor in the brain, with a density of binding sites comparable to those of the two major neurotransmitters, glutamate and γ-aminburtyric acid (GABA) (Devane *et al.*, 1988; Herkenham *et al.*, 1990; Katona and Freund, 2008). The CB_1 receptor is located on the presynaptic side of the synapse at nearly all types of central nervous system synapses (Freund *et al.*, 2003; Katona and Freund, 2008). Presynaptic CB_1 localization was first noted on GABAergic terminals, though more recent evidence has shown CB_1 receptors to be just as abundant on glutamatergic projections (Katona and Freund, 2008). Glutamatergic projections with CB_1 receptors have been discovered on neocortical (Lafourcade *et al.*, 2007), hippocampal (Katona *et al.*, 2006; Kawamura *et al.*, 2006), hypothalamic (Wittmann *et al.*, 2007) and cerebellar (Kawamura *et al.*, 2006) neurons. Other recent evidence also

suggest CB_1 activity exists in subcortical ascending pathways, such as cholinergic (Degroot *et al.*, 2006), noradrenergic (Oropeza *et al.*, 2007), or serotonergic (Balazsa *et al.*, 2008) axons. Endcannabinoids may not be primarily involved in basal and tonic intra- or inter-cellular communication, instead acting mainly as a on-demand intercellular signaling system (Katona and Freund, 2008). This would suggest that only precisely timed and positioned physiological stimuli evoke endocannabinoid biosynthesis and release from a selected subdomain of the cell surface, allowing for a crucial role in neuronal regulation, especially in disease states such as epilepsy (Katona and Freund, 2008).

Other intracellular effects in neurons after binding of agonists to CB_1 receptors are inhibition of adenylyl cyclase, leading to decreased levels of intracellular cAMP, and stimulation of potassium channels (A-type and inwardly rectifying), leading to an increased efflux of potassium (Lutz, 2004). Pharmacological and kinetic studies suggest that CB_1 activation inhibits presynaptic Ca^{2+} channels through direct G protein inhibition, in a process termed depolarization induced suppression of inhibition (DSI) and it's analogous depolarization induced suppression of excitation (DSE). Cannabinoid-sensitive inputs are unusual among CNS synapses in that they use N-, but not P/Q-type Ca^{2+} channels for neurotransmitter release, indicating that the endocannabinoids are highly selective, rapid modulators of hippocampal inhibition. Collectively, CB_1 receptor agonists render neurons less excitable. Endocannabinoids inhibit neurotransmitter release from nerve terminals that synapse on the endocannabinoid-generating cell. A major mechanism of synaptic inhibition is the suppression of presynaptic voltage-gated calcium channels, as shown by exogenous cannabinoids ability to block calcium currents (Alger,

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2004; Twitchell *et al.*, 1997). During DSI and DSE, the suppression of synaptic transmission is transient, lasting for only seconds. This indicates that DSI can facilitate induction of long term potentiation (LTP). Since the strength of GABAergic inhibition usually regulates the ability of excitatory synapses to induce LTP, DSI can induce weak excitatory potentials that can induce LTP. Indeed, LTP does not occur if CB_1 receptors are blocked (Alger, 2004; Carlson *et al.*, 2002). Hence, the endocannabinoid system may play an important role in regulating synaptic plasticity.

The endocannabinoids AEA and 2-AG are thought to be synthesized on demand as a result of neuronal excitation. The synthesis of anandamide is thought to take place in two steps. First, N-arachidonyl phosphatidyl ethanolamine is formed from the precursor phosphatidyl ethanolamine by the enzyme N-acyltransferase. Next, anandamide is synthesized from N-arachidonoyl phosphatidyl ethanolamine by phospholipase D (Lutz, 2004). 2-AG is also thought to be synthesized in two steps from the precursor phosphatidyl inositol, though two different pathways have been described. 1,2-diacylglycerol and lysophosphatidyl inositol have been found to be intermediate products. Two diacylglycerol lipase enzymes (DGL-alpha and DGL-beta) catalyze the synthesis of 2-AG from 1,2-diacylglycerol (Lutz, 2004). Synthesis of 2-AG and anandamide can be initiated by an increase in intracellular calcium concentration, however a single action potential does not increase calcium concentration enough for endocannabinoid production. Bursts of action potentials, such as those seen in seizures and other excitatory events, can lead to endocannabinoid production (Alger, 2004).

The on-demand synthesis of endocannabinoids allows them to be used as a means of local retrograde signaling. Because they can easily partition into and diffuse

throughout cellular membranes, lipid messengers, such as AEA, are not easily contained by physical boundaries, such as the vesicles that store cationic neurotransmitters. AEA synthesis seems to be triggered by depolarization of the cell and consequent Ca^{2+} influx (Reggio, 2006). Endocannabinoid synthesis may also be triggered by activation of group I metabotropic glutamate receptors (mGluRs). Calcium does not seem to play a role in this pathway, suggesting that depolarization and mGluR activation represent two separate pathways to endocannabinoid synthesis, increasing the magnitude of DSI (Reggio, 2006). With its ability to readily partition, the termination of AEA signaling poses another problem. Termination cannot rely on uptake and compartmentalization as with classical neurotransmitters. Instead, signaling termination must rely on chemical transformation. It has been proposed that there is a specific transporter on both neurons and glia that mediates endocannabinoid uptake, though others suggest that the process is by simple diffusion (Reggio, 2006). Once taken into the cell, endocannabinoids are broken down by membrane bound enzymes on the endoplasmic reticulum known as fatty acid amide hydrolysis (FAAH) for the degradation of AEA (Di Marzo *et al.*, 1998; Reggio, 2006) and monoglyceride lipase (MAGL) for the degradation of 2-AG. FAAH is found predominantly in the postsynaptic cell somata and dendrites, whereas MAGL is in presynaptic nerve terminals (Alger, 2004) (Figure 2).

Recent developments of specific inhibitors for FAAH and MAGL have catapulted endocannabinoid research in the past few years. The ability to specifically inhibit each enzyme will allow for greater therapeutic opportunities in regulating specific endocannabinoid channels (Petrosino and Di Marzo, 2010). The ability to modulate the hydrolytic enzymes that control AEA and 2-AG levels potentially allows for more

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specific methods to modulate cannabinoid receptor activity without the unwanted psychotropic effects that are typical of Cannabis preparations. A specific FAAH inhibitor, such as Org-231295 has been suggested by preclinical studies as a possible analgesic for inflammatory and neuropathic pain, and as a possible therapeutic agent for depression and anxiety (Petrosino and Di Marzo, 2010). The specific MAGL inhibitors, OMDM-169 (Bisogno *et al.*, 2009) and JZL-184 (Long *et al.*, 2009) have all been suggested as possible analgesic agents in models of inflammatory or acute pain (Petrosino and Di Marzo, 2010). These agents have yet to be examined in seizure models, but the ability to specifically control endocannabinoid levels is enticing for future studies.

Figure 2.

Schematic representation of the endocannabinoid system in pre- (top) and postsynaptic (bottom) neurons. EMT – endocannabinoid membrane transporter, MAGL – monoacylglyceride lipase, DAGL – DAG lipase, AEA – anandamide, NarPE – N-arachidonyl phosphatidylethanolamine, NAT – N-acyltransferase. Taken from(Pacher *et al.*, 2006).

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Figure 2
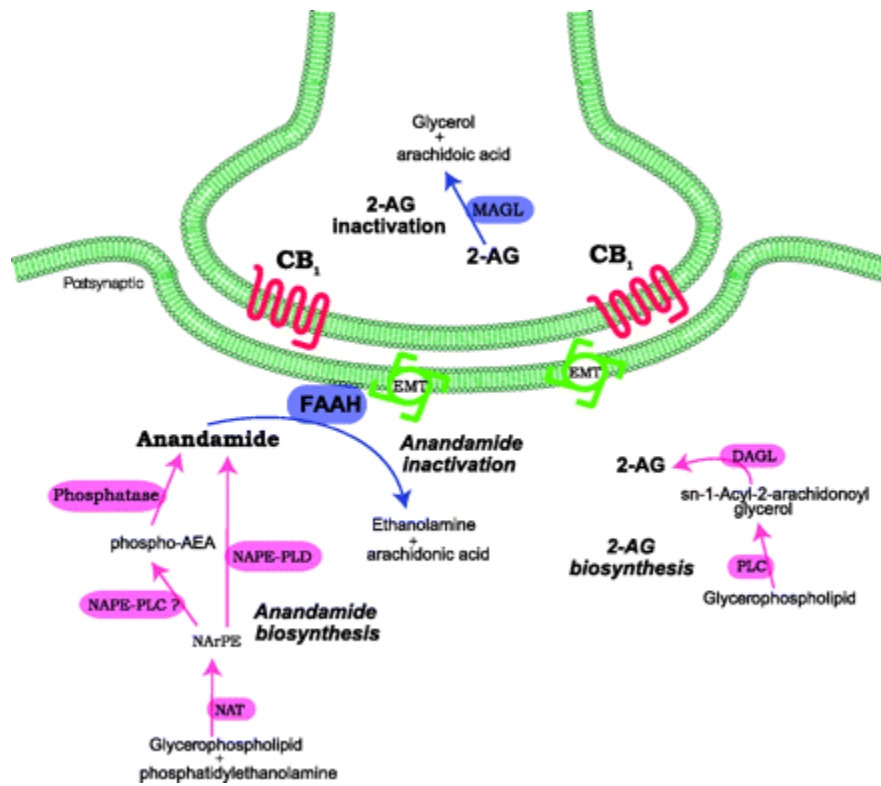
I. Depolarization induced suppression of inhibition (DSI) and excitation (DSE)

In the early 1990's, it was reported that brief depolarization of the CA1 pyramidal cells in the hippocampus caused a reduction in the amplitude of the GABa ergic IPSCs that lasted for several seconds (Alger, 2002; Pitler and Alger, 1992). This phenomenon came to be known as depolarization induced suppression of inhibition and it has also been recorded in Purkinje cells of the cerebellum (Alger, 2002; Llano *et al.*, 1991; Vincent *et al.*, 1992) CA3 pyramidal cells (Morishita and Alger, 2000), dentate gyrus granule cells (Isokawa and Alger, 2005) and neocortical pyramidal cells (Zilberter, 2000). Various studies were done to isolate DSI as a retrograde signaling process, narrowing the site of induction to the post-synaptic cell (Alger, 2002). However, the retrograde messenger responsible for DSI remained unknown until strong evidence was reported that the messenger was likely to be an endocannabinoid in the hippocampus (Alger, 2002; Ohno-Shosaku *et al.*, 2001; Wilson and Nicoll, 2002). DSI in the hippocampus occurs when the stimulation of the postsynaptic cell leads to the on demand production and release of the endocannabinoid (Alger, 2002). The endocannabinoid then diffuses across the synapse (or is carried) and binds with CB_1 receptors on CCK-positive interneurons that act at pre-synaptic cells. This in turn decreases release of GABA from the interneurons, thereby suppressing the inhibition to the post-synaptic cell. It is important to note that CB_1 receptors are only found on CCK-positive interneurons (Hajos *et al.*, 2000; Katona *et al.*, 1999) and therefore endocannabinoids are not capable of blocking all GABA release surrounding a given neuron (Alger, 2002). In the hippocampus, DSI is probably mediated by the endocannabioid 2-AG (Alger, 2002). Calcium is essential to the expression of DSI, as it is initiated by voltage-dependent Ca^{2+} influx. Other research has

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shown that DSI disappears when the inward Ca^{2+} current disappears (Llano *et al.*, 1991), increases when $[Ca^{2+}_{(out)}]$ is increased (Lenz *et al.*, 1998) and is prevented by the removal of extracellular Ca^{2+} during the voltage step (Ohno-Shosaku *et al.*, 1998). Other studies have shown that the magnitude of DSI induced by a given voltage step is directly dependent on the concentration of the intracellular Ca^{2+} chelator (Alger, 2002; Lenz *et al.*, 1998).

Depolarization induced suppression of excitation (DSE), the excitatory correlate to DSI, was observed in several brain regions by employing a similar protocol as DSI, however it could not be induced in the hippocampus following those protocols. Ohno-Shosaku et al (Ohno-Shosaku *et al.*, 2002) discovered that DSE does in fact occur in the hippocampus, but the voltage step of the post-synaptic cell must be approximately 10 times longer than the voltage step necessary to induce DSI. It was also discovered that DSE was not as robust as DSI in the hippocampus, as EPSC levels were only reduced by 30% compared to a 50% reduction of IPSCs in DSI. Studies of DSE in the hippocampus have recently been shown to be rather complicated, as DSE was expressed at Schaffer collateral synapses, but not Perforant path synapses in the same CA1 neuron (Xu *et al.*, 2010), suggesting different functional expression of CB_1 receptors at the two pathways. The fact that DSE in the hippocampus requires a long voltage step indicates that DSE may not be a physiologically relevant phenomena in normal cell signaling. However, under hyperexcitably conditions such as an epileptic seizure, DSE may play a valuable role in the control of neuronal excitability (Figure 3).

Figure 3.

Schematic representation of the effects of cannabinoid signaling on excitatory and inhibitory events. The traces below the figure represent excitatory and inhibitory post-synaptic potentials (EPSPs and IPSPs) before and after a depolarization of the post-synaptic cell. The decreased amplitude of the EPSP indicates that DSE has occurred following 2-AG signaling. The decreased amplitude of the IPSP is representative of DSI. Figure adapted from (Basavarajappa, 2007).

Figure 3.

Glutamatergic neuron

Presynaptic neuron

GABAergic neuron

G-Protein (+) Ca²⁺ (-)

Ca²⁺ (+) (-) G-Protein

Glutamate AMPAR

Ca²⁺ Channel
CB1R
Depolarization

GABA
GABA$_A$R

Na⁺

2-AG

Ca²⁺

Cl⁻

(+)

Postsynaptic neuron (Pyramidal Cell)

Post synaptic cell = 0mV

-60mV

EPSPs

IPSCs
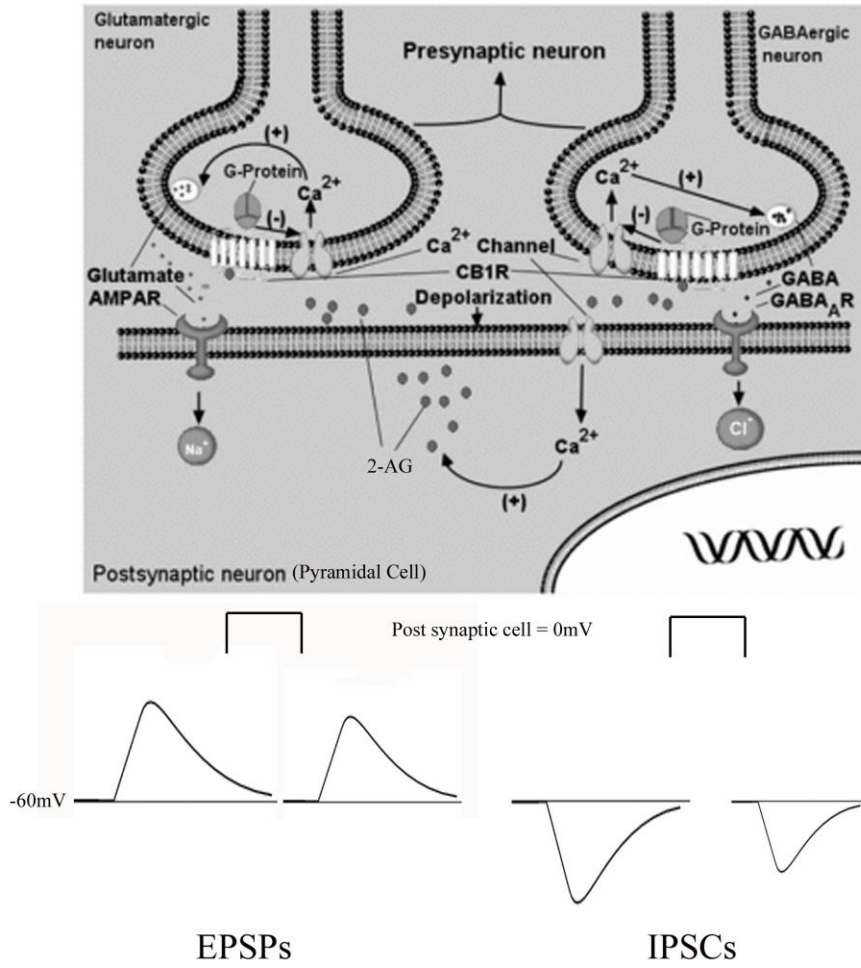
J. Δ-9-THC's anti-seizure effects

Though reports from the 1800s eluded to the anti-seizure properties of cannabinoids, they were not explored in biomedical research until the 1940s. As early as 1947, Loewe and Goodman documented the anticonvulsant effect of *Cannabis* extracts in the mouse maximal electroshock (MES) model (Mechoulam, 1986). After the isolation of Δ-9-THC in 1964 (Mechoulam and Gaoni, 1965), followed by several other cannabinoids, including cannabinol, and cannabidiol (CBD), the use of cannabinoids as anticonvulsants was further explored. Cannabidiol does not bind to cannabinoid receptors, it is not psychotropic, and the molecular targets have not yet been identified (Lutz, 2004), yet it has shown anticonvulsant effects in several studies. Wada's group was among the first to report the acute antiepileptic effects of Δ-9-THC in the rat model of kindled seizures (Corcoran *et al.*, 1973; Wada *et al.*, 1973). Around the same time, Karler's group showed the anticonvulsant effect of Δ-9-THC and CBD, in the MES model (Karler *et al.*, 1973; Karler *et al.*, 1974b; Karler *et al.*, 1974d). In this model, an electric current is applied via the cornea to produce tonic hind limb extension. The suppression of the hind limb extension is considered as a measure of the anti-convulsive activity of the tested compound (Lutz, 2004). Sofia et al. also used the MES model to show that Δ-9-THC was more potent in increasing hind-limb extension latency and preventing mortality than the other anticonvulsants phenytoin, phenobarbital, and chlordiazepoxide (Sofia *et al.*, 1976; Sofia and Barry, 1977). The acute antiseizure effects of cannabinoids and their metabolites were also shown in several other models in various species, including cats, baboons, gerbils, frogs, and chickens (Cox *et al.*, 1975; Dwivedi

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and Harbison, 1975; Johnson *et al.*, 1975; Karler *et al.*, 1974c; Wada *et al.*, 1975a; Wada *et al.*, 1975b).

Human studies of cannabinoids and epilepsy have been inconclusive. An early clinical study from 1948 supported the anticonvulsant actions of two isomers of DMHP (a synthetic analogue of THC), $\Delta6\alpha,10\alpha$-THC dimethylheptyl isomer, when administered to institutionalized children with epilepsy (Mechoulam, 1986). These five children had generalized seizures inadequately controlled with phenobarbital and phenytoin. Two of the five became entirely seizure free with the synthetic $\Delta6\alpha,10\alpha$-THC dimethylheptyl isomer treatment (Mechoulam, 1986). Several small clinical studies have examined the effect of CBD on seizure frequency, since CBD does not have the psychoactive effects of THC. These studies found either some reduction of frequency or no significant reduction compared to placebo (Gordon and Devinsky, 2001). However, these studies used rather low doses of CBD, suggesting that further studies are needed at higher doses. Another epidemiologic study found that marijuana use appeared to be a protective factor against first seizures in men (Gordon and Devinsky, 2001; Ng *et al.*, 1990). Gordon and Devinsky have also performed informal interviews of patients with active epilepsy who have used marijuana intermittently or regularly and found that the majority (90.2%) did not identify a relationship between their marijuana use and seizure frequency or severity (Gordon and Devinsky, 2001). Another study at Harlem Hospital Center between 1981 and 1984 showed that heroin use is a risk factor and marijuana is a protective factor in new-onset seizures (Zagnoni and Albano, 2002). Furthermore, a survey of the medical use of *Cannabis* products in Germany showed that epilepsy (3.6%) was among the most frequently mentioned indications (Zagnoni and Albano, 2002). Several case reports

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implicate the recreational use of marijuana in the regulation of seizure events. In one case report, a patient suddenly developed ictal symptoms upon withdrawal from frequent marijuana use (Ellison *et al.*, 1990), leading to further questions of tolerance to cannabinoids and the effects of withdrawal (to be discussed later). These inconclusive results in human cases of epilepsy demonstrate the need to further explore the role of the endocannabinoid system in various types of CNS insults, especially those known to lead to epilepsy.

K. Endocannabinoid system: protector against excitotoxicity

Based on its nature as a retrograde messenger during excitatory events, it has been proposed that the role of the endocannabinoid system is to protect the brain against excitotoxicity (Mechoulam and Lichtman, 2003). The endocannabinoid system has been studied in a variety of CNS disease models. Several pathological models support the neuroprotective role of the on demand synthesis of endocannabinoids. Kainic acid (KA) induced seizures show a 3-fold increase in anandamide, whereas models of traumatic brain injury see 2.5 and 10-fold increases of anandamide and 2-AG respectively (Bahr *et al.*, 2006). Other pathologic models showing a significant increase in endocannabinoid levels include models of Parkinson's disease, multiple sclerosis, stress and schizophrenia (Bahr *et al.*, 2006). Bahr et al reviewed the literature of the effects of cannabinoid agents on various models of CNS insult. They conclude that in models of excitotoxicity, reduced neuronal damage is seen with application of Δ-9-THC, cannabidiol, anandamide, methanandamide, WIN-55212-2, CP-55,940, HU-210, AM374 (FAAH inhibitor), and AM404 (transport inhibitor). Reduced seizure activity is seen with treatment of UCM707

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(transport inhibitor) and cellular and functional protection occurs with a combination of AM374 and AM404 (Bahr *et al.*, 2006). Furthermore, in another mouse model, an intrahippocampal injection of AMPA produced excitotoxicity. Modulation of both FAAH and anandamide transport were shown to enhance endocannabinoid responses to the injury, resulting in cellular protection as well as improved behavioral and memory performance (Karanian *et al.*, 2005; Karanian and Bahr, 2006). The role of the endocannabinoid system in neuroprotection has been further supported by Marsicano and colleagues. They used two mouse models: animals deficient in receptors and those lacking CB_1 receptors in the principal glutamatergic (excitatory) neurons of the forebrain but not the GABAergic (inhibitory) interneurons of cortex. Both groups of mice exhibited more severe seizures after treatment with kainic acid than did control animals. The CB_1 receptor antagonist SR-141716A augmented seizures to a much greater extent in CB_1 heterozygous mice than in wild-type mice but failed to influence the high seizure activity of mice lacking CB_1 receptors in principal neurons. They were able to show that endogenous activation of CB_1 receptors on principal neurons promote cell survival during excitotoxicity while CB_1 receptors on GABAergic cells exert a negligible function. This shows that cell-type specificity and dynamic regulation appear to be fundamental features of the highly efficient physiological protection provided by the endocannabinoid system (Marsicano *et al.*, 2003). It also supports the hypothesis that the antiseizure effects are CB_1 mediated, as shown in other models.

L. Anti-seizure effects are CB$_1$ mediated

In vitro and *in vivo* seizure models have been employed to explore the role of the endocannabinoid system and CB$_1$ receptors in particular. One *in vitro* model uses primary hippocampal neuronal cultures. By removing magnesium from the culture medium, the cells show high-frequency, continuous firing until the magnesium is returned to the medium. This electrical behavior models the continuous excitatory period similar to that seen in status epilepticus. After a period of epileptogenesis, the cells continue to show spontaneous recurrent epileptiform discharges (SREDs) for the life of the cultures (DeLorenzo *et al.*, 1998). Cannabinoids have been shown to be effective anti-convulsants in this model and their effects have been shown to be CB$_1$ mediated (Blair *et al.*, 2006; Deshpande *et al.*, 2007d). They have also been shown to be more effective than standard anticonvulsants during the period of high-frequency, status epilepticus-like activity (Deshpande *et al.*, 2007b). This is relevant to SE in humans, since 20% of cases of SE are refractory to the initial treatment of two or more antiepileptic drugs, leading to significant morbidity and mortality (Deshpande *et al.*, 2007b). In addition, the model has also shown that pharmacoresistance did not develop to the anticonvulsant effects of cannabinoids, while pharmacoresistance did develop to benzodiazepines, which are commonly used to treat status epilepticus (Deshpande *et al.*, 2007a).

In vivo models have also implicated the anti-seizure effect of cannabinoids to be CB$_1$ mediated. Wallace et al demonstrated that anandamide and its analog O-1812 have significant anticonvulsant effects in the MES model in mice and that these could be blocked by SR141716A (Wallace *et al.*, 2001; Wallace *et al.*, 2002). Other studies from the DeLorenzo laboratory have employed the pilocarpine model of temporal lobe

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epilepsy to demonstrate that the anticonvulsant effects of cannabinoids and endocannabinoids are mediated through CB_1 receptor activation. The pilocarpine model represents a refractory epileptic condition, as the seizures are not easily treated by conventional AEDs. This model has been employed by the DeLorenzo laboratory to show that Δ-9-THC and WIN55212-2 completely blocked spontaneous seizure activity. It was shown that it was, in fact CB_1 mediated by administering the CB_1 antagonist SR141716A, which potentiated seizure duration and frequency, even to the level of SE (Wallace *et al.*, 2003).

In the kainic acid induced seizure model of temporal lobe epilepsy, hippocampal glutamatergic neurons, where CB_1 receptors are present at low but detectable levels, are the central mediators of on-demand endocannabinoid-dependent protection against acute excitotoxic seizures (Lutz, 2004). In this model, kainic acid (KA), an excitatory amino acid found in seaweed, is injected systemically to induce strong activation of excitatory pathways, inducing the hippocampus as the center of the seizures generated (Lutz, 2004). Recent studies with this model have established that CB_1 receptors expressed on glutamatergic neurons are important mediators of the protective function in the KA induced epileptiform seizures (Marsicano *et al.*, 2003), that the endogenous cannabinoid system is activated on demand when strong neuronal activation occurs, and that BDNF is a central mediator of the long-term neuroprotective function (Khaspekov *et al.*, 2004; Lutz, 2004). In addition to studies implicating the endocannabinoid system as protective in epileptogenesis, other studies have explored the role of cannabinoid compounds in another common cause of seizures. CP 55940, WIN 55212-2, and anandamide were able to significantly reduce lethality and seizure scores in cocaine-induced seizures (Hayase *et*

al., 2001a; Hayase *et al.*, 2001b). The authors suggest that dopamine receptors might also play a role in this protective effect. Cocaine-induced lethality and seizures are closely correlated with dopamine receptors and can be antagonized by dopamine antagonists (Hayase *et al.*, 2001a; Hayase *et al.*, 2001b). Other studies have shown co-localization of cannabinoid and dopamine receptors, suggesting a functional relationship between the two receptor types in signaling protection. Another recent study by Naderi, et al showed a synergistic anticonvulsant effect between the CB_1 receptor agonist WIN55212-2 and the classical anticonvulsant, diazepam, in the electroshock-induced seizure model in mice. This suggests an interaction between the cannabinoidergic and GABAergic systems in the modulation of seizures in mice (Naderi *et al.*, 2008). The study also showed that inhibition of FAAH by URB597 also produced significant antiepileptic effects in this model (Naderi *et al.*, 2008). By studying cannabinoid receptor function in various models of seizures, it was also found that these receptors exhibit profound plasticity in their distribution in models of long-term seizure activity.

M. CB_1 receptor plasticity in disease states

CB_1 receptor mRNA is present in the hippocampus of the rat brain as early as gestational day 16. mRNA levels continue to rise until postnatal day 1, when levels then decrease to their adult levels (Berrendero *et al.*, 1999; Fernandez-Ruiz *et al.*, 2000). However, [^{35}S]GTPγS binding studies have revealed that the mRNA levels present at early gestational ages do not signify functional receptors. CB_1 receptor binding continues to increase during development until it reaches adult levels (Berrendero *et al.*, 1999; Fernandez-Ruiz *et al.*, 2000). Interestingly, hippocampal CB_1 mRNA levels decrease in

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aged rats (2 years +) compared to young adults (3 months). However, there is no difference in functional binding in the hippocampus of adult or aged rats (Berrendero *et al.*, 1998). The endocannabinoid system plays an important role in axonal guidance during CNS development (Berghuis *et al.*, 2007), which explains why mRNA levels may be high during development.

Healthy adults maintain rather stable levels of CB_1 receptors. However, cannabinoid receptors have the ability to adapt in certain disease states, such as Huntington's disease, neuropathic pain, cerebral ischemia, and epilepsy. There is a loss of CB_1 receptor expression in the substantia nigra in Huntington's disease (Glass *et al.*, 1993; Glass *et al.*, 2000), with suggestions that it may be a protective and compensatory response to the disease state (Glass *et al.*, 2000). In Huntington's disease, loss of CB_1 receptor binding in key brain regions is detected early in human postmortem tissue (Glass *et al.*, 2000). A mouse transgenic model of Huntington's disease (Lastres-Becker *et al.*, 2002) has shown similar decreases in CB_1 mRNA in the basal ganglia. A detailed analysis of the transgenic mouse model reveals that the CB_1 receptor expression begins to decrease before motor symptoms begin, suggesting a therapeutic time point in intervention (Dowie *et al.*, 2009). It has also been shown that CB_1 receptor expression is upregulated in a rat model of chronic neuropathic pain, indicating that the increased CB_1 receptor expression may underlie the increased antinociceptive efficacy of cannabinoids in chronic pain conditions (Siegling *et al.*, 2001). In this rat model, CB_1 receptors have been shown to be upregulated in the contralateral thalamus after the unilateral axotomy of the tibial branch of the sciatic nerve (Siegling *et al.*, 2001). The authors suggest that this

CB_1 receptor upregulation contributes to the increased analgesic efficacy of cannabinoids in chronic pain conditions (Siegling *et al.*, 2001).

In a rat middle cerebral artery occlusion mode of stroke, Jin et al (Jin *et al.*, 2000) showed that CB_1 receptor expression increased beginning by 2 hours and persisting for 72 hours or more after ischemia. Western blot and immunohistochemical studies showed the CB_1 receptor expression increased at the arterial boundary zone of the cortical mantle. A more recent study used quantitative PCR to measure CB_1 receptor mRNA content following transient middle cerebral artery occlusion in mice and showed similar results: the mRNA for the CB_1 receptor in the brain ipsilateral to the occlusion was significantly elevated at 1 hour, and maximally elevated 6 hours after the ischemia (Zhang *et al.*, 2008). In contrast, 5 hours of permanent middle cerebral artery occlusion had no effect on CB_1 receptor binding site density in male rats (Sommer *et al.*, 2006). This suggests that the increase in CB_1 receptor following transient ischemia is a result of reperfusion rather than ischemia.

CB_1 receptors have also been demonstrated to show plasticity changes in epilepsy in several studies. In the febrile seizure model of epilepsy, blockade of CB_1 receptors seems to play a protective role against CB_1 reorganization. In the model, rats are exposed to heat at postnatal day 10, increasing their core temperature to 41-42°C. When monitored several weeks later, the rats show alterations in inhibitory neurotransmission in the hippocampus, attributed to a potentiation of endocannabinoid signaling that was caused by an increase in the number of presynaptic CB_1 receptors on GABAergic interneurons (Chen *et al.*, 2003; Chen *et al.*, 2007; Lutz, 2004). This model is clinically relevant as there is a potential clinical association of prolonged, complex febrile seizures

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in infancy to temporal lobe epilepsy in adults. In this model, DSI was selectively enhanced, but no changes of glutamatergic transmission were observed, implicating a protective role of the endocannabinoid system (Chen *et al.*, 2003; Chen *et al.*, 2007; Lutz, 2004). They were also able to show that blocking the induction of this plasticity with CB_1 receptor antagonists (SR141716A) abolished the long term effects of prolonged febrile seizures in the developing brain (Chen *et al.*, 2007). Notably, they also showed that SR141716A had an acute proconvulsant effect, with beneficial chronic effects on seizure thresholds (Chen *et al.*, 2007). The DeLorenzo laboratory has also shown that there is a long-term plasticity change in the expression of the CB_1 receptor with the induction of epilepsy in the pilocarpine model of acquired epilepsy in the rat. They demonstrated that status epilepticus causes a unique "redistribution" of hippocampal CB_1 receptors, consisting of specific decreases of CB_1 expression and corresponding functional changes, while other areas showed increases in expression and function (Falenski *et al.*, 2007). These results demonstrated that status epilepticus causes a unique and selective reorganization of the CB_1 receptor system that persists as a permanent hippocampal neuronal plasticity change associated with the development of acquired epilepsy (Falenski *et al.*, 2007). A more recent study has shown that these changes occur in a time-dependent manner. One week following SE injury, there is a prominent loss of CB_1 receptor immunoreactivity throughout the hippocampus. When rats began displaying spontaneous seizures at 1 month post-SE, the CB_1 receptor distribution was characteristic of that observed in long term epileptic rats (Falenski *et al.*, 2009). Specifically, the study noted decreases in CB_1 receptor immunoreactivity in the stratum pyramidale neuropil and dentate gyrus inner molecular layer, and increases in the strata oriens and radiatum of

CA1-3 (Falenski *et al.*, 2009). These studies suggest that the dysregulation of CB_1 receptor expression has a temporal correlation with the emergence of recurrent seizures. The localization of the CB_1 receptor changes indicates that the upregulation of CB_1 receptors is at the glutamatergic terminals, suggesting that the increase in CB_1 receptors may act as a compensatory mechanism to control epileptic seizures and prevent SE. In addition, a downregulation of CB_1 receptors at the GABAergic synapses would allow for greater inhibition during seizure events.

Other studies of reorganization of CB_1 expression have been demonstrated in human tissue. A recent study of by Ludanyi et al. used hippocampal tissue from epileptic and seizure free humans (Ludanyi *et al.*, 2008). The study used expression profiling and quantitative electron microscopic analysis to show a major downregulation of CB_1 receptors in the hippocampal formation of patients with intractable temporal lobe epilepsy. This downregulation was mostly seen on the glutamatergic synapses of the hippocampus, with a portion of the CB_1 downregulation occurring in mossy cells. The net effect of downregulation of CB_1 at these excitatory synapses is that excitatory synapses innervating granule cell dendrites will remain without negative feedback control in the epileptic dentate gyrus (Ludanyi *et al.*, 2008). They also found no significant changes in the number of CB_1-positive GABAergic interneurons, confirming findings from earlier animal studies. These results suggest that the patients with intractable epilepsy may not have had the compensatory upregulation of CB_1 receptors at glutamatergic synapses to aid in the control of seizures that is observed in the rat pilocarpine model.

N. Cannabinoid pro-convulsant effects and tolerance

The large number of studies implicating cannabinoid compounds as
anticonvulsants are in direct conflict with several studies and reports that implicate
cannabinoids as proconvulsant agents. In the early anticonvulsant studies of the 1970s,
administration of Δ-9-THC produced convulsions in a population of rabbits (Consroe *et
al.*, 1977) and epileptic beagle dogs (Mechoulam, 1986). However, convulsions do not
occur in these animals with CBD and other nonpsychoactive cannabinoids, even with
higher doses. Mechoulam reviewed literature in which Δ-9-THC, given by intravenous,
oral, and inhalation routes caused convulsions in non-seizure susceptible animals, though
he notes that these convulsions occurred only with lethal or near-lethal doses given
acutely or high doses given repeatedly for months (Mechoulam, 1986). In some
experimental models of seizure, namely the iron- or cobalt-induced epileptic rat, the
aluminum-induced epileptic cat, and the limbic-kindled epileptic rat, low doses of Δ-9-
THC and some THC compounds can elicit convulsions, while similar or higher doses of
CBD do not (Chiu *et al.*, 1979; Mechoulam, 1986). How can cannabinoids act in opposite
fashion in different models? This paradox can be reconciled in part by considering the
dual role that endocannabinoids are known to play in the brain. Endocannabinoids are
able to elicit inhibitory effects by blocking glutamate release or excitatory effects by
blocking GABA release, depending on which neural circuits are activated (Mechoulam
and Lichtman, 2003). FAAH knockout mice represent one model that shows a
proconvulsant effect. FAAH is responsible for breaking down anandamide in the brain,
therefore FAAH knockouts show an increased endogenous level of anandamide. These
mice show a lower seizure threshold and FAAH regulated proconvulsant and neurotoxic

activity (Clement *et al.*, 2003). This effect is reasonable due to the fact that the activation of the endogenous cannabinoid system was not cell-type specific as it is active in both GABAergic and glutamatergic neurons and not in an appropriate time-course, as CB_1 receptors were activated for a long period of time. Nakatsuka et al also demonstrated that cannabinoids can potentiate epileptiform activity where CB_1 receptors are localized to GABAergic terminals in patch clamp experiments using granule cells of the human dentate gyrus (Nakatsuka *et al.*, 2003). They showed that the activation of CB_1 receptors could suppress inhibitory synaptic activity. Bath application of WIN 55212-2 suppressed the frequency of spontaneous inhibitory post-synaptic currents and AM251 completely blocked these effects, suggesting that they were mediated by CB_1 receptors (Smith, 2005).

In considering cannabinoids as a potential treatment of seizures, the issue of drug tolerance must also be considered. In some of Karler's early studies of cannabinoids in the MES model, they noted the development of tolerance to the anticonvulsant effects of cannabinoids (Colasanti *et al.*, 1982; Karler *et al.*, 1974a; Karler and Turkanis, 1980). Complete tolerance of Δ-9-THC to MES anticonvulsant effects has been reported in as early as 3-4 days. Cross-tolerance of Δ-9-THC tolerant animals to phenytoin and phenobarbital also occurred (Karler *et al.*, 1974a). It was also shown that withdrawal after repeated high-dose administration might increase seizure susceptibility in some epileptic animals (Karler and Turkanis, 1980). Chronically administered cannabinoids produce tolerance to their acute behavioral effects, in which the potency or efficacy of the drug changes such that the physiological and/or psychological consequences of the same drug dose are significantly diminished with repeated use (Martin *et al.*, 2004). However, this

tolerance to *in vivo* cannabinoid effects does not appear to result from alterations in cannabinoid pharmacokinetics (Martin, 1986; Martin, 2005). Continued administration of cannabinoids can also lead to dependence in humans, as well as in increased levels of dopamine in the nucleus accumbens of animals (Smith, 2005). The issues of tolerance and dependence have not been thoroughly explored in regards to the use of cannabinoids as anti-epileptics, but they deserve further attention as the prevalence of *Cannabis* use among American adults is about 4%, with 30% of users reaching levels of dependence (Compton *et al.*, 2004). Recreational marijuana users with epilepsy may represent a significant patient population. Furthermore, there may be a statistically unknown number of patients with epilepsy who choose to use cannabinoids as a means of "self-medication" for their condition.

O. Future directions, potential therapies

The exploitation of the brain's endogenous system of controlling excess hyperexcitability is logically a potential target for epilepsy therapeutics. Because of its ubiquitous nature, it is important that future studies of cannabinoids in the treatment of epilepsy must be focused on the brain regions and cells at the focal point of the seizures. The potential problem in delivering a cannabinoid drug to treat epilepsy is the inability to control its actions at different cannabinoid receptors regulating the release of different neurotransmitters. Animal studies, as well as information from human studies have shown conflicting results as to cannabinoids pro- or anti-convulsant effects. The effect may be mediated by the type and focal point of the seizures, as well as origin, whether it be genetic in nature or precipitated by a neurological insult. The location of CB_1

receptors at either glutamatergic or GABAergic synapses helps to explain the dual actions of cannabinoids in regards to anti-seizure activity. As there are many types and causes of epilepsy, it is clear that the endocannabinoid system may not be an effective target for all forms of epilepsy. Lutz proposes that a potential therapeutic endocannabinoid target lies in the uptake inhibitors (Lutz and Monory, 2008). By inhibiting anandamide uptake, CB_1 receptors would remain activated in the synapses that are producing the endocannabinoids on demand. This mechanism would more specifically target the glutamatergic synapses, while having less of an effect on the pro-convulsant GABAergic synapses. Several uptake inhibitors have been characterized, AM404, UCM707, OMDM-1, and OMDM-2. He reasons that targeting uptake may be more beneficial than prolonged application of CB_1 receptor agonists, since prolonged application may actually lead to a down-regulation in CB_1 receptor level. Interestingly, these inhibitors evoke much less or even no psychotropic effects as compared to CB_1 receptor agonists (Lutz, 2004). Further research needs to be done with these compounds, as current studies have produced mixed results (Marsicano *et al.*, 2003; Naderi *et al.*, 2008). Enhancing endocannabinoid tone is perhaps a safer approach than using exogenous endocannabinoids. Recent studies suggest that the unique pharmacology of endocannabinoids reduces the cellular changes underlying tolerance and allows for a greater control over CB_1 responsiveness (Bahr *et al.*, 2006). Endogenously released cannabinoids can lead to markedly different outcomes in synaptic physiology compared to exogenously applied CB_1 agonists. In addition, Lutz and Monory have recently proposed targeting CB_1 receptors to prevent the development of epilepsy following febrile seizures in infants (Lutz and Monory, 2008). They reason that acute blockade of

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CB$_1$ on GABAergic terminals during neuronal excitability of febrile seizures leads to a decrease of hyperexcitability in these cells, preventing the upregulation of CB$_1$ that leads to a lowered seizure threshold. However, they note that acutely blocking CB$_1$ on glutamatergic terminals is pro-convulsant during a critical period of initial neuronal insult. Still, the idea of manipulating the endocannabinoid system during or shortly after the precipitating event of epileptogenesis is intriguing as a possible mechanism to prevent the plasticity changes seen in CB$_1$ receptor organization in epileptic animals and humans. Further research is needed to examine the potential of targeting the endocannabinoid system to prevent epileptogenesis and to control seizures in those suffering from the debilitating disease. As noted by Robert Christison in 1848 as he investigated the many beneficial effects of *Cannabis*, it "is a remedy which deserves a more extensive inquiry…" (Alger, 2004; Piomelli, 2004).

P. Summary and rationale

Cerebral ischemia is a catastrophic event with significant morbidity and mortality. It is the leading cause of acquired epilepsy in persons 35 and older (Forsgren *et al.*, 1996), yet little is known about the underlying basic mechanisms leading to stroke induced epilepsy. Compared to the general population, patients who have suffered a cerebral infarct have a 17-fold greater risk of developing recurrent seizures (Warlow *et al.*, 2003). In addition, 30% of patients with epilepsy do not obtain satisfactory control of their seizures with the medical and pharmacological interventions currently available (Morimoto *et al.*, 2004), underscoring the need for greater research about stroke induced epilepsy.

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Models of stroke induced epilepsy are difficult to come by. An *in vitro* model utilizing hippocampal neuronal cultures has successfully modeled many aspects of the ischemic penumbra (Sun *et al.*, 2001) and has developed electrographic seizures following the injury. *In vivo* models of stroke induced epilepsy have been more difficult to study. Acute non-convulsive seizures follow middle cerebral artery occlusion in adult rats (Hartings *et al.*, 2003) and perinatal models of ischemia have been shown to produce acquired epilepsy (Kadam *et al.*, 2010). However, there haven't been any good models for stroke acquired epilepsy *in vivo*, therefore a more intact *in vitro* system is appealing to study the molecular changes following stroke.

Organotypic hippocampal slice cultures have been utilized for years in various studies of stroke and seizures. They have a distinct advantage over HNC *in vitro* models in that they maintain normal cellular morphology and architecture and continue to develop in culture (Bahr, 1995), while allowing for easy manipulation. They are between HNC and *in vivo* models in terms of complexity and can assist in answering many basic physiological and anatomical questions. OHSCs have been studied extensively in oxygen-glucose deprivation and glutamate injuries to mimic the effects of ischemia. Most studies have focused on manipulating the system to prevent cell death, while few have looked at the long term physiological outcomes. In addition, few studies have utilized OHSCs to produce spontaneous recurrent seizures as a model of epilepsy (Bausch *et al.*, 2006; Routbort *et al.*, 1999). With this in mind, it seems that OHSCs would be an ideal substrate to study the physiological consequences of a stroke-like injury.

There is a plethora of experimental data to suggest that modulation of the endocannabinoid system plays a role in epilepsy. Our laboratory has extensively studied

the endocannabinoid system in the rat pilocarpine model of epilepsy. Studies have found that there is a unique, functional redistribution of CB_1 receptors in the hippocampus during the development of epilepsy (Falenski *et al.*, 2007; Wallace *et al.*, 2003), with an overall increase in CB_1 receptor expression. Evidence also exists that the endocannabinoid system undergoes upregulation in cerebral ischemia (Hillard, 2008). A model of stroke induced acquired epilepsy would provide an ideal system for studying the endocannabinoid system following stroke-like injury. Studies have shown the OHSCs express CB_1 receptors similarly to *in vivo* tissue (Boscia *et al.*, 2008), indicating that OHSCs are an appropriate system to study CB_1 receptor changes *in vitro*. In addition, the ability to maintain OHSCs for long periods of time after a manipulation is an advantage to using such an *in vitro* system to study long-term changes after an injury. The ease of manipulation also makes an OHSC model ideal for performing electrophysiological and immunohistochemical studies. Based on strong evidence from *in vitro* and *in vivo* studies, we propose the development of an OHSC model of stroke-induced acquired epilepsy to further study the anatomical and physiological changes associated with the endocannabinoid system.

Q. Central hypothesis

We hypothesize that status epilepticus and stroke like injuries will cause increased neuronal excitability in organotypic hippocampal slice cultures, rendering a model of acquired epilepsy. In addition, we hypothesize that the endocannabinoid system plays a modulatory role in stroke-induced acquired epilepsy in our OHSC model. Addressing the following experimental questions in this dissertation will rigorously test our hypotheses.

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1. Does a status epilepticus like injury induce spontaneous recurrent epileptiform discharges in our organotypic hippocampal slice cultures?

2. Does a stroke-like injury induce spontaneous recurrent epileptiform discharges in adult-equivalent organotypic hippocampal slice cultures?

3. Are CB_1 receptors upregulated in OHSCs that display seizure activity? What is the distribution of the CB_1 receptors?

4. Is there a functional difference in CB_1 receptor activity following a stroke-like injury?

An organotypic hippocampal slice culture protocol has been established in our laboratory specifically to address the questions raised in this dissertation. Experimental results from this dissertation establish the use of OHSCs as a model of acquired epilepsy following a status-epilepticus like injury or a stroke-like injury. Electrophysiological and cell death assays were employed to study the injuries and assess the physiological consequences to neuronal hyperexcitability. Immunohistochemical and western blot techniques were utilized to examine the CB_1 receptor distribution in OHSCs following stroke-like injury. Electrophysiological analysis of DSI and DSE following stroke-like injury indicate that the CB_1 receptor distribution changes following glutamate injury is functional and possibly plays a role in controlling neuronal excitability in an epileptic state.

CHAPTER 1: 4-AMINOPYRIDINE TREATMENT CAUSES RECURRENT, SPONTANEOUS SEIZURES IN AN ORGANOTYPIC HIPPOCAMPAL SLICE CULTURE MODEL OF ACQUIRED EPILEPSY

A. Introduction

Epilepsy is a significant problem in neurology affecting approximately 2.7 million people in the United States and costing at about \$15.5 billion a year in medical costs and lost or reduced earnings and productivity (Kobau *et al.*, 2005). Epilepsy is a debilitating disease with a significant impact on quality of life. Furthermore, approximately 30% of patients living with epilepsy have seizures that are not adequately controlled via medical intervention. Among cases of epilepsy, epidemiological studies have revealed that the cause of epilepsy was unknown in 68% of the cases and preceded by a brain insult in 31% of the cases (Shneker and Fountain, 2003). The brain insults that are known to lead to acquired epilepsy (AE) include stroke, CNS infection, traumatic brain injury, and status epilepticus (DeLorenzo, 2006). Acquired epilepsy is a serious clinical problem, but it has been challenging to develop good experimental models in the laboratory to carefully study the neurological changes that occur during the development of epilepsy (epileptogenesis) after a brain insult. Because of the severity of this problem worldwide, it is important to develop new models of acquired epilepsy to allow for screening of novel anti-epileptic drugs that may provide seizure relief for patients with refractory

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seizures while elucidating possible molecular targets to prevent epileptogenesis following brain insult.

Organotypic hippocampal slice cultures (OHSCs) are a useful tool in epilepsy research as they are easy to manipulate, can be maintained in culture for long periods of time and are more cost effective than animal models while still maintaining normal anatomical morphology (Stoppini *et al.*, 1991; Sundstrom *et al.*, 2005; Zimmer and Gahwiler, 1984). OHSCs have been used for years to study acute epileptiform activity. To induce this activity, epilepsy researchers have used convulsant drugs such as pilocarpine, kainic acid, and 4-aminopyridine (Bausch and McNamara, 2000; Gutiérrez *et al.*, 1999; Wahab *et al.*, 2010a; Wahab *et al.*, 2010b). In addition, serum deprivation and perfusion with a media containing no magnesium has also been shown to produce acute epileptiform activity in OHSCs (Albus *et al.*, 2008). However, few studies have used OHSCs to produce a model of acquired epilepsy wherein spontaneous epileptiform events occur following an injury. Spontaneous seizure events are known to occur in a small number of OHSCs after long term in culture (Bausch and McNamara, 2000; McBain *et al.*, 1989; Routbort *et al.*, 1999), but few studies have shown an injurious manipulation leading to an increase in seizure activity. Bausch et al (Bausch *et al.*, 2006) showed an increase in seizure activity in OHSCs after long-term treatment with TTX and subsequent removal of the sodium channel blocker. Activity deprivation causing AE led to interesting insights of morphological changes in the seizing OHSC, but is not representative of a brain injury leading to AE. Similarly, the same group employed kainic acid as a method of injuring OHSCs. In this model, they found no significant difference in spontaneous seizure activity between injured slices and controls after 40 days *in vitro*,

however they were able to show increased hyperexcitability in granule cells of injured OHSCs (Bausch and McNamara, 2004).

Because of their usefulness in epilepsy research, other methods of injury are worth exploring for their potential to cause AE in OHSCs. The classic convulsant 4-aminopyridine, (4-AP) has been used in animal models of SE wherein i.p. injection causes tonic-clonic seizure activity in mice (Medina-Ceja *et al.*, 2008). In addition, 4-AP has recently been shown to acutely produce seizures in OHSCs that are refractory to most standard anti-epileptic drugs (AEDs) (Albus *et al.*, 2008). 4-AP interferes with several types of K^+ channels that include the D type and A type K^+ currents and a subportion of delayed rectifier currents (Wahab *et al.*, 2010a). Blocking K^+ channels interferes with the cell's ability to repolarize, prolonging action potentials and increasing the amount of neurotransmitter released. Since exposure to 4-AP acutely caused drug refractory seizures in OHSCs, we sought to explore the long term effects of using 4-AP to produce a SE-like injury.

B. Methods

Organotypic hippocampal slice culture preparation

Slice cultures were prepared using the method of Stoppini et al. (Stoppini *et al.*, 1991), as previously reported by (Schanuel *et al.*, 2008). All animal use protocols are in strict accordance with the National Institute of Health guidelines and are approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Postnatal day 8 (P8) Sprague-Dawley rat pups (Harlan, Frederick, MD, USA) were deeply anesthetized with isoflurane and decapitated. The brains were removed, and

hippocampi were dissected out and cut into 350-µm transverse sections using a McIlwain

tissue chopper (Brinkmann Instruments, Canada) and placed into Hank's balanced salt

solution [HBSS – Gibco BRL (Invitrogen, Carlsbad, CA, USA)] supplemented with 0.5%

sucrose and 1% penicillin-streptomycin. The middle four to six slices of each

hippocampus, including part of the entorhinal cortex, were placed onto tissue culture

membrane inserts (Millipore, Bedford, MA) in a 6-well tissue culture dish containing

medium consisting of 50% minimum essential medium, 25% horse serum and 25%

HBSS, 1% penicillin-streptomycin [all from Gibco BRL (Invitrogen, Carlsbad, CA)] and

supplemented with 36 mM glucose, and 25 mM HEPES (Sigma, St. Louis, MO, USA)

(pH 7.2). Cultures were maintained at 37°C under room air +5% CO_2. After 1 day in

culture, culture medium was replaced with fresh medium containing no antibiotics.

Culture medium was replaced two times a week thereafter.

Field potential recording with acute 4-AP application

To observe the acute effects of 4-AP, several OHSCs were recorded at DIV 10. A

portion of the tissue culture insert membrane containing a single cultured slice was placed

into an interface slice recording chamber (Harvard Apparatus, Holliston MA) mounted on

a vibration table and viewed through a dissecting microscope (Diagnostic Instruments,

Sterling Heights, MI). Slice cultures were perfused (2–3 ml/min, gravity feed system)

with artificial cerebrospinal fluid a minimal essential medium (MEM) (containing in

addition to amino acids and vitamins (in mM): NaCl 105, KCl 3, NaH_2PO_4 1.25, $MgSO_4$

1.8, $CaCl_2$ 1.6, glucose 10, $NaHCO_3$ 26.3 mM, pH 7.40, osmolality 265 mosm)

equilibrated with 95% O_2-5% CO_2. Recording pipettes were pulled on a Flaming Brown

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P-80 PC Micropipette puller (Sutter Instrument Co, Novato, CA) filled with 3.5 M NaCl for extracellular recordings to achieve a resistance of 2-4 MΩ. Data were collected with an AxoClamp 2B (Molecular Devices, Union City, CA) recording amplifier and FLA-01 Bessel filter unit (Cygnus Technologies, Inc, Delaware Water Gap, PA) and pCLAMP 9.0 software (Molecular Devices). After a 15 minute equilibration period in aCSF, 200µM 4-AP was added to the perfusant and field potentials were recorded at 34°C in the CA3 pyramidal cell layer. OHSCs were perfused with the drug for 45 minutes before wash-out with normal MEM.

4-AP injury

After 10 days *in vitro* (DIV10), slice culture media was removed and replaced by media containing 200µM 4-AP for 24 hours. Drug containing media was then removed and slice cultures were washed with HBSS at 37°C two times before returning to normal media. Control slices received normal media (without glutamate) during the 24 hour time period, followed by washing with HBSS twice before returning to normal media.

Neuronal death assay

Neuronal death was assessed 24 hours, 5 days and 14 days after 4-AP treatment by measuring uptake of the fluorescent dye Propidium Iodide (PI), using established procedures (Lipski *et al.*, 2007; Pomper *et al.*, 2001). Greater PI uptake indicates greater cell death. Cultures were exposed to 2 µM PI for 4 hours in normal culture media before injury to assess pre-injury cell death. PI concentration was maintained in the culture during injury and for the following 14 days. Before recording dye uptake under fluorescent light, a phase contrast picture of each slice was taken so that orientation in the

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field could be maintained in subsequent recordings. Dye uptake was recorded on an Olympus CK40 inverted microscope coupled to a QColor 3 camera and QCapture software (Olympus, Center Valley, PA). All optical parameters (illumination aperture, neutral density filters etc.) as well as the camera exposure time and electronic gain, were standardized and kept constant. The fluorescent signal was measured densitometrically using the Image-J software (NIH). The perimeter of each slice was outlined (identified in phase contrast image) and the mean pixel value (0–255) was recorded and converted to a scale of 0-100. All measurements were made after subtracting background fluorescence (region positioned immediately outside the culture). Cell death was expressed as a percent of mean pixel value of age matched controls.

Field potential recording for delayed seizures

At DIV 25-26, a portion of the tissue culture insert membrane containing a single cultured slice was placed into an interface slice recording chamber. Field potential recording parameters were the same as reported for acute 4-AP application. After a 15 minute equilibration period in MEM, field potentials were recorded at 34°C in the CA3 pyramidal cell layer. If spontaneous activity was not observed in the first few minutes, slices were tested for viability by hilar stimulation (0.3-ms square pulse, 0.03 Hz, 50–150 µA) using a bipolar concentric electrode (FHC, Inc., Bowdoin, ME) and a stimulator (World Precision Instruments, Sarasota, FL). OHSCs were first tested for viability by stimulating in the dentate gyrus while recording in the CA3 cell layer (Figure 4). Slices were considered acceptable if stimulation elicited an action potential spike that immediately followed the stimulus artifact with a response threshold ≤150 µA (Bausch and McNamara, 2000; Bausch *et al.*, 2006). Neither the amplitude of the spike nor the

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shape of the waveform was used as criterion for acceptable recordings. Each slice culture was recorded at 2 minute intervals (2 minutes on, 2 minutes off) over a 20 minute time period, for a total of 10 minutes of recorded activity. Recordings were scored for seizure events by two separate individuals who were blinded to the treatment group.

Data analyses

Data are expressed as mean ± SEM. To determine significance between treatment groups for neuronal death assays, Student's t-tests were used. For extracellular recordings, z- test was used. In each experiment, n refers to the number of OHSCs. For neuronal death assays, glutamate treated slices were normalized to control slices harvested from the same animal. All experiments were performed over the period of several weeks to months so that the results were representative of multiple cultures. A *P*-value < 0.05 was considered significant. Statistical analysis was performed using SigmaStat 2.0 software (Systat Software, San Jose, CA, USA) and graphs were drawn using Microsoft Excel for Mac software (Microsoft corporation).

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Figure 4. A. Phase contrast photo (4X) of an OHSC at DIV 21 - Cell layers of the dentate gyrus (DG), CA3 and CA1 are clearly visible. **B. Field potential recording of OHSC-** To test viability of OHSCs during field potential recording, the stimulating electrode is placed in the DG while the recording electrode is placed over the CA3 cell region. The trace shows an example of an evoked field potential recording from a viable OHSC. The stimulus artifact is followed by the field potential.

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Figure 4

a.

Stimulus

Record

CA1

CA3

DG

b.

0.1mV

50 msec
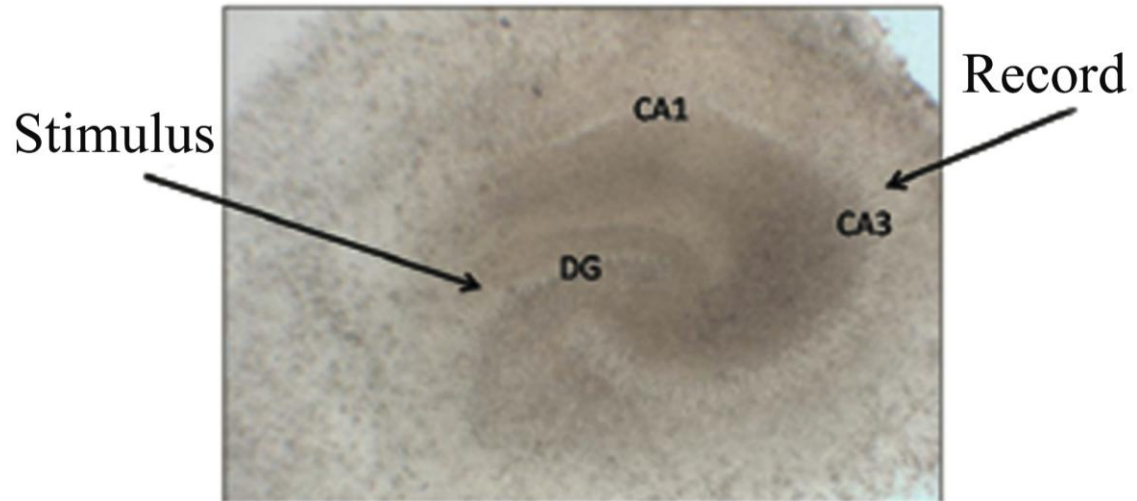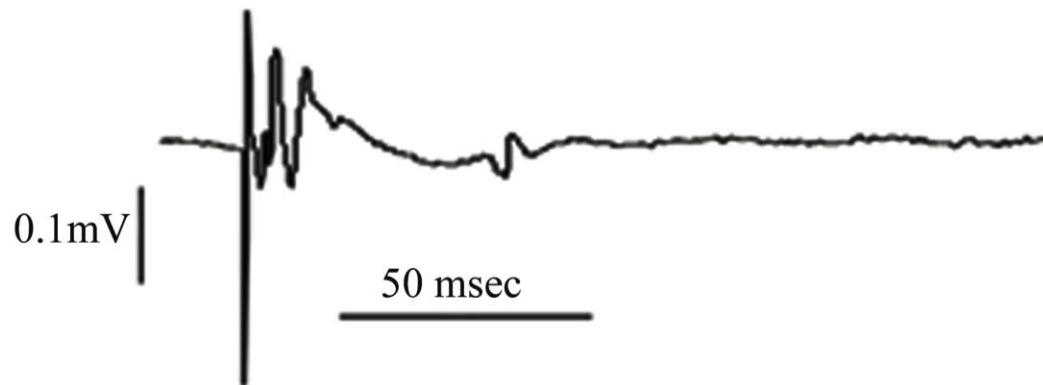
C. Results

4-AP acutely causes seizure activity

At DIV10, perfusing OHSCs with MEM containing 200 µM 4-AP caused high frequency electrical discharges as observed with field potential recording. Recording of the CA3 cell area demonstrated low frequency bursting, generally beginning within the first 10 minutes of 4-AP perfusion (Figure 5). This bursting evolved into high frequency tonic-clonic like discharges (Figure 6) that continued in the presence of 4-AP. These events were comparable to *in vivo* SE, as one event followed another or evolved into a state of continuous activity. These high-frequency events lasted up to an hour in these experiments and have been reported to last up to 120 minutes (Albus *et al.*, 2008) in the literature.

4-AP exposure causes cell death

Addition of 200µM 4-AP to the slice culture media for a 24 hour period significantly increased PI uptake just prior to removal of the drug. Propidium iodide uptake was measured again at 5 and 14 days following the injury. When expressing PI uptake as a percent of control, 4-AP treated OHSCs showed a $171\pm18.6\%$ (n=24) increase in PI uptake (control = $100 \pm18.8\%$, n=12, P=0.023) at the end of the injury period. However, the difference in PI uptake was not significantly different between the two groups at 5 days [treated =$121.8 \pm 6.5\%$ (n=17), control=100 ± 9.8 (n=8), P=0.078] or 14 days [treated = $120.1 \pm 7.0\%$ (n=17), control = 100 ± 8.23 (n=8), P=0.106] following 4-AP treatment. This indicates that the majority of the cell death from the excitotoxic

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injury occurs acutely, which is similar to what is observed in other *in vitro* models of SE induced acquired epilepsy (Deshpande *et al.*, 2007c) (Figures 7 and 8).

4-AP induces long-term changes in OHSC excitability.

To investigate changes in OHSC excitability, we obtained field potential recordings from the CA3 region of OHSCs 14 days after 4-AP injury to allow for a period of epileptogenesis. Slices were recorded in normal physiological media without the addition of pharmacological agents or the use of stimulation to induce seizure activity. Representative field potential recordings of control and 4-AP treated OHSCs are shown in Figure 9. Seizure events were defined as the abrupt onset of a high amplitude burst of rhythmic activity superimposed on a field depolarization shift that lasted ≥ 3 s, during which the waveforms evolved over time and terminated abruptly (Bausch *et al.*, 2006; Routbort *et al.*, 1999). Field potential recordings showed seizure events in 62% of 4-AP treated OHSCs (n= 37) compared to 30% of control slices (n= 20, P=0.006, z-test) (Figure 10). While some control and injured slices displayed individual action potentials and brief depolarizing shifts, these slices were not considered to have seizure events unless the burst of activity occurred for greater than 3 seconds. These electrographic seizures displayed both ictal and interictal activity, similar to what is observed in *in vitro* and *in vivo* seizure models, as well as in EEG recordings of human epilepsy.

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Figure 5 – 4-AP perfusion, 5 minutes

Field potential recording of a DIV 10 OHSC after 5 minutes of 200μM 4-AP perfusion.

Short bursts begin to appear within the first 10 minutes of perfusion with 4-AP. High pass

filtered offline at 10Hz.

Figure 5

10 sec

0.2 mV

1 sec
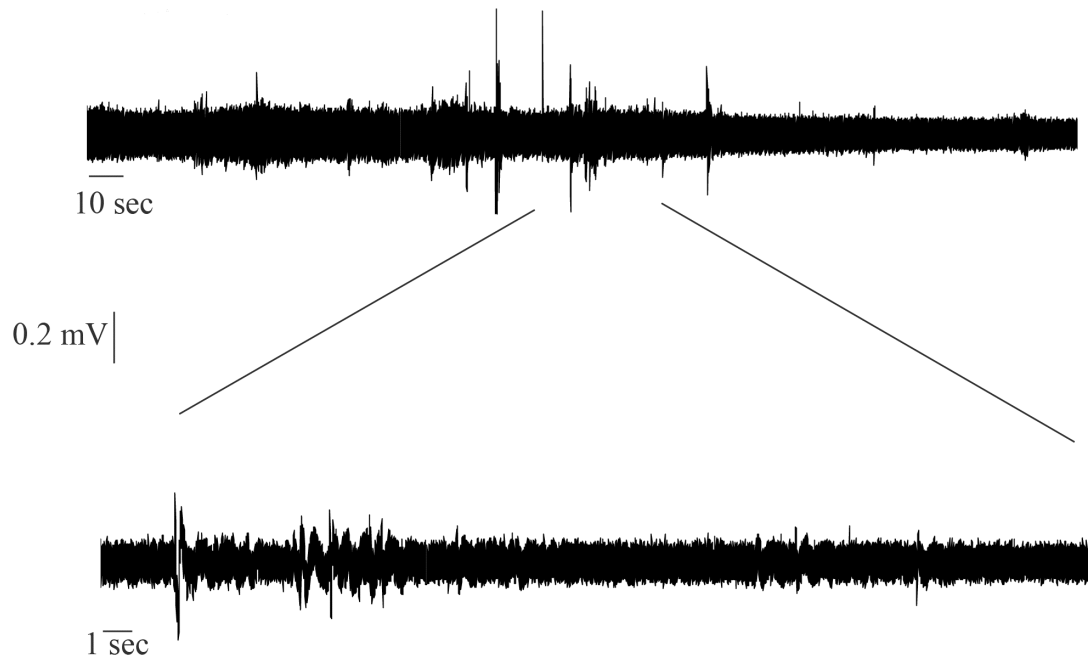
Figure 6 - 4-AP perfusion, 25 minutes.

Field potential recording of a DIV 10 OHSC after 25 minutes of 200µM 4-AP perfusion.

Ictal and inter-ictal bursting patterns are observed, characteristic of the refractory model

of seizures described by Albus et al. (Albus *et al.*, 2008). Traces were high pass filtered

offline at 10Hz.

Figure 6

0.2 mV

10 sec

2 sec

0.1 sec
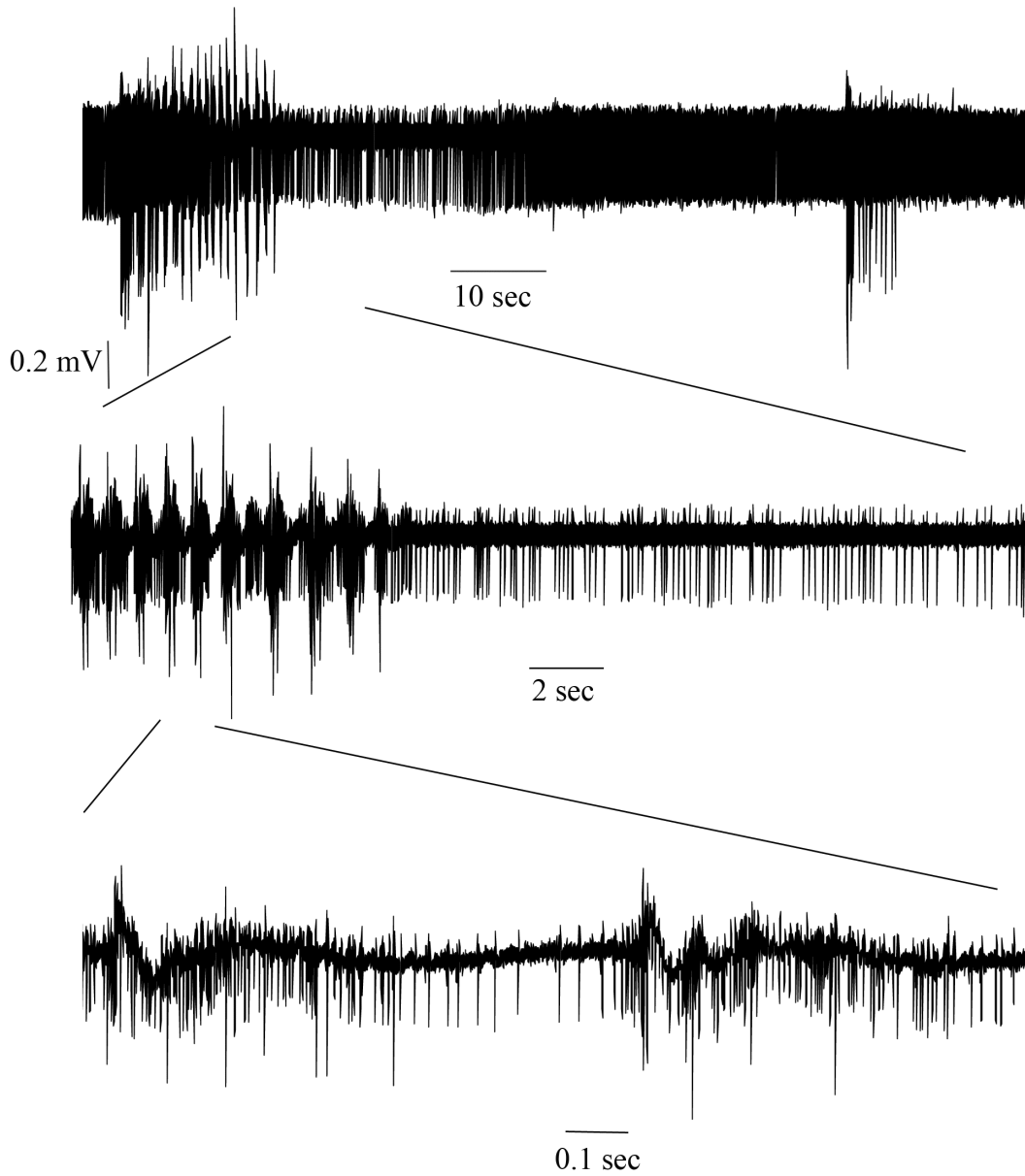
Figure 7 – Propidium iodide uptake following 4-AP treatment

4-AP causes cell death, indicated by the red staining of Propidium Iodide (PI). PI uptake is greatly increased in OHSCs following treatment with 24 hours of 4-AP (bottom panels) compared to control slices (top panels). Staining is especially notable in the pyramidal and granule cell layers of OHSCs.

Figure 7

24 hrs   5 days   14 days

Control
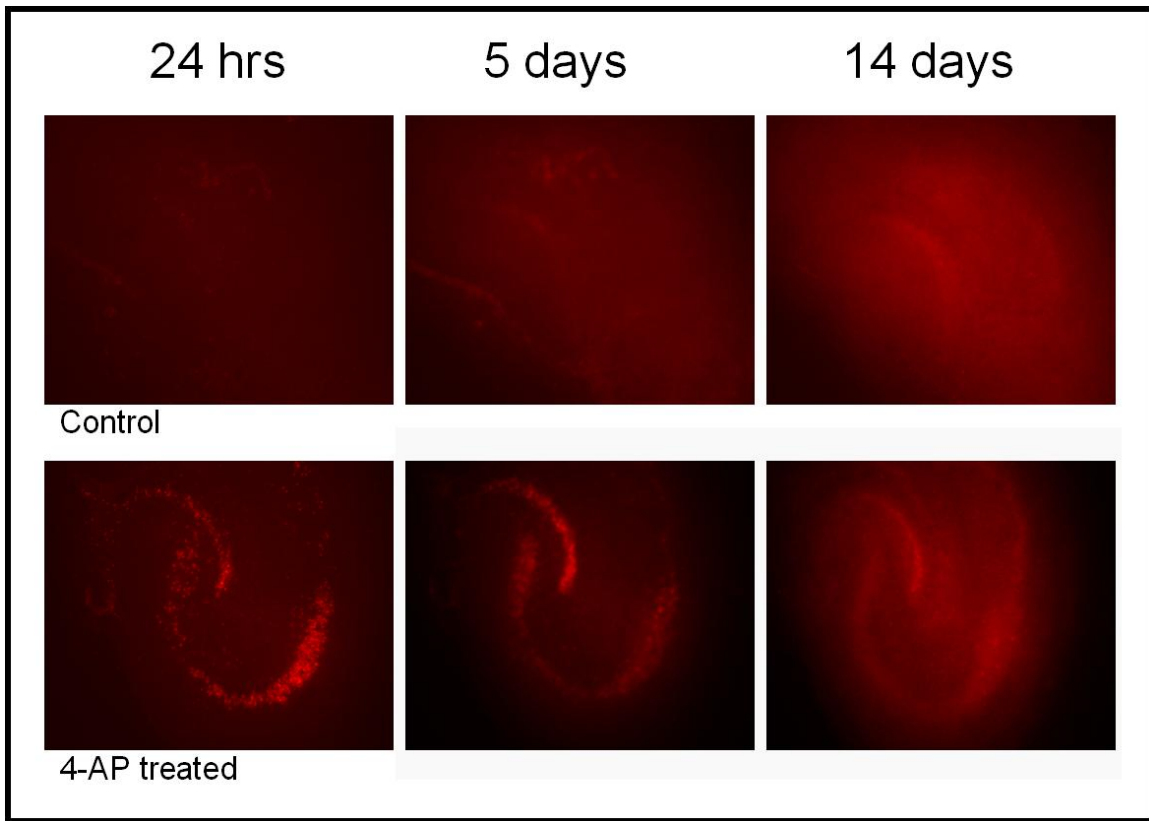
4-AP treated

Figure 8 – Bar graph of PI staining

Bar graph representing the densitometric analysis of PI uptake in OHSCs following 4-AP injury. There is significantly more cell death in OHSCs that have undergone 4-AP treatment than controls at the 24 hour time point (*$p<0.05$). However, these differences are not significant at 5 days or 14 days following the injury, indicating that most of the cell death occurs acutely.

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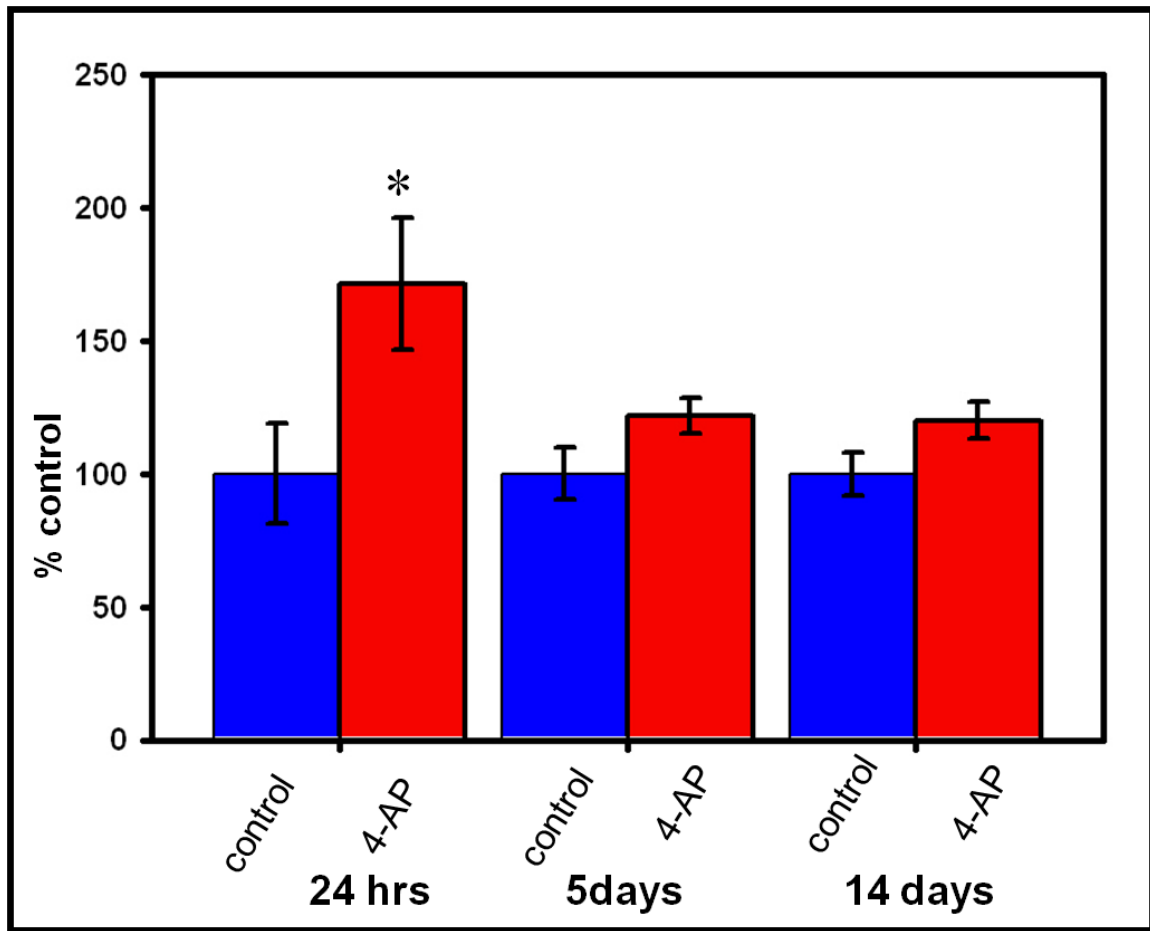
Figure 8

Figure 9 – 4-AP treatment causes long term seizure activity

Field potential recording from CA3 14 days after 4-AP injury reveals a significant number of OHSCs that display seizure activity. An in vitro seizure is defined as an electrographic event with an abrupt onset of a high amplitude burst of rhythmic activity superimposed on a field depolarization shift that lasted ≥3 s, during which the waveforms evolved over time and terminated abruptly

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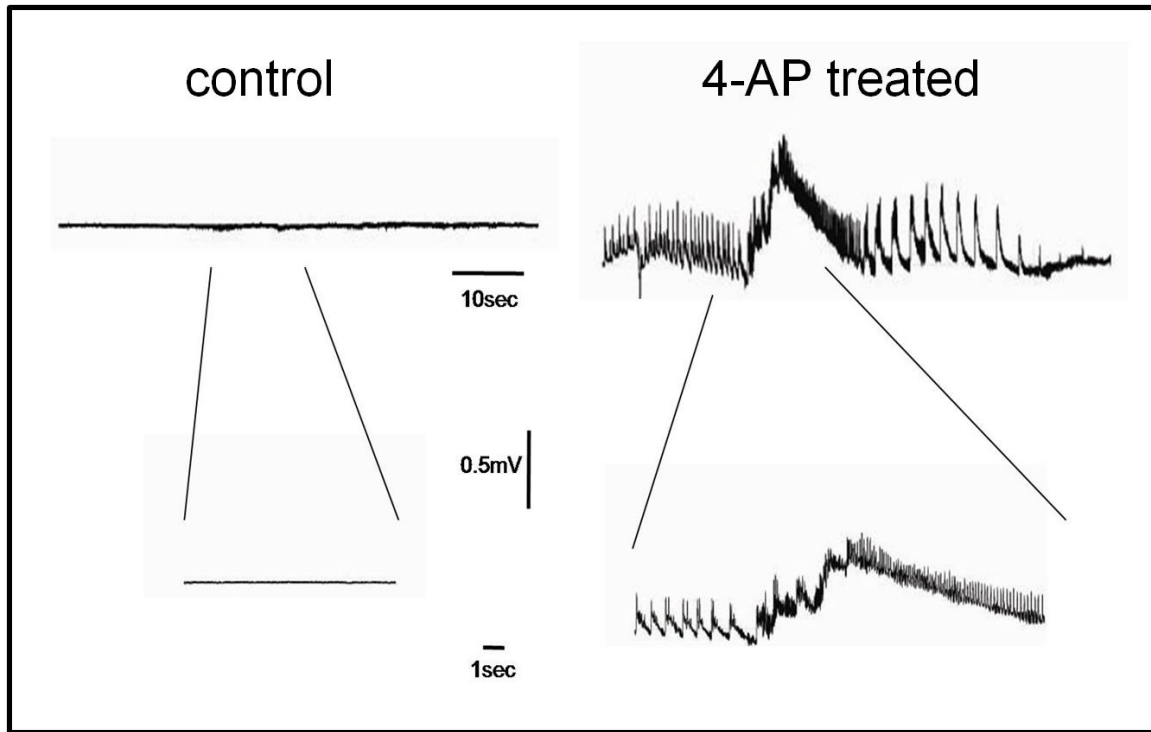
Figure 9

Figure 10 – Bar graph of seizure rates

Bar graph representative of the percentage of OHSCs displaying spontaneous recurrent

epileptiform discharges (SREDs, seizures) in each treatment group. Fourteen days after

4-AP injury, 62% of treated cultures showed seizure activity compared to 30% of

controls (p=0.006, z-test).

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Figure 10
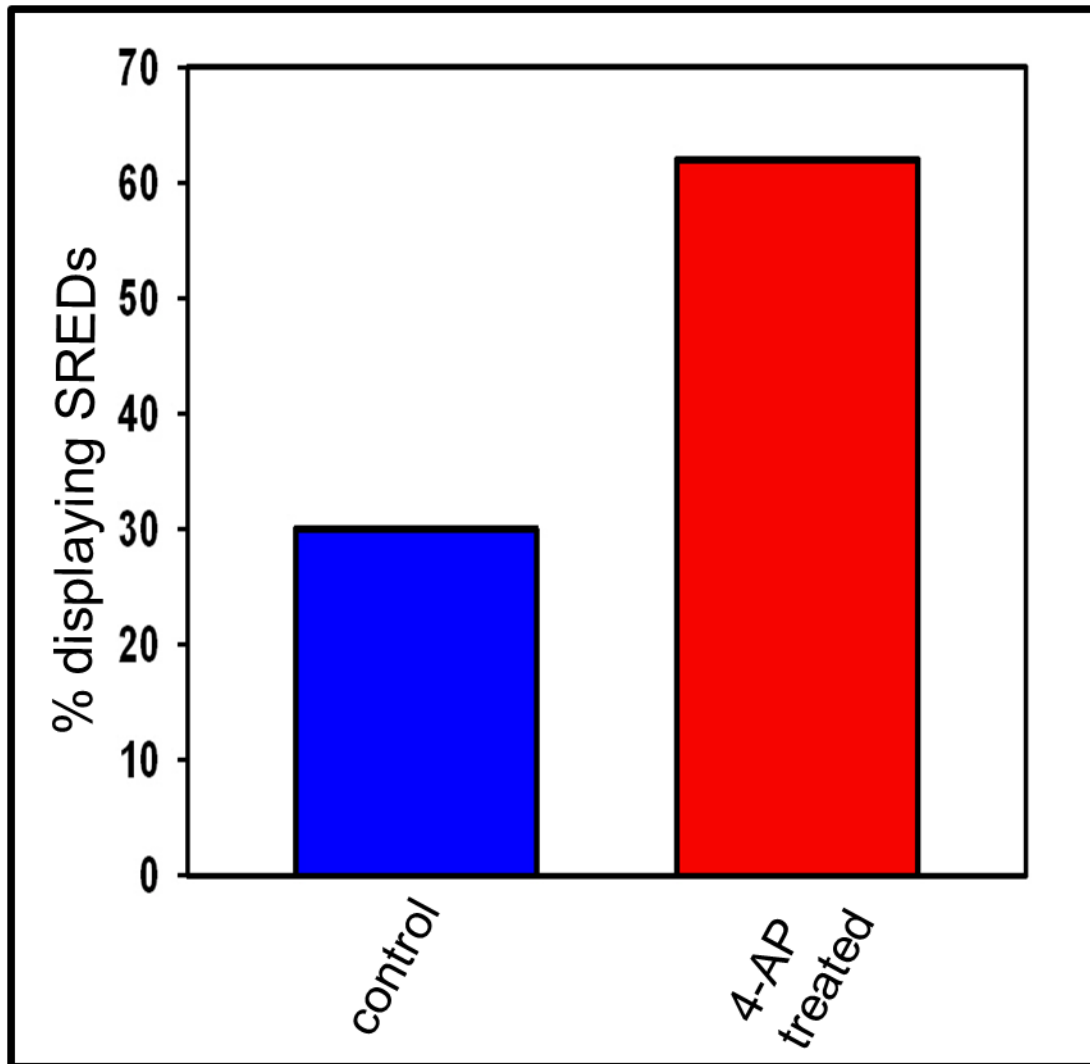
D. Discussion

We have successfully cultured and maintained viable organotypic hippocampal slice cultures in our laboratory. In the first part of this experiment, we established that OHSCs in our hands would acutely respond to 4-AP as previously shown in the literature (Albus *et al.*, 2008). It was important to establish that our culture system responded to 4-AP at DIV 10 to demonstrate that K^+ channels were functional in our OHSCs and could therefore respond to the drug injury. Albus et al. recently demonstrated that acute 4-AP treatment in OHSCs yields a model of pharmacoresistant epilepsy. They found that tonic–clonic seizure-like events induced by low magnesium or by 4-aminopyridine, are refractory to standard antiepileptic drugs such as valproic acid, carbamazepine, phenytoin, phenobarbital and 1,4-benzodiazepines (Albus *et al.*, 2008). Though the acute effects of 4-AP treatment are well studied, the long term physiological consequences were unknown in an OHSC model.

We therefore utilized 4-AP to induce a longer, status epilepticus like injury. The addition of 200μM 4-AP to culture media for a 24 hour period led to a significant amount of cell death acutely following the injury period. However, cell death was not significantly different from controls in the time periods after the injury period (5 and 14 days). The pattern of cell death appeared to mostly occur in the glutamatergic cells in the pyramidal cell and dentate granule cell layers, though future immunohistochemical studies will have to be carried out to confirm cell type. However, this follows the pattern of cell death observed in animal models of SE (Poirier *et al.*, 2000). Our temporal specific finding also agrees with the periods of cell death seen in other models of

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acquired epilepsy. In the pilocarpine rat model of AE, there is a 10% loss of cells in the first week after pilocarpine injury, but there is no further cell death during the period of epileptogenesis that follows (Raza *et al.*, 2004). In the low magnesium model of acquired epilepsy in primary hippocampal neuronal cultures, our laboratory has demonstrated that approximately 30% of the cells die during the status epilepticus injury, but there is no significant cell death during the period of epileptogenesis (Deshpande *et al.*, 2007c).

Following injury with 4-AP, we observed a significant increase in spontaneous seizure activity in OHSCs that underwent 4-AP treatment, with 62% of cultures displaying seizure activity in the CA3 region. This is significantly higher than what has been reported in OHSCs treated with kainic acid in which 29% of OHSCs displayed seizures in the dentate gyrus (Bausch and McNamara, 2004) However, in our experiments, we also noted a high percentage of control OHSCs that showed spontaneous seizure activity. Though it has been reported that OHSCs in long term culture develop spontaneous seizures, our observation of a 30% seizure rate at DIV 24 is equivalent to other reported seizure rates at DIV 40 (Bausch and McNamara, 2004). This may be explained by the fact that we recorded from the CA3 region, whereas previous studies have examined the seizure-resistant dentate gyrus. These discrepancies may also be due to our recording protocol for PI fluorescence. Studies have shown that PI is not neurotoxic and can be suitable to maintain in long term culture (Kristensen *et al.*, 2007), but in our protocol, OHSCs were out of the incubator for extended periods during PI recording while each slice in the 6 well plate was imaged, which could have inadvertently increased OHSC injury. However, the significant difference in seizure activity between control and 4-AP treated OHSCs cannot be solely attributed to the PI protocol. We

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therefore conclude that treatment with 4-AP for 24 hours acted as an epileptogenic insult in our OHSC model.

Slice cultures have been utilized to induce excitability using various pharmacological manipulations. McBain et al (McBain *et al.*, 1989) utilized the roller tube method of culture and found epileptiform activity characteristic of both interictal and ictal events in intracellular recordings in the absence of manipulation in long-term cultures (up to 12 weeks). They concluded that long-term culturing in this manner lead to alterations in the balance of excitatory and inhibitory synaptic activity (McBain *et al.*, 1989). More recently, Bausch and McNamara (Bausch and McNamara, 2000) have extensively studied granule cell hyperexcitability in OHSCs cultured on membranes via the interface method (Stoppini *et al.*, 1991). They found that long-term culturing in this method also led to excessive seizure activity in granule cells. At 40-60 days *in vitro*, 22% of the cultures displayed electrographic seizures. They also did extensive morphological studies in their OHSCs and found that some dentate granule cells may form direct reciprocal synaptic connections with neurons in all hippocampal subfields including CA1 and that these reciprocal synapses contribute to dentate granule cell hyperexcitability (Bausch and McNamara, 2000). The formation of reciprocal synapses may also contribute to the expression of seizures in both control and 4-AP treated OHSCs. Though it is interesting to note that OHSCs develop seizure activity after long periods in culture without pharmacological manipulation, it is important to emphasize that our model utilized cultures much earlier in their *in vitro* life. Our extracellular recordings took place at DIV 24, well before others have reported high seizure rates at DIV 40-60.

Another *in vitro* model utilizing primary hippocampal neuronal cultures involves the removal of Mg^{2+} for excessive activation of the NMDA receptor (Sombati and Delorenzo, 1995). 4-AP acts by blocking the delayed rectifier type K^+ channels, allowing for excessive neuronal depolarization. Though these two injuries act on different receptors, they both allow for extended increases in intracellular Ca^{2+}, which is thought to play an essential role in epileptogenesis (DeLorenzo *et al.*, 2005). Organotypic hippocampal slice cultures provide a substrate with a complexity between primary cultures and animal models. Establishing a reliable model of acquired epilepsy in OHSCs provides an additional mechanism to study the molecular changes that occur during epileptogenesis while allowing for high-throughput screening of future anti-epileptic and antiepileptogenic agents. We have successfully established an organotypic hippocampal slice culture model of spontaneous recurrent epileptiform discharges after cellular injury by a status epilepticus-like excitotoxic injury. In the 4-AP injury model, most of the cell death appears to occur acutely after injury. The surviving, but injured cells undergo the molecular changes that result in expression of seizures following a latent period of epileptogenesis. This model provides us with a unique, intact hippocampal culture system in which we can study the cellular and molecular changes that occur after brain injury and how these changes may lead to acquired epilepsy. The ability to maintain and record several parameters of excitotoxicity in slice cultures will allow us to further use this system to evaluate other types of excitotoxic injury and to model and evaluate other neurotraumas that lead to epileptogenesis.

CHAPTER 2: AN ORGANOTYPIC HIPPOCAMPAL SLICE CULTURE MODEL OF EXCITOTOXIC INJURY INDUCED SPONTANEOUS RECURRENT EPILEPTIFORM DISCHARGES

A. Introduction

The association between stroke and epilepsy is well recognized, but it is not well understood. Stroke is one of the most common neurological conditions, affecting over half a million Americans per year (Taylor et al., 1996) and is a leading cause of acquired epilepsy (AE), most commonly in the elderly (Annegers et al., 1996; Armstrong et al., 2009; Herman 2002). The development of AE after a stroke has been shown to increase both the morbidity and mortality of the stroke patient (Menon and Shorvon 2009). Thus it is important to prevent the development of AE after stroke. Despite this, the mechanisms underlying the development of epilepsy (epileptogenesis) following stroke-like injury are poorly understood.

Stroke refers to the brain injury that occurs following cerebral ischemia (Sharp et al., 1998). It has been suggested that acute ischemia increases extracellular concentrations of glutamate (Buchkremer-Ratzmann et al., 1998), reduces GABAergic function, and causes functional or structural impairment of GABAergic interneurons (Menon and Shorvon 2009). In addition, injury from stroke also causes selective cell death and apoptosis, changes in membrane potential properties, mitochondrial changes, receptor changes, deafferentation or collateral sprouting (Menon and Shorvon 2009). Together

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these impaired functional and structural relationships may lead to epileptogenesis- a process that causes a permanent neuronal plasticity change in previously normal brain tissue, leading to the onset of spontaneous recurrent epileptiform discharges (SREDs) (DeLorenzo et al., 2007). Certainly, this period of epileptogenesis represents an important clinical time-frame wherein intervention may be able to inhibit the plasticity changes that lead to the development of seizures.

Development of an *in vivo* model of stroke-induced AE has met limited success (Karhunen et al., 2005). Our lab has utilized the *in vitro* hippocampal neuronal culture model of stroke-induced AE (Sun et al., 2001; Sun et al., 2004). The *in vitro* model utilizes glutamate exposure to produce an injury similar to that seen secondary to ischemic stroke. Subsequent to injury, surviving neurons manifest SREDs analogous to epileptic seizures (Sun et al., 2001; Sun et al., 2004). While this model provides insight into some of the molecular mechanisms involved in epileptogenesis following stroke, it is limited by lack of normal anatomical morphology and circuitry that is important in excitatory feedback in the brain. In contrast, animal models provide proper morphology and neuronal feedback, but they are often time consuming and cost restrictive for use in rapid screening of novel therapeutic compounds. Some of these limitations could be overcome while still using an *in vitro* system by utilizing organotypic hippocampal slice cultures (OHSCs). OHSCs have been shown to manifest intact neuronal morphology, cellular and anatomical relations and network connections (Noraberg et al., 2005; Sundstrom et al., 2005; Zimmer and Gahwiler 1984). OHSCs have been used to study the acute physiological effects (Albus et al., 2008; Wahab et al., 2010) and some of the morphological changes that occur following excitotoxic injury (Routbort et al., 1999;

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Thomas et al., 2005). Glutamate toxicity is an important aspect of the ischemic cascade (Buchkremer-Ratzmann et al., 1998). OHSC models have also established similarities in cell death patterns in oxygen glucose deprivation and glutamate injury, suggesting NMDA mediated cell death in both injuries (Lipski et al., 2007, Noraberg et al., 2005). However, a thorough characterization of the physiological changes that occur after excitotoxicity particularly its effect on seizure genesis has not been explored.

In this paper, we describe development of an OHSC model of glutamate injury induced AE. Our novel model utilizes a glutamate injury paradigm to induce a stroke-like injury in OHSCs (Lipski et al., 2007). After a period of epileptogenesis, field potential and intracellular recordings revealed expression of SREDs, the *in vitro* correlate of "seizures", in glutamate treated slices as compared to untreated sham control slices. Pharmacological studies using standard anticonvulsant drugs have also been described.

B. Methods

Organotypic hippocampal slice culture preparation

Slice cultures were prepared using the method of Stoppini et al. (Stoppini et al., 1991), as previously reported by (Schanuel et al., 2008). All animal use protocols are in strict accordance with the National Institute of Health guidelines and are approved by the International Animal Care and Use Committee of Virginia Commonwealth University. Postnatal day 8 (P8) Sprague-Dawley rat pups (Harlan, Frederick, MD, USA) were deeply anesthetized with isoflurane and decapitated. The brains were removed, and hippocampi were dissected out and cut into 350-µm transverse sections using a McIlwain tissue chopper (Brinkmann Instruments, Canada) and placed into Hank's balanced salt

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solution [HBSS – Gibco BRL (Invitrogen, Carlsbad, CA, USA)] supplemented with 0.5%

sucrose and 1% penicillin-streptomycin. The middle four to six slices of each

hippocampus, including part of the entorhinal cortex, were placed onto tissue culture

membrane inserts (Millipore, Bedford, MA) in a 6-well tissue culture dish containing

medium consisting of 50% minimum essential medium, 25% horse serum and 25%

HBSS, 1% penicillin-streptomycin [all from Gibco BRL (Invitrogen, Carlsbad, CA)] and

supplemented with 36 mM glucose, and 25 mM Hepes (Sigma, St. Louis, MO, USA) (pH

7.2). Cultures were maintained at 37°C under room air +5% CO_2. After 1 day in culture,

culture medium was replaced with fresh medium containing no antibiotics. Culture

medium was replaced two times a week thereafter.

Glutamate injury

After 21 days *in vitro* (DIV21), slice culture media was removed and replaced by

media containing 3.5mM glutamate and cultures were returned to the incubator for a

period of 35 minutes (Lipski et al., 2007). Glutamate containing media was then removed

and slice cultures were washed with HBSS at 37°C two times before returning to normal

media. Control slices received normal media (without glutamate) during the 35 minute

time period, followed by washing with HBSS twice before returning to normal media.

Neuronal death assay

Delayed neuronal death was assessed 24 and 72 hours after glutamate treatment

by measuring uptake of the fluorescent dye Propidium Iodide (PI, Sigma), using

established procedures (Lipski et al., 2007; Pomper et al., 2001). Greater PI uptake

indicates greater cell death. Cultures were exposed to 2 µM PI for 4 hours in normal culture media. Before recording dye uptake under fluorescent light, a phase contrast picture of each slice was taken so that orientation in the field could be maintained in subsequent recordings at 24 and 72 hours. Dye uptake was recorded on an Olympus CK40 inverted microscope coupled to a QColor 3 camera and QCapture software (Olympus, Center Valley, PA). All optical parameters (illumination aperture, neutral density filters etc.) as well as the camera exposure time and electronic gain, were standardized and kept constant. The fluorescent signal was measured densitometrically using the Image-J software (NIH). The perimeter of each slice was outlined (identified in phase contrast image) and the mean pixel value (0–255) was recorded and converted to a scale of 0-100. All measurements were made after subtracting background fluorescence (region positioned immediately outside the culture). Cell death was expressed as a percent increase of mean pixel value of date matched controls. To assess differential cell death by region, cell regions were identified and circled as regions of interest in the phase contrast image. Propidium iodide staining was then measured densitometrically in each of the 3 cell regions (CA1, CA3, DG). As in whole hippocampus recordings, cell death was expressed as a percent increase of mean pixel value of each cell region in age date-matched controls.

Field potential recording

At DIV 30-33, a portion of the tissue culture insert membrane containing a single cultured slice was placed into an interface slice recording chamber (Harvard Apparatus, Holliston MA) mounted on a vibration table and viewed through a dissecting microscope

(Diagnostic Instruments, Sterling Heights, MI). Slice cultures were perfused (2–3 ml/min) with artificial cerebrospinal fluid (aCSF) composed of (in mM) 120 NaCl, 3.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1.25 NaH_2PO_4, 25.6 $NaHCO_3$, and 10 glucose, equilibrated with 95% O_2-5% CO_2. Recording pipettes were pulled on a Flaming Brown P-80 PC Micropipette puller (Sutter Instrument Co, Novato, CA) filled with 3.5 M NaCl for extracellular recordings to achieve a resistance of 2-4 MΩ. Data were collected with an AxoClamp 2B (Molecular Devices, Union City, CA) recording amplifier and FLA-01 Bessel filter unit (Cygnus Technologies, Inc, Delaware Water Gap, PA) and pCLAMP 9.0 software (Molecular Devices). After a 15 minute equilibration period, field potentials were recorded at 34°C in the CA3 pyramidal cell layer. If spontaneous activity was not observed in the first few minutes, slices were tested for viability by hilar stimulation (0.3-ms square pulse, 0.03 Hz, 50–150 µA) using a bipolar concentric electrode (FHC, Inc., Bowdoin, ME) and a stimulator (World Precision Instruments, Sarasota, FL). Slices were considered acceptable if stimulation elicited an action potential spike that immediately followed the stimulus artifact with a response threshold ≤150 µA (Bausch and McNamara 2000; Bausch et al., 2006). Neither the amplitude of the spike nor the shape of the waveform was used as criterion for acceptable recordings. Each slice culture was recorded at 2 minute intervals (2 minutes on, 2 minutes off) over a 20 minute time period, for a total of 10 minutes of recorded activity. Recordings were scored for seizure events by two separate individuals who were blinded to the treatment group.

Intracellular recording

Whole-cell current-clamp recordings were performed on visually identified pyramidal neurons in the CA3 region of OHSCs (one cell per slice). A portion of the

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culture membrane containing a single OHSC was excised and transferred to a recording chamber mounted on a Axioskop 2 FS Plus upright microscope 9 (Carl Zeiss, Inc., Thornwood, NY), equipped with an IR/DIC camera. Slices were perfused at 1-2ml/min with aCSF using a Dynamax peristaltic pump (Rainin, Oakland CA). Patch microelectrodes had a resistance of 2 to 4 MΩ resistance when filled with an internal solution of (in mM) 140 K^+ gluconate, 1MgCl$_2$, 10 HEPES, 1.1 Ethylene glycol-bis (ß-aminoethyl ether)-*N, N,N',N'*-tetraacetic acid (EGTA), 4 Na$_2$ ATP, 15 Tris Phosphocreatine, pH 7.2 with NaOH and osmolarity adjusted to 310 mOsm with sucrose. Recordings were obtained using MultiClamp 700B amplifier in current clamp mode. Data were digitized by a DigiData 1440A, low-pass filtered at 2 kHz, sampled at 10 kHz and recorded using pClamp 10.0 software. Cells were acceptable for recording if they maintained a membrane potential of -40mV or more hyperpolarized and were able to spike when a current pulse was injected. Cells were omitted if series resistance was not stable within 15% during recording.

Drug perfusion

To determine efficacy of AEDs in this model, phenobarbital, phenytoin, and ethosuximide (all from Sigma) were added to the aCSF during perfusion and field potential recording at DIV 30-33. Drugs were dissolved as stock solutions and working solutions were prepared fresh daily. Phenobarbital and ethosuximide stocks were dissolved in sterile water while phenytoin was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the bath solution was less than 0.01%. Drugs were perfused for 20 minutes followed by a 20 minute wash out period with aCSF. The order

of drug perfusion was randomized for each slice. Field potential recording was continuous for 40 minutes, rather than intermittent as in previous recordings. Drug effect was analyzed by evaluating average field potential frequency and amplitude for 2 minutes before drug perfusion began and again in the final 2 minutes of drug perfusion. Drug effect is expressed as a percent decrease in average field potential frequency and amplitude during AED perfusion, as compared to the values before drug perfusion.

Data analyses

Data are expressed as mean ± SEM. To determine significance between treatment groups for neuronal death assays, Mann-Whitney Rank sum or one way ANOVA tests were employed with a Fisher's post-hoc where appropriate. For extracellular recordings, Chi-square analysis or Fisher's exact test was used. For intracellular recording, Fisher's exact test was used to evaluate seizure occurrence, while Mann-Whitney Rank sum and Student's t-tests were used to evaluate additional electrophysiological properties. In all assays, n refers to number of OHSCs. For neuronal death assays, glutamate treated slices were normalized to control slices harvested from the same animal. For intracellular recording, 1 neuron was patch-clamped per OHSC. All experiments were performed over the period of several weeks to months so that the results were representative of multiple cultures. A *P*-value < 0.05 was considered significant. Statistical analysis was performed using SigmaStat 2.0 software and graphs were drawn using SigmaPlot 11 (Systat Software, San Jose, CA, USA).

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C. Results

Glutamate exposure produced hippocampal neuronal injury

Figure 11a shows PI staining in OHSCs after injury with 3.5 mM glutamate. PI uptake was measured in optical density units of the whole slice. At 24 hours, glutamate injured OHSCs showed an increase in PI uptake of $151.17 \pm 5.364\%$ (n= 174) of age-matched controls ($100 \pm 3.09\%$, n= 121, $p<0.001$, Mann-Whitney Rank sum test). The increase in PI staining in glutamate treated slices was still significant at 72 hours, with optical density measurements of $123.5 \pm 2.89\%$ (n=154) of controls ($100 \pm 2.535\%$, n=108, $p<0.001$, Mann-Whitney Rank sum test) (Figure 11b). PI staining displayed that the glutamate treatment killed some, but not all neurons, producing a mixed population of live and dead neurons. The CA1 region showed significantly higher cell death than the dentate gyrus at 24 hours ($133.0 \pm 3.8\%$ vs. $115.8 \pm 2.4\%$, n=154 for both, $p<0.05$, one way ANOVA with a Fisher's post-hoc analysis), though this effect was not significant at 72 hours (Figure 11c). The CA3 region did not show a significant difference in cell death when compared to either CA1 or DG at either time point.

Glutamate injury induced alterations in OHSC excitability

To investigate changes in OHSC excitability, we obtained field potential recordings from the CA3 region of OHSCs 9-12 days after glutamate injury. OHSCs were first tested for viability by stimulating in the dentate gyrus while recording in the CA3 cell layer. Representative field potential recordings of control and glutamate treated OHSCs are shown in Figure 12. Seizure events were defined as the abrupt onset of a high amplitude burst of rhythmic activity superimposed on a field depolarization shift that

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lasted ≥3 s, during which the waveforms evolved over time and terminated abruptly (Bausch *et al.*, 2006; Routbort *et al.*, 1999). Field potential recordings showed seizure events in 46.25% of glutamate treated OHSCs (n= 80) compared to 7.14% of control slices (n= 28, p<0.001, Chi-square analysis). OHSCs displaying seizures had an average of 3.73 ± 0.57 seizure events per 2 minute recording, lasting an average of 14.26 ± 3.11 sec. The average maximal amplitude shift was 0.78 ± 0.14 mV. While some control and injured slices displayed individual action potentials and brief depolarizing shifts, these slices were not considered to have seizure events unless the burst of activity occurred for greater than 3 seconds.

Spontaneous recurrent epileptiform discharges in glutamate-injured OHSCs

To further confirm seizure activity in glutamate treated OHSCs, whole-cell current clamp methods were employed on CA3 cells in glutamate treated and control slices. Cells in glutamate treated slices displayed SREDs while cells in control slices did not (Figure 13). SREDs were defined as a burst of activity with a spike frequency ≥ 3Hz for durations of ≥ 20 sec (Sun *et al.*, 2001). This electrographic recording is analogous to electrographic seizures observed with EEG recordings (McNamara, 1994). Cells in control slices showed spontaneous firing as well, but it did not meet the criteria for a SRED. SREDs were observed in 43.7% of glutamate treated slices (n=16), compared to 0% of control slices (n=9, P= 0.027, Fisher exact test). Cells in control slices had an average spike frequency of 0.0637 ± 0.0495 Hz over 257 minutes of recording. Cells from all glutamate treated slices had an average spike frequency of 0.8406 ± 0.545154 Hz over 211 minutes. Of those glutamate treated slices that displayed SREDs, average spike frequency was 1.54384 ± 0.9357 Hz over 105 minutes (P=0.014 compared to control,

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Mann-Whitney Rank Sum Test), with frequencies over 3 Hz during SRED events. The resting membrane potential of control cells was -63.9 ± 2.8 mV (n=9) and this was not significantly different from all glutamate treated cells (-59.45 ± 2.5 mV, n=16, p=0.25, Student's t-test) or those cells displaying SREDs (-55.1 ± 3.8 mV, n=7, p=0.08, Student's t-test). Input resistance was also not significantly different between the two groups (226.87 ± 21.3 MΩ for control, 258.5 ± 53.3 MΩ for glutamate treated cells (p=0.6, Student's t-test).

SREDs lasted for the life of the OHSCs

Field potential recordings were performed at different time points after glutamate injury to confirm the optimal time of recording. In glutamate treated slices seizure activity was not observed immediately after the injury, at DIV 21. However, seizure activity was present at DIV 24, 3 days after injury, in 40% of slices. Seizure activity continued in approximately 50% of treated slices until DIV 40 (Figure 14). Consistent with literature findings (Bausch and McNamara, 2000; McBain *et al.*, 1989), control OHSCs began showing more spontaneous seizure activity as time in culture increased, with 12.5% showing seizure activity between 34 and 40 days *in vitro*. At this point in culture, the expression of spontaneous epileptiform activity is thought to arise from an alteration in the balance of excitatory and inhibitory synaptic activity, resulting increased synaptic activity (McBain *et al.*, 1989). Therefore, we established DIV 30-33 as an acceptable window for field potential recording as there is a significant difference in the frequency of seizure events in injured and control slices.

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Degree of cell death did not determine development of SREDS

To determine if the varying degree of cell death played a role in the development of seizure events, we analyzed PI staining in the glutamate treated slices that had been used in field potential recordings. As shown in Figure 15, slices displaying SREDs showed a 45.89 ± 11.956% (n=34) increase in PI uptake 24 hours after injury when compared to matched controls, while slices that did not show seizure activity showed a 48.5 ± 9.231% (n=39) increase. Similarly, at 72 hours, seizing slices maintained a 45.5 ± 9.23% (n=34) increase while non-seizing slices showed a 31.3 ± 7.445% (n=39) increase over controls. These values are not significantly different (p=0.862 and 0.175, respectively, Student's t-test) from one another, suggesting that magnitude of cell death following initial injury does not play a significant role in the development of seizure activity in this model. In addition, there was no significant difference in cell death in individual cell areas among OHSCs that displayed seizure activity and those that did not (all P>0.198, data not shown).

Phenobarbital and phenytoin but not ethosuximide inhibited epileptiform activity

To better understand the characteristics of the *in vitro* seizure events, we employed several standard AEDs to see their effect on seizure activity in this model. Given the refractory nature of other seizure models in OHSCs, chosen AED concentrations were slightly higher than equivalent anticonvulsant plasma concentrations in rats (Albus et al., 2008). Perfusion of phenytoin (200 µM) during extracellular recording reversibly reduced the amplitude and frequency of the field potential seizure activity (Figure 16a). After 18 minutes of drug perfusion, phenytoin decreased the field potential amplitude by 23.4 ± 9.3 % (P=0.045) and frequency by 83.8 ± 4.6% (p<0.001,

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n=4). Phenobarbital (400 µM) also acted as a reversible anti-seizure agent in this model, though the onset of its action was longer (Figure 16b). Field potential amplitude decreased by $23.3 \pm 8.5\%$ (p=0.035) and frequency decreased by $77.3 \pm 12.7\%$ (p<0.001) following 18 minutes of drug perfusion (n=4). On the other hand, ethosuximide (1 mM), a T-type, voltage gated calcium channel blocker effective in treating generalized absence seizures (Rogawski and Porter 1990), had no effect on seizure activity in this model (Figure 16c). Amplitude ($3.7 \pm 3.6\%$ decrease) and frequency ($27.6 \pm 29.6\%$ increase) remained unchanged (p>0.05, n=4). Thus, the seizure activity induced by glutamate injury in our model responded to therapeutically relevant concentrations of anticonvulsants analogous to the setting of generalized tonic clonic and partial complex seizures (Macdonald and Kelly 1995).

Figure 11. A. Propidium Iodide uptake - Propidium Iodide (PI) uptake in OHSCs following 35 minute treatment with 3.5mM glutamate. PI staining indicates increased cell death in glutamate treated slices as compared to controls at both 24 and 72 hours following treatment. Note that significant cell death is observed in the CA1 cell region. (4X magnification). **B. Comparison of PI uptake -** Quantification of PI uptake in OHSCs at 24 and 72 hours after injury, expressed as a percent change from control. PI uptake was measured as mean optical density of the whole slice and normalized to age-matched controls. PI staining indicated a significant increase in cell death at both 24 (n=121, 174 for control and glutamate treated, respectively) and 72 hours (n=108, 154 for control and glutamate treated, respectively) after glutamate treatment (*$p<0.001$, Mann-Whitney Rank Sum test). **C. PI uptake by cell region** - At 24 hours, cell death is significantly higher in the CA1 cell region than it is in the dentate gyrus (DG) (*$p<0.05$, Kruskall-Wallis One Way ANOVA On Ranks). The CA3 region did not have a significant difference in PI uptake from either the CA1 or DG. At 72 hours, there is no significant difference in PI uptake between cell regions. (n=154)

90

Figure 11

a.
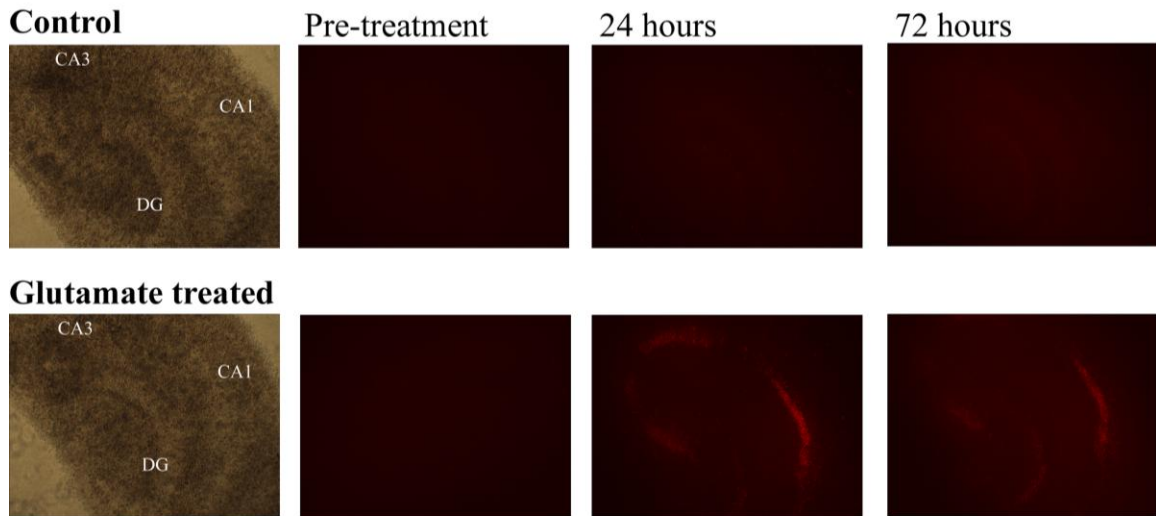
b.
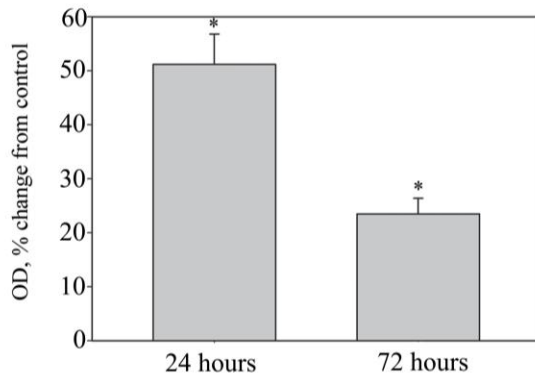
c.
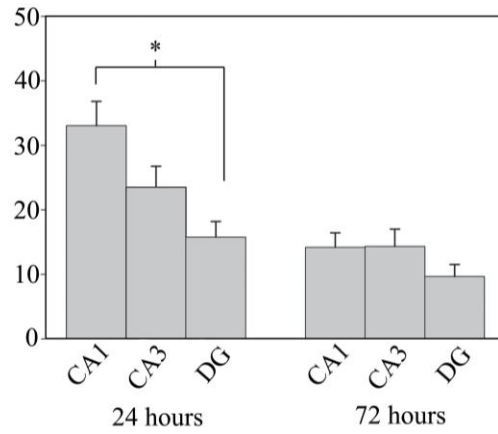
Figure 12. **Extracellular recordings -** Representative extracellular field potential recordings of CA3 cell layer of OHSCs at DIV 30. **A. Control** - Control OHSCs do not display seizure activity in aCSF. **B. Glutamate treated** - Representative field potential recording from an OHSC that was subjected to glutamate injury at DIV 21. Seizure activity is characterized by repetitive bursts of activity that last for longer than 3 seconds, changes over time and terminates. All vertical bars = 0.5mV

Figure 12

a. Control
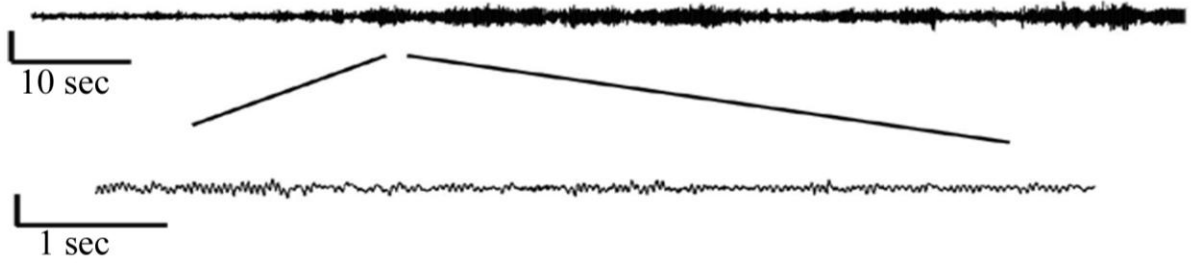
b. Epileptic
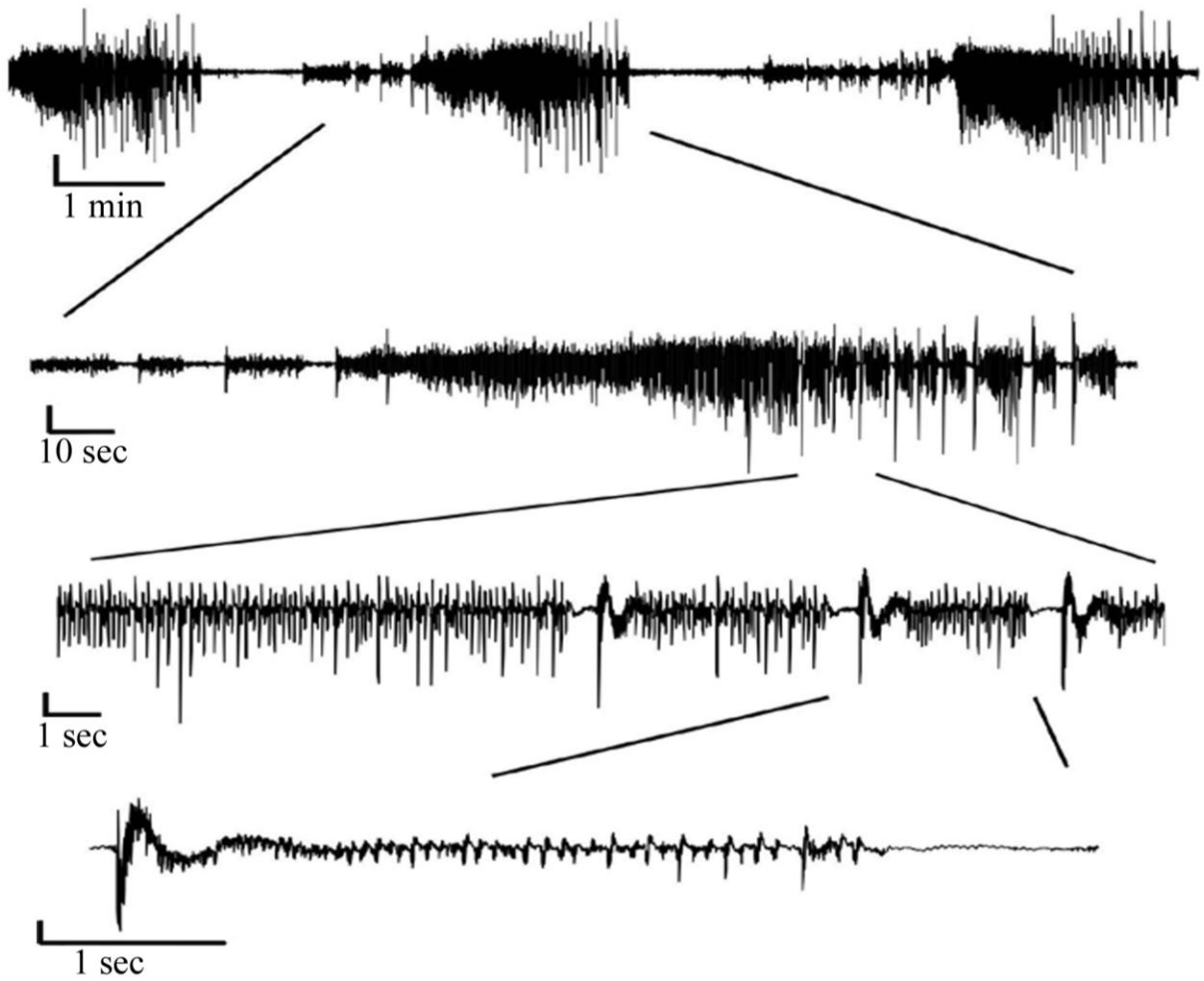
Figure 13. **Intracellular recording -** Representative intracellular whole cell current clamp recordings of CA3 pyramidal cells at DIV 30. **A. Control -** Cells from control slices did not display repetitive bursting (n=9) **B. Glutmate treated** - Cells from glutamate treated slices displayed spontaneous recurrent epileptiform discharges (SREDs), similar to activity seen in extracellular field potential recordings. SREDs were observed in 43% of slice cultures following glutamate injury at DIV 21 (n=16).

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Figure 13

a. Control

20 mV
2 min

10 sec

b. Epileptic

20 mV
2 min
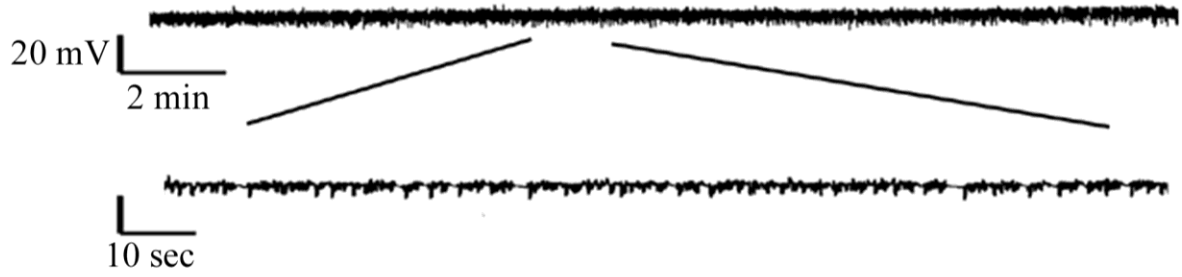
10 sec
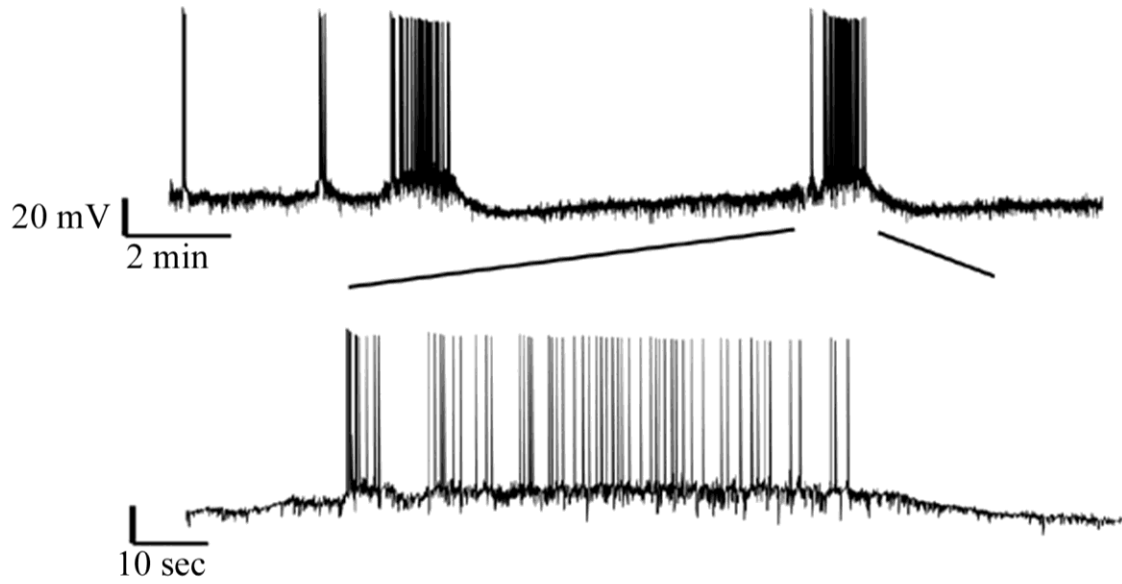
Figure 14. Development of seizures after injury - After glutamate injury (solid line), a significant percentage of OHSCs display seizure activity in field potential recordings as compared to age matched controls (dashed line). This change is long lasting and was observed up to 40 days *in vitro*. Within hours of injury (DIV 21), OHSCs did not display seizure events in either the control or injured groups (n=6 each). Between 3 and 8 days after injury (DIV 24-29), 40% of injured OHSCs and 0% of controls displayed seizure events (n=10 each, $*p<0.05$, Fisher's exact test). In our established experimental time frame, DIV 30-33, we observed seizure events in 46.25% of glutamate injured OHSCs and 7.14% of controls (n=80, 28, $**p<0.001$, Chi-square analysis). By DIV 34-40, seizure events were observed in 50% of injured slices and 12.5% of controls (n=8, 9).

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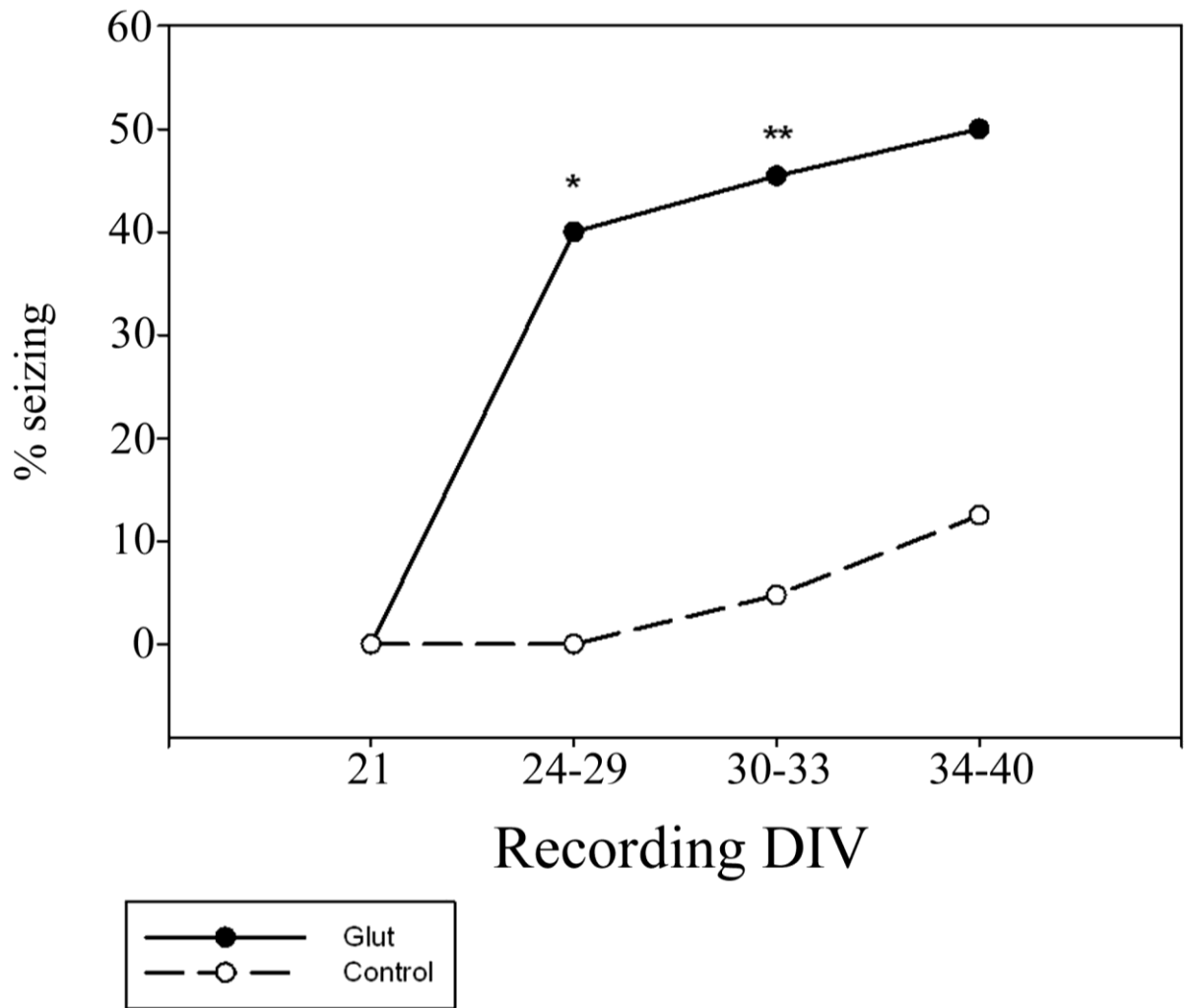
Figure 14

Figure 15. **Degree of cell death does not determine seizure activity -** After glutamate injury, OHSCs displayed slightly varying degrees of cell death. However, the degree of cell death observed does not appear to play a role in the development of seizure activity. OHSCs were evaluated for cell death at 24 and 72 hours after glutamate injury on DIV 21. They were returned to the incubator until field potential recording at DIV 30-33. OHSCs that did not display seizure activity (white bars, n=39) did not significantly differ in PI uptake from OHSCs displaying seizures (gray bars, n=34).

Figure 15
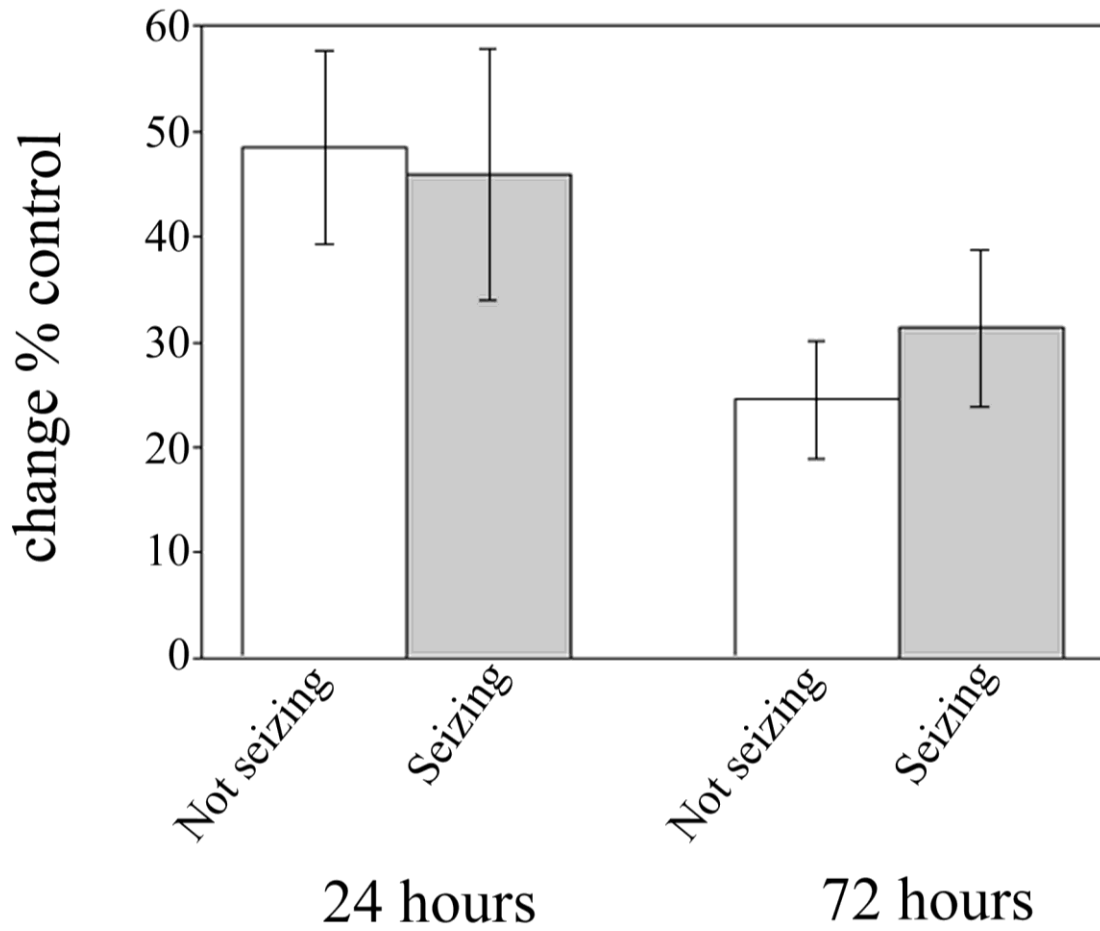
Figure 16. **Standard AEDs are effective at blocking seizure activity -** Representative field potential recordings of the effect of standard anti-epileptic drugs on seizure activity in glutamate treated slices at DIV 30. **A. Phenytoin** (200μM) is effective at blocking seizure activity during a 20 minute perfusion and can be washed out. On average, phenytoin decreased field potential amplitude by 23.4 ± 9.3 % (P=0.045) and frequency by 83.8 ± 4.6% (p<0.001, n=4) **B. Phenobarbital** (400μM) is also effective at blocking seizure activity during the 20 minute period of perfusion, amplitude decreased by 23.3 ± 8.5% (p=0.035) and frequency decreased by 77.3 ± 12.7% (p<0.001) following 15 minutes of drug perfusion (n=4). **C. Ethosuximide** (1 mM), is not effective at blocking seizure activity in glutamate injured OHSCs (p>0.05, n=4). All vertical bars = 0.1mV

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Figure 16

a.

Phenytoin (200µM)

2 min

5 sec

b.

Phenobarbital (400µM)

2 min

5 sec

c.

Ethosuximide (1mM)

2 min

5 sec

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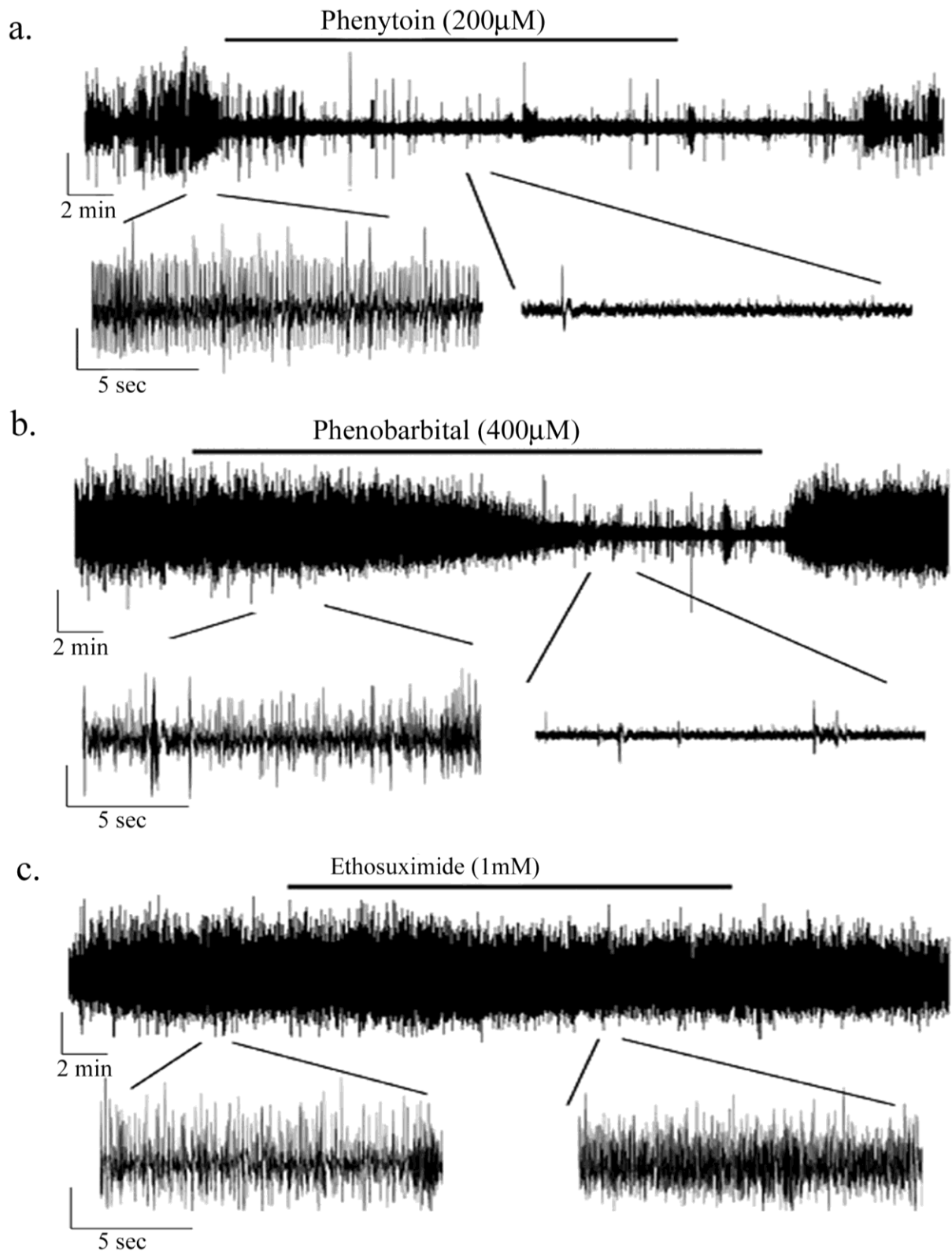
D. Discussion

The experiments in this study document a novel model of epileptogenesis in OHSCs following glutamate injury. Similar to the excitotoxic injuries associated with both ischemic and anoxic stroke events, glutamate injury in this model produced a mixed population of neurons characterized by both cell survival and cell death. As suggested by our initial hypothesis, neurons that survived the glutamate exposure become the substrate for the development of epileptogenesis as indicated by increased excitability of the CA3 cell layer in a significant number of OHSCs. The OHSC model of glutamate injury induced spontaneous seizure events may provide insights into the development of stroke induced AE. It also offers a powerful tool to screen potential pharmacological agents to treat seizures and develop therapeutic interventions to prevent the development of AE after stroke.

The seizure events seen in extracellular recordings expressed many characteristics of overt electrographic epileptic seizures. Seizure events started and terminated spontaneously and were synchronized in nature, as they represented a population of synchronized neurons (Figure 12). The seizure events produced by excitotoxic glutamate injury manifested larger spike amplitude than would be seen if the activity originated from a single neuron, suggesting that the activity occurred in a group of neurons. The typical seizure pattern observed in OHSCs after glutamate injury also included an abrupt onset of activity superimposed on a large field potential shift, consistent with the classic paroxysmal depolarizing shift associated with electrographic seizure discharges in both *in vitro* and *in vivo* models of epilepsy (Bausch et al., 2006; Dichter and Ayala 1987). Finally, the seizures produced by glutamate injury responded to the anticonvulsant drugs

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phenobarbital and phenytoin, but not to ethosuximide (Figure 16). These results demonstrated that OHSCs subjected to injury by glutamate exposure could be transformed into neuronal networks manifesting seizures for the life of the culture, producing an *in vitro* model of epilepsy.

Various studies of OHSCs have confirmed that cells develop and mature similar to age-matched cells *in vivo* (Bahr et al., 1995, Stoppini et al. 1991). In this respect, OHSCs are often used at times comparable to the appropriate *in vivo* age. Our OHSCs are cultured at P8 and injured at DIV 21, thereby showing the equivalent cellular maturity of young adult rats at P29. OHSCs from P8 pups have been shown to develop mature synaptic properties within a few days in culture (Muller et al., 1993). In addition, OHSCs have been shown to respond to seizure inducing stimuli over a wide range of time points anywhere from 7-56 days in culture (Albus et al., 2008). We therefore believe our slice cultures are at an appropriate age to respond to a glutamate injury at DIV 21.

Organotypic hippocampal slice cultures have been utilized in many models of excitotoxicity, including kainic acid (Routbort et al., 1999), NMDA (Ring et al., 2010), and oxygen glucose deprivation injuries (Lipski et al., 2007). However, few of these studies have examined the long-term physiological changes that occur in OHSCs after excitotoxic injury, thereby exploring their potential as a model of acquired epilepsy. Recently, Bausch and McNamara (Bausch and McNamara 2004) used a kainic acid injury paradigm in OHSCs and found no significant difference in seizure rates from controls when recording from granule cells in physiological buffer 30 days after injury. They were however, able to show that the granule cells were more hyperexcitable and there was considerable mossy fiber sprouting in the injured slices. Various other studies

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have used excitotoxic injuries to study neuroprotective agents and morphological changes (Boscia et al., 2006; Cho et al., 2007; Lipski et al., 2007; Ring et al., 2010; Routbort et al., 1999; Thomas et al., 2005). Our model takes this a step further by characterizing the physiological changes after an excitotoxic injury with glutamate. A study by Lahtinen et al. (Lahtinen et al., 2001) utilized a higher concentration of glutamate (10 mM) to examine acute electrophysiological consequences of glutamate injury in OHSCs. Within 2 hours of the injury, they found increased hyperexcitability in the CA3 cell region. In addition, they reported attenuation of cell death with TTX following glutamate injury, indicating that some cell death during the initial 24 hour period may be caused by neuronal excitability following the initial injury. Indeed, clinical and *in vivo* studies have shown that seizures often occur acutely following an ischemic injury (Menon and Shorvon, 2009, Karhunen et al., 2006), but this does not necessarily lead to epileptogenesis and acquired epilepsy. This would suggest that the post-insult activity observed by Lahtinen et al. is not necessarily indicative of chronic epileptogenesis. In contrast, our study employs a more moderate glutamate injury (3.5mM glutamate for 35 minutes) and we examine cell death at both 24 hours and 72 hours. We establish that cell death subsides following the 24 hour period, as the PI uptake is significantly decreased at 72 hours. In addition, we examine epileptogenesis in OHSCs 9-12 days following glutamate injury indicating long lasting changes in neuronal excitability following glutamate injury. It is important to stress this issue, since our study is the first demonstration of the development of spontaneous recurrent epileptiform discharges (epilepsy) in the OHSCs and represents the first model of post injury acquired "epilepsy" in this model.

Our model utilizes glutamate as a mode of excitotoxicity, as excessive glutamate concentration is an important aspect of the ischemic prenumbra in stroke (Davalos et al., 1997). The involvement of glutamate in epileptogenesis has been implicated in whole animal, (Croucher et al., 1988; Croucher and Bradford 1990; Rice and DeLorenzo 1998) slice, (Anderson et al., 1990; Stasheff et al., 1989) and cell culture (Sombati and Delorenzo 1995; Sun et al., 2001) models of epilepsy. To induce epileptogenesis, these models all used continuous neuronal spiking produced by seizures, (Rice and DeLorenzo 1998) repeated high-frequency excitation (Croucher et al., 1988; Croucher and Bradford 1990; Stasheff et al., 1989), or low extracellular magnesium environments(Anderson et al., 1986; Sombati and Delorenzo 1995) while only one used a glutamate-induced prolonged, reversible depolarization as used in this study (Sun et al., 2001; Sun et al., 2004). Many of these models have implicated activation of the *N*-methyl-D-aspartate receptor (NMDAR) for epileptogenesis (Croucher et al., 1988; Croucher and Bradford 1990; DeLorenzo et al., 1998; Rice and DeLorenzo 1998; Stasheff et al., 1989). Interestingly, epileptiform discharges have also been produced by growing OHSCs in culture in the presence of tetrodotoxin (TTX) to block activity or D(−)-2-amino-5-phosphonopentanoic acid (D-APV) to block NMDAR activation (Bausch et al., 2006). Removal of these agents resulted in the expression of seizure-like activity (Bausch et al., 2006). This distinctly different culture model uses the inhibition of glutamate receptors to induce hyperexcitability. Control OHSCs displayed a low occurrence of seizures, possibly as a result of collateral synaptic connections that are known to form after long term culture of hippocampal tissue (Bausch and McNamara 2000; McBain et al., 1989). Although the mechanism producing hyperexcitability in this model has not been fully

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delineated, it has been shown that inhibition of glutamate receptors in neurons in culture produces alterations in NMDAR subunit expression that are regulated by synaptic activity during development (Hoffmann et al., 2000; Yashiro and Philpot 2008). It is possible that alterations in NMDAR subunit expression may underlie the development of hyperexcitability in this model. Though glutamate exposure may induce changes in receptor subunit expression in the glutamate injury–induced epileptogenesis model, these potential changes probably occur through a separate mechanism. It will be interesting to investigate the mechanism of glutamate injury-induced epileptogenesis in future studies.

The potential role of selective neuronal death in glutamate injury–induced epileptogenesis requires further investigation, especially in light of the fact that inhibitory neurons are typically less vulnerable to excitotoxicity than excitatory neurons (Tecoma and Choi 1989). Although differential cell death may affect the balance between the number of inhibitory and excitatory neurons, resulting in a larger number of surviving inhibitory neurons, (Tecoma and Choi 1989) the glutamate-induced injury produced "epilepsy" in the OHSCs despite the potential alterations in neuronal subpopulations. Our experiments suggest that the severity of injury is not a large factor in epileptogenesis, as there was no difference in the degree of cell death in those OHSCs that developed seizures and those that did not (Figure 15). Further studies are needed to determine the role of selective cell death in this model. In addition, the possible roles of gap junctions (Dudek et al., 1998), ischemia-induced alterations in second-messenger systems, and gene changes (Morris et al., 2000) in mediating epileptogenesis represent important future directions for research that can be conveniently studied in this system.

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The association between stroke and epilepsy has been demonstrated clinically, and stroke is the most common cause of acquired epilepsy in adults (Hauser et al., 1991). However, the mechanisms by which cerebral ischemia initiates epileptogenesis are not understood. The glutamate injury produced in this model of epileptogenesis resembles some of the phenomena associated with stroke. Increases in extracellular glutamate, (Bullock et al., 1995; Davalos et al., 1997) excitotoxic delayed neuronal death (Choi 2000) associated with the ischemic penumbra (Dirnagl et al., 1999), and a delayed period of epileptogenesis are all present in this model. To our knowledge, this study demonstrates, for the first time, spontaneous, recurrent, epileptiform activity in organotypic hippocampal slice cultures induced by glutamate injury. This model of glutamate injury–induced epileptogenesis may offer new insights into the development and maintenance of the epileptic condition after a neurological trauma such as stroke and therefore may provide therapeutic strategies to develop both novel anti-epileptogenic and anticonvulsant agents to prevent stroke-induced epilepsy.

CHAPTER 3: CB₁ RECEPTOR CHANGES AFTER GLUTAMATE INJURY IN ORGANOTYPIC HIPPOCAMPAL SLICE CULTURES

A. Introduction

Stroke is one of the most common neurological emergencies, affecting over half a million Americans per year (Taylor *et al.*, 1996) and it is a leading cause of acquired epilepsy (AE) among the elderly. Many of the physiological mechanisms surrounding stroke are well studied, but the molecular changes that occur in the brain during the development of epilepsy, a process known as epileptogenesis, are not widely understood. Acquired epilepsy is known to develop after brain injuries such as traumatic brain injury, status epilepticus, or CNS infection, as well as stroke. Several *in vitro* (Sombati and Delorenzo, 1995; Sun *et al.*, 2001) and *in vivo* (Cavalheiro *et al.*, 1991; Karhunen *et al.*, 2005; Racine, 1972) models of acquired epilepsy have been developed to study the molecular events that occur during epileptogenesis. Our laboratory has utilized the rat pilocarpine model of acquired epilepsy to extensively study changes in the endocannabinoid system in AE (Falenski *et al.*, 2007; Falenski *et al.*, 2009; Wallace *et al.*, 2001; Wallace *et al.*, 2002; Wallace *et al.*, 2003).

It's been reported in the scientific literature for several decades that cannabinoids have anticonvulsant properties (Karler and Turkanis, 1981). More recently, these anticonvulsant effects have been demonstrated to be cannabinoid type I (CB₁) receptor

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mediated (Wallace *et al.*, 2003). Our laboratory has utilized the rat pilocarpine model of AE to show that endocannabinoid tone regulates seizure frequency and duration via a CB_1 receptor mechanism. The CB_1 receptor is the most abundant G-protein coupled receptor in the brain and several studies have shown a significant upregulation in CB_1 protein in epileptic tissue (Chen *et al.*, 2003; Falenski *et al.*, 2007). Recently, our laboratory has utilized the rat pilocarpine model of AE to further investigate the cellular distribution of CB_1 upregulation in epileptic animals (Falenski *et al.*, 2007). Interestingly, in addition to an overall upregulation of CB_1 protein in the epileptic hippocampus, studies also revealed a unique redistribution of CB_1 receptor histology whereby CB_1 receptor density actually increased in the stratum oriens, stratum radiatum and outer molecular layer of the dentate gyrus and decreased on the CA1 and CA3 pyramidal cells and inner molecular layer of the dentate gyrus (Falenski *et al.*, 2007). These findings suggest a possible compensatory mechanism after the development of epilepsy by increasing cannabinoid tone on excitatory synapses and decreasing it on inhibitory synapses, thereby controlling seizure severity.

These fascinating findings require further study as the possibility of pharmacological manipulation of CB_1 receptors may hold keys to the prevention of acquired epilepsy or for the control of seizures. However, animal studies of this magnitude are costly and labor intensive, so the development of an organotypic hippocampal slice culture (OHSC) model of acquired epilepsy would be much better suited to high throughput screening and evaluation of changes in the endocannabinoid system. In addition, OHSCs have been utilized in several studies involving the physiological signaling of endocannabinoids (Lafourcade *et al.*, 2009) and the

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neuroprotective role of various cannabinoid compounds (Khaspekov *et al.*, 2004; Kreutz *et al.*, 2009), but not until recently has the molecular distribution of the CB_1 receptors been fully characterized in OHSCs. A recent study of the distribution of CB_1 and mGlu1a receptors in OHSCs that had been in culture for 14 days found that the pattern of CB_1 distribution was similar to what is observed in healthy adult animals (Boscia *et al.*, 2008). This study confirmed that the OHSC system is a valid model to further explore the molecular changes of the endocannabinoid system after an injury.

Our recent development of a stroke-like injury model of AE in OHSCs (Chapter 2) provides an ideal model to compare the changes of CB_1 receptor expression to those seen in well established animal models. This study was initiated to examine the effects of glutamate injury on CB_1 receptor expression in order to compare these effects to other established models of acquired epilepsy, and further validate the use of OHSCs in studying the molecular changes associated with epileptogenesis. Western blot and immunohistochemical analyses were used to examine CB_1 receptor expression in OHSCs after glutamate injury, establishing that they do, indeed show similarities in CB_1 receptor changes as those observed in *in vivo* models.

B. Methods

Organotypic hippocampal slice culture preparation

Slice cultures were prepared using the method of Stoppini et al. (Stoppini *et al.*, 1991), as previously reported by (Schanuel *et al.*, 2008). All animal use protocols are in strict accordance with the National Institute of Health guidelines and are approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

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Postnatal day 8 (P8) Sprague-Dawley rat pups (Harlan, Frederick, MD, USA) were deeply anesthetized with isoflurane and decapitated. The brains were removed, and hippocampi were dissected out and cut into 350-μm transverse sections using a McIlwain tissue chopper (Brinkmann Instruments, Canada) and placed into Hank's balanced salt solution [HBSS – Gibco BRL (Invitrogen, Carlsbad, CA, USA)] supplemented with 0.5% sucrose and 1% penicillin-streptomycin. The middle four to six slices of each hippocampus, including part of the entorhinal cortex, were placed onto tissue culture membrane inserts (Millipore, Bedford, MA) in a 6-well tissue culture dish containing medium consisting of 50% minimum essential medium, 25% horse serum and 25% HBSS, 1% penicillin-streptomycin [all from Gibco BRL (Invitrogen, Carlsbad, CA)] and supplemented with 36 mM glucose, and 25 mM HEPES (Sigma, St. Louis, MO, USA) (pH 7.2). Cultures were maintained at 37°C under room air +5% CO_2. After 1 day in culture, culture medium was replaced with fresh medium containing no antibiotics. Culture medium was replaced two times a week thereafter.

Glutamate injury

After 21 days *in vitro* (DIV21), slice culture media was removed and replaced by media containing 3.5mM glutamate for a period of 35 minutes (Lipski *et al.*, 2007). Glutamate containing media was then removed and slice cultures were washed with HBSS at 37°C two times before returning to normal media. Control slices received normal media (without glutamate) during the 35 minute time period, followed by washing with HBSS twice before returning to normal media.

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Western blot protocol

Fresh OHSC tissue was harvested on DIV 32 and kept on ice. In order to obtain a sufficient amount of protein, four OHSCs per sample were homogenized in 50 mM Tris, pH 7.5, 6 mM EGTA, 320 mM sucrose, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride. Before electrophoresis, whole homogenate samples were thawed on ice, and protein concentration per sample was calculated using the MicroBradford reagent system (Bio-Rad, Hercules, CA) quantified using a UV-2101PC ultraviolet spectrophotometer (Shimadzu, Kyoto, Japan). Samples were balanced to 30 μg protein/gel lane and denatured in beta-mercaptoethanol and loading dye buffer. Samples were then heated to 90°C for 5 min in a programmable thermal controller PTC100 (MJ Research, Watertown, MA) and allowed to cool to room temperature before loading onto a 4-12% Bis-Tris Gel (InVitrogen, Life Technologies, Carlsbad, CA). A colorimetric molecular mass marker including standards ranging from 10 to 182 kDa (Bio-Rad) was loaded onto the last lane of the gel to aid in determining protein size. Gels were assembled into a MiniProtean II Electrophoresis System (Bio-Rad) and resolved for 90 min at constant 200 V in running buffer (Invitrogen). After electrophoresis, gels were Western blot transferred to nitrocellulose membranes (Millipore Corp., Bedford, MA) for 2 h at 4°C using a Genie transfer apparatus (IDEA Scientific, Minneapolis, MN) at a constant 100 V in transfer buffer (NuPage Transfer Buffer, Invitrogen). After transfer, the Western blot was stored in phosphate-buffered saline at 4°C overnight. Membranes were stained for protein and quantitated for actin protein levels as described previously (Morris *et al.*, 2000b).

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Immunostaining of the Western blot was performed by first blocking the membrane in buffer composed of 3% blotting grade blocker (Bio-Rad) and 0.05% Tween 20 in phosphate-buffered saline for 45 min at room temperature. Rabbit (polyclonal) anti-cannabinoid CB_1 receptor primary antibodies generalted to the N-terminus residues 1-77 (N^{1-77}, generously donated by Dr. Ken Mackie, Indiana University) or the C-terminus residues 461-473 ($C^{461-473}$, generously donated by Dr. Maurice Elphick, Queen's College) were added to the blocking solution at a concentration of 1:500 and 1:5000 respectively, and the membrane was incubated for 90 min at room temperature. After primary antibody incubation, the membrane was washed for a total of 15 min (three times for 5 min each) in phosphate-buffered saline. The membrane was then reblocked in fresh blocking buffer for 30 min. Anti-rabbit IgG-horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was then added to the blocking solution in a 1:5000 dilution, and the membrane was incubated for a final 45 min. Western blots were washed (three times for 5 min each) in PBS and incubated for 2 min in SuperSignal (Pierce Chemical, Rockford, IL) for enhanced chemiluminescent analysis. Chemiluminescent images were visualized using Kodak X-Omat Blue XB-1 X-ray film (Eastman Kodak, Rochester, NY) and developed using a Kodak M35A X-Omat Processor (Eastman Kodak). Film images were digitized using a gel scanner and analyzed using Image J software (NIH). Using the molecular mass marker as reference, the protein band was determined to correspond to a mass of around 53 kDa. To normalize protein levels for each band, Actin was probed as a protein standard. The blot membrane was then washed with PBS for 30 minutes, followed by re-blocking in blocking buffer. The membrane was then incubated in Actin primary antibody (1:4000 dilution, Sigma) for 60

minutes. It was washed in PBS (3x5 minutes) before being incubated in anti-mouse HRP secondary antibody (1:500), followed by another wash in PBS (3x5 minutes). The membrane was incubated with SuperSignal for 2 minutes and visualized as above. Actin bands were visualized at 43kDa. The CB_1 protein band densitometry readings were normalized to their corresponding actin protein band densitometry readings. Previous studies in our laboratory utilized the specific peptide block for each CB_1 antibody in rat tissues. These studies revealed no immunostaining with addition of the peptide block, indicating antibody specificity.

Immunohistochemistry

Following field potential recording to assess for seizure activity (see chapter 2), OHSCs attached to culture membranes were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS). Fixed cultures were washed as floating samples in 24-well plates in PBS before blocking and permeabilizing in SuperBlock® blocking buffer (Pierce, Rockford, IL) containing 0.2% Triton X-100 (SBBT) for 60 min at room temperature, followed by a 72 h incubation at 4 ºC with polyclonal antibody to the C-terminus of the CB_1 receptor (1:1000) (Egertova and Elphick, 2000) in SBBT. After antibody incubation, OHSCs were washed four times in PBS containing 0.2% Triton X-100 (PBST). Cultures were then incubated with anti-rabbit fluorochrome-conjugated secondary antibody (1:200, Alexa Fluor® 488; Invitrogen Corp., Eugene, OR) in SBBT for 60 min. After four PBST washes, stained OHSCs were counterstained for 5 min with DAPI (300 nM in PBS). Stained cultures were washed three times in PBS, mounted on slides, coated with ProLong Gold® anti-fade agent (Invitrogen Corp., Eugene, OR) and cover slipped. Control experiments without primary antibodies were carried out in an identical manner.

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Stained cultures were evaluated with a TCS-SP2 AOBS confocal microscope with a 10X or 40X objective (Leica Microsystems GmbH, Wetzlar, Germany) and lasers with wavelengths at 405nm (DAPI) and 488nm (FITC). Confocal gain and offset settings were optimized for each set of tissue so that glutamate treated slices could be normalized to controls from the same session. Digital images were converted to 16-bit grayscale and analyzed with Image J software (NIH). Hippocampal regions were visually identified and circled as regions of interest to analyze individual areas. Glutamate treated areas were normalized to matched control areas. No-primary control OHSCs were not incubated in CB_1 primary, but were treated the same otherwise.

Statistical analyses.

Results are given as means ± S.E.M. Statistical comparisons were made using SigmaStat software (Systat software, Chicago IL). The Student's t test was used to compare control to glutamate treated slices. N refers to number of OHSCs. Graphs were generated using Sigmaplot 11 software (Systat software). $P < 0.05$ was considered significant.

C. Results

There is an overall increase of CB_1 receptor protein following glutamate injury

Western blot analysis revealed an overall increase in CB_1 receptor protein at DIV 32 in whole homogenates of OHSCs that had been treated with glutamate. The N-terminus and C-terminus antibodies showed similar results when run with the same tissue samples, therefore the results of all blots were included for analysis, regardless of

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antibody used. Densitometric analysis showed a $128 \pm 10.2\%$ increase in CB_1 protein in glutamate treated slices as compared to matched controls ($100 \pm 5\%$, P=0.02, Student's t-test, n=15 each). This analysis reveals an increase in CB_1 receptor protein relative to total protein in each sample in OHSCs that had undergone treatment with 3.5 mM glutamate (Figures 17 and 18).

CB_1 receptors are upregulated in stratum oriens, stratum radiatum, and dentate gyrus

Similar to what has been described *in vivo* (Falenski *et al.*, 2007) and *in vitro* (Boscia *et al.*, 2008), confocal imaging of both control and glutamate treated OHSCs stained for CB_1 receptors revealed a dense plexus of CB_1-immunoreactive fibers that surrounded the immunonegative principal cells. Densitometric analysis of confocal images obtained from OHSCs stained for CB_1 receptors showed a relative overall increase in several cell regions following glutamate treatment. The CA3 region had staining that was 1.72 ± 0.19 (n=5) times the relative control OHSCs (1 ± 0.106, p=.036, n=4, Student's t-test). Similarly, the dentate gyrus showed a relative increase of 1.38 ± 0.114 (n=7) times the control staining (1 ± 0.05, n= 8, p=0.014, Student's t-test). The increase in CB_1 density in the CA1 region did not show statistical significance (control, 1 ± 0.18, n=4, glutamate, 1.032 ± 0.164, n=5, p=0.97, t-test). However, separate analysis of the stratum pyramidale, stratum oriens and stratum radiatum revealed a significant increase in CB_1 staining in the stratum oriens of the CA1 region (control, 1 ± 0.09, n=6, glutamate treated, 1.63 ± 0.290, n=4, p=0.038) and the stratum radiatum of the CA3 region (control, 1 ± 0.06, n=4, glutamate treated, 1.33 ± 0.15, n=5, p=0.032) (Figures 19 and 20). The spread of the granule cells layers in OHSCs (Caeser and Aertsen, 1991)

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made it difficult to delineate layers of the dentate gyrus in all samples, therefore it was only evaluated as a whole region. OHSC staining also found that the no primary controls showed minimal staining (Figure 21), indicating specificity of our secondary antibody and recording parameters.

Figure 17 – Western blot anaysis of CB$_1$ receptor protein. Whole homogenates of OHSCs were collected at DIV 32 and analyzed for CB$_1$ receptor protein expression. Two different CB$_1$ receptor antibodies were used. The N-terminus antibody **(a.)** and the C-terminus antibody **(b.)** showed similar results when normalized to actin. Both antibodies revealed an increase in CB1 protein in samples that had undergone glutamate treatment at DIV21.

Figure 17 a.

N-terminus Antibody

Control   Glut

CB1

Actin

Figure 17b.

C-terminus Antibody

Control   Glut

CB1

Actin
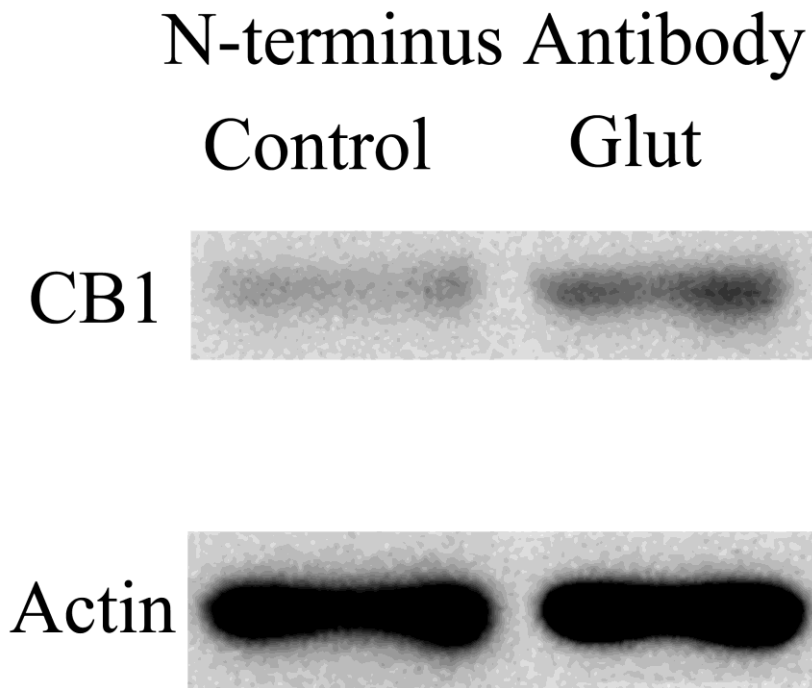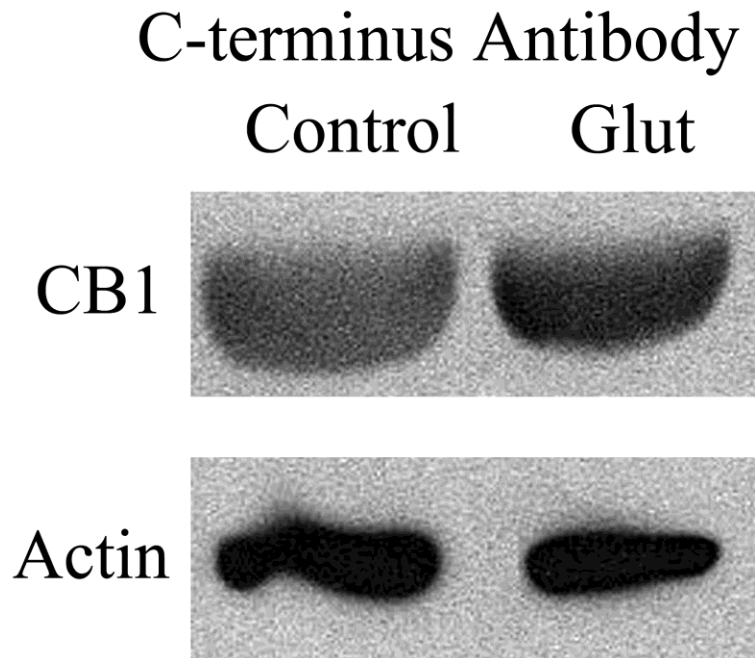
Figure 18 – There is an increase in CB$_1$ receptor protein after glutamate treatment.
Densitometric analysis showed CB1 protein levels at $128 \pm 10.2\%$ of matched controls
($100 \pm 5\%$, P=0.02, Student's t-test, n=15 each) in glutamate treated slices. This analysis
reveals an increase in CB$_1$ receptor protein relative to total protein in each sample in
OHSCs that had undergone treatment with 3.5 mM glutamate.

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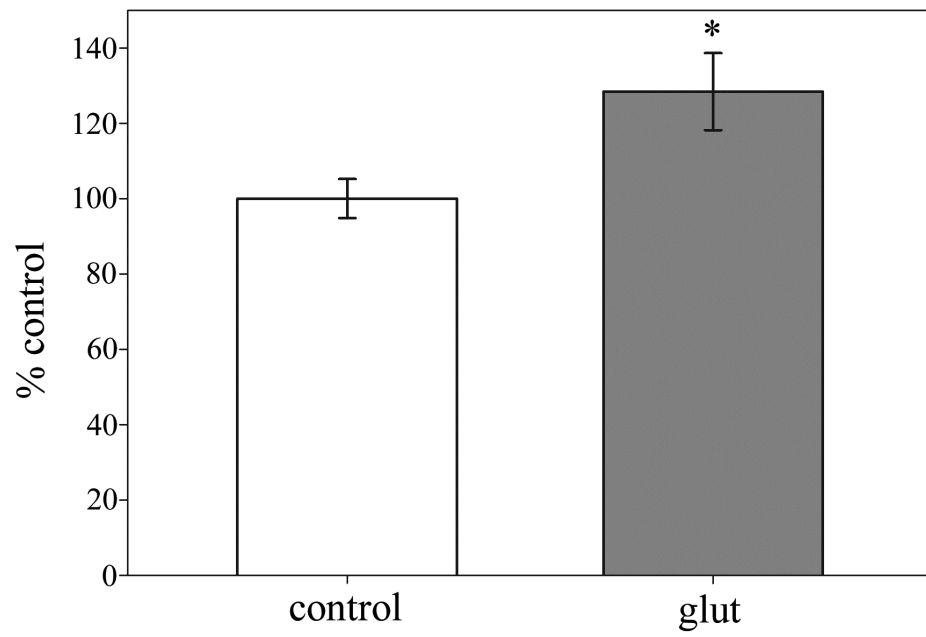
Figure 18

Figure 19 - Immunohistologic staining of the CB$_1$ receptor at 10X. Immunohistologic staining for the CB$_1$ receptor using the C-terminus antibody confirms increases in CB$_1$ receptor expression in glutamate treated OHSCs. Confocal microscopy was utilized to analyze CB$_1$ staining in individual cell areas of the slice cultures. Densitometric analysis revealed significant increases in CB$_1$ staining in the CA3 and DG regions when compared to their matched controls.

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Figure 19

CB1 staining - 10X magnification

control	glut

CA1

CA3

DG
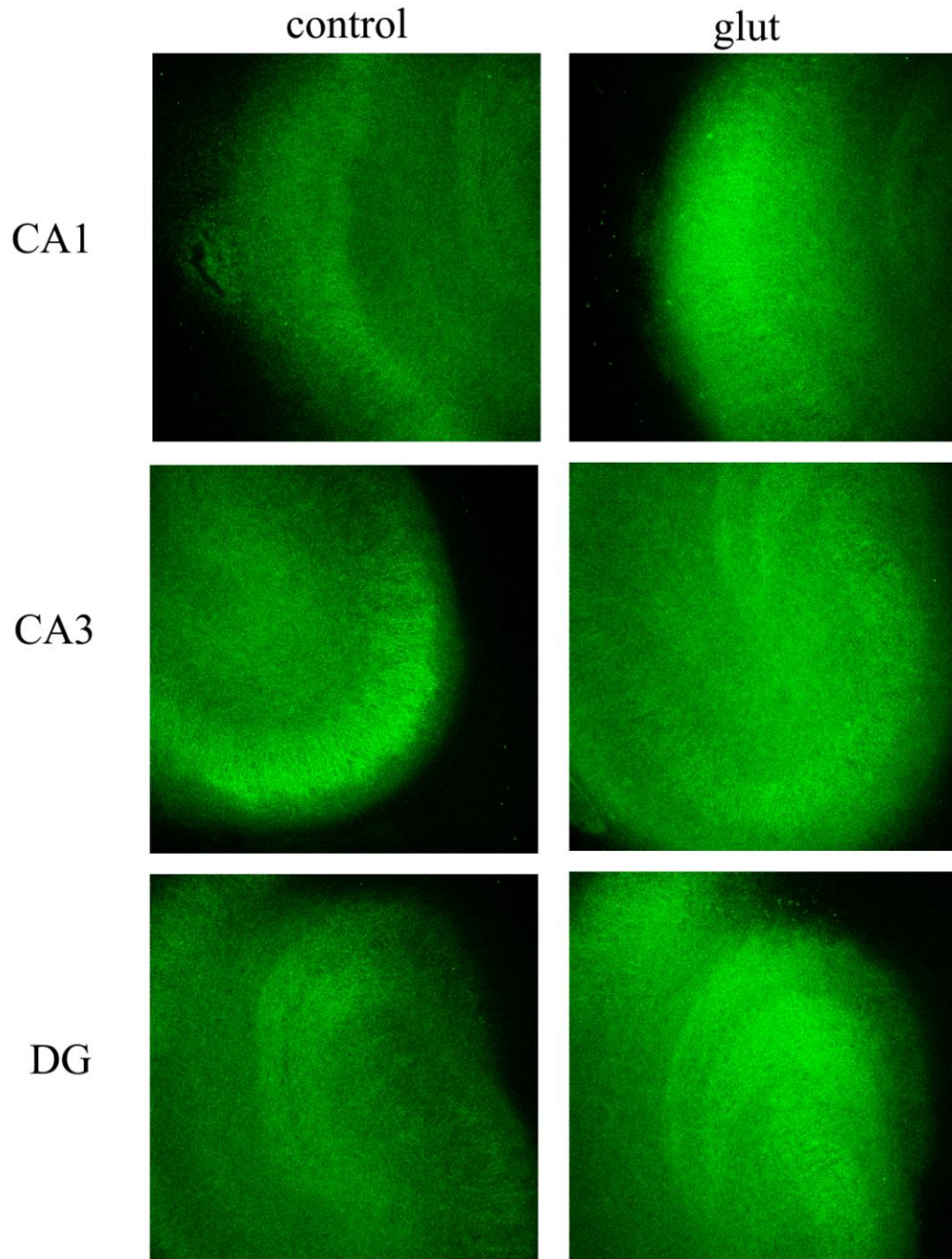
Figure 20 – Confocal imaging of CB$_1$ receptor stained OHSCs – 40X magnification.

Higher magnification of OHSCs stained for CB$_1$ receptor protein show considerable punctate staining, especially in around cell bodies. When comparing to control (left panels) OHSCs, the overall increase in CB$_1$ receptor expression is especially apparent in glutamate treated (right panels) OHSCs at this magnification.

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Figure 20

CB1 receptor staining - 40X

control   glut

CA1

CA3

DG
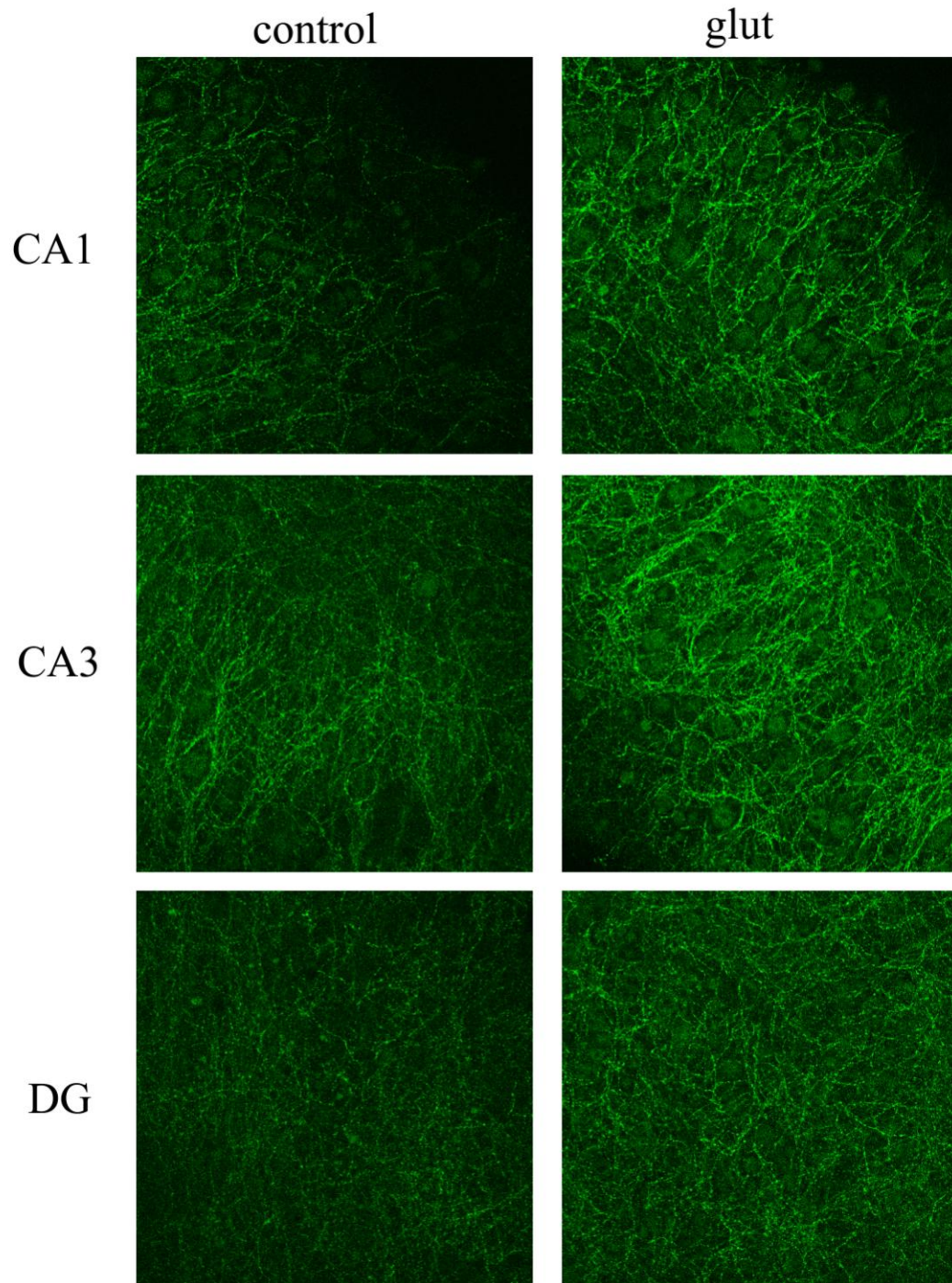
Figure 21 – No primary control. To ensure that our fluorescent antibody bound specifically to the primary antibody, we omitted the primary antibody from the staining protocol for control OHSCs. The observed fluorescence from the no primary controls was minimal, indicating specific binding. (10X magnification)

Figure 21
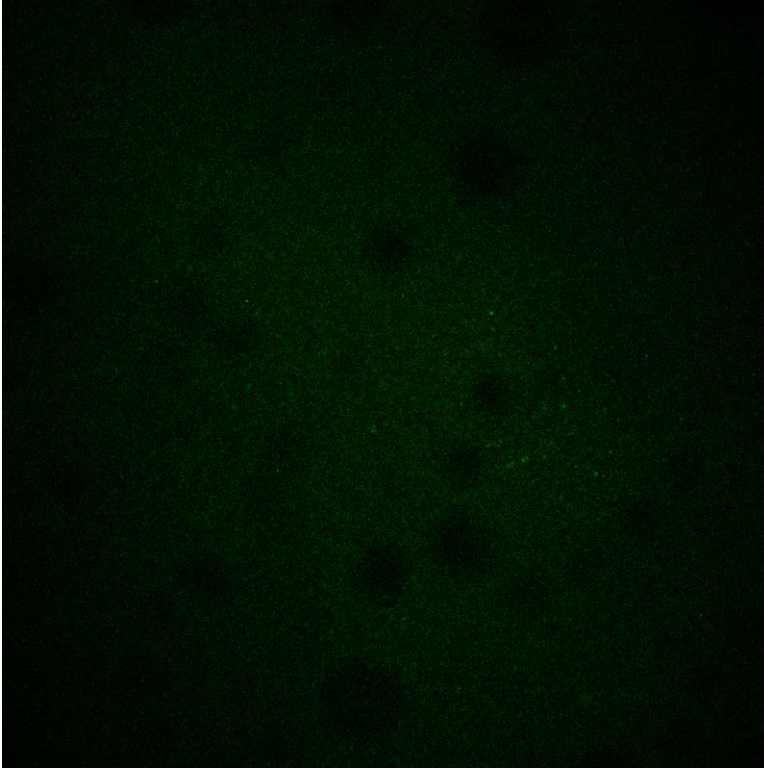
D. Discussion

Results from this study demonstrate that the CB_1 receptor undergoes changes in expression in the OHSC model of stroke acquired epilepsy. This supports our hypothesis that the OHSC model has many similarities to the molecular changes observed in *in vivo* models of AE. We have found that the CB_1 receptor expression rises to 128% of control levels following treatment with glutamate, compared to a 183% rise in CB_1 receptor expression observed in the hippocampus of epileptic rats (Wallace *et al.*, 2003). Glutamate treated OHSCs were collected for protein analysis at DIV 32 at a point when previous studies (Chapter 2) have shown that approximately 46% of OHSCs would be displaying seizure activity. Since OHSCs were not tested for seizure activity, we would expect only about 46% of them would have undergone the changes in CB_1 receptor expression, which would contribute to a less robust change in CB1 receptor expression than what is observed in animal models. In addition, 7% of control OHSCs at that time point display seizures, indicating that they may have undergone some of the molecular changes associated with seizures and possibly have a greater expression of CB_1 receptors than what is observed in control animal tissue. Nonetheless, the upregulation of CB_1 receptors observed in this study provides additional support of the utility of the OHSC model of stroke acquired epilepsy. Stroke acquired epilepsy has been challenging to model *in vivo* and current *in vitro* models have utilized primary hippocampal neuronal cultures (Sun *et al.*, 2001), which lose normal anatomical connections that play an important role in the ischemic penumbra. The use of organotypic hippocampal slice cultures provides a model that is between the organizational complexity of *in vitro* primary neuronal cultures and *in vivo* models.

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Densitometric analysis of immunohistochemical staining of OHSCs showed a specific increase in the stratum oriens of the CA1 and stratum radiatum of the CA3 region in OHSCs that displayed seizure activity. This finding further supports similarity to the *in vivo* pilocarpine model of acquired epilepsy (Falenski *et al.*, 2007; Falenski *et al.*, 2009). It also supports the hypothesis that the CB_1 receptor upregulation observed in Western blot analysis is specifically located at the glutamatergic terminals. CB_1 receptor distribution in OHSCs under normal conditions has been shown to be similar to what is observed in normal rats (Boscia *et al.*, 2008). Specifically, previous studies have shown that the entire hippocampal slice culture is decorated with a dense plexus of CB_1-immunoreactive fibers that surrounded the immunonegative principal cells. In CA1, an extensive perisomatic axonal meshwork is evident in the stratum pyramidale (Boscia *et al.*, 2008). Our immunostaining experiments in control OHSCs showed a similar CB_1 profile, with intense staining surrounding the pyramidal cells and decreased staining in the stratum oriens and radiatum. Following glutamate treatment, seizing OHSCs displayed significantly increased intensity in CB_1 staining in the stratum radiatum and stratum oriens regions. These results are in agreement with the histological organization of CB_1 receptors in animals with AE. Recent studies from our laboratory suggest a selective reorganization of CB_1 receptors among the excitatory and inhibitory terminals of the hippocampus (Falenski *et al.*, 2007). Utilizing the rat pilocarpine model of AE, immunohistochemical studies revealed an increase in CB_1 staining of the stratum radiatum and stratum oriens along with the outer molecular layer of the dentate gyrus in the epileptic hippocampus. In addition, there was decreased staining of the CA1 and CA3 pyramidal cell layers and inner molecular layer of the dentate gyrus (Falenski *et al.*,

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2007). These findings further support the hypothesis that the CB_1 receptor changes are a compensatory mechanism to control seizures. An upregulation at glutamatergic terminals, as observed in these studies, would play a role in dampening excessive neuronal excitation, whereas a decrease in CB_1 at inhibitory terminals would allow for an increased inhibitory tone. Interestingly, a recent study of the GABAergic terminals in the mouse pilocarpine model of AE has suggested that there is actually a preferential loss of GABAergic innervation from CCK-containing basket cells, with an increased contribution from PV-containing interneurons (Wyeth *et al.*, 2010). This would explain the decrease in CB_1 staining observed at GABAergic terminals without a loss of GABAergic terminals on CA1 pyramidal cells.

The rat pilocarpine model of AE is not the only model that displays an upregulation of CB_1 receptors as CB_1 receptor protein increases have also been noted in the febrile seizure model (Chen *et al.*, 2003), and kainic acid model. In addition, a recent study of the hippocampus of the Amazonian, Proechimys, has shown that these rodents are resistant to developing spontaneous seizures following epileptogenic precipitating events such as pilocarpine, kainic acid, or kindling (Arida *et al.*, 2005; de Amorim Carvalho *et al.*, 2003). Evaluation of the Proechimys hippocampus revealed an elevated level of CB_1 receptor protein that is over 1.3 times greater than what is observed in Wistar rats (Araujo *et al.*, 2010). Another intriguing study of human, sclerotic, epileptic tissue actually revealed a downregulation of CB_1 protein in the epileptic hippocampus (Ludanyi *et al.*, 2008). These studies provide an interesting view of the role of the endocannabinoid system in epilepsy. They suggest that the upregulation of CB_1 receptors in animal models of AE might actually be a compensatory mechanism to attempt to

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control seizure severity after a seizure precipitating event. The studies of human tissue suggest that this compensatory mechanism does not develop in humans with intractable epilepsy.

In vivo studies in the rat pilocarpine model of AE have utilized [^{35}S] GTPγS autoradiography to assess the functionality of the CB_1 receptor reorganization following the development of epilepsy. These studies confirmed the functionality of the CB_1 receptors in this model (Falenski *et al.*, 2007). An additional advantage to the development of a OHSC model of AE is that we will readily be able to assess the functionality of the observed receptor upregulation utilizing electrophysiological techniques. These studies will further elucidate the physiological changes associated with an overall increase in CB_1 receptor expression after epileptogenic injury.

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CHAPTER 4: PHYSIOLOGICAL CHANGES OF THE ENDOCANNABINOID SYSTEM FOLLOWING GLUTAMATE INJURY

A. Introduction:

Epilepsy is a common and debilitating neurological condition characterized by spontaneous recurrent seizures. It affects over 2.7 million people in the United States (Kobau *et al.*, 2005). Epilepsy can be cryptogenic in nature, in which there is likely a genetic cause, or it can be an acquired epilepsy (AE) which develops after a precipitating incident such as traumatic brain injury, CNS infection, status epilepticus or stroke (DeLorenzo *et al.*, 2005; Elger and Schmidt, 2008). After a brain trauma, a cascade of events causes many neuronal changes, including transcriptional changes, mossy fiber sprouting, and neurogenesis (Rakhade and Jensen, 2009) during the period known as epileptogenesis. These neuronal changes can then lead to the expression of the epileptic phenotype. The changes that occur during epileptogenesis are important to study to further advance our knowledge of AE so we may develop novel treatments and therapies to prevent or better treat this debilitating condition. Among receptor systems that undergo plasticity changes during epileptogenesis, the endocannabinoid system has come to the forefront of neuroscience research.

The endocannabinoid system includes the CB_1 and CB_2 receptors, their endogenous ligands, N-arachidonyl ethanolamine (anandamide, AEA) (Munro *et al.*, 1993) and 2-arachidonylglycerol (2-AG) (Mechoulam *et al.*, 1995) and the protein

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machinery to synthesize, reuptake and degrade the endocannabinoids. In the CNS, the CB_1 receptor is the most prevalent G-protein coupled receptor and it is expressed at high levels in the hippocampus, cortex, cerebellum, and basal ganglia (Herkenham *et al.*, 1990; Herkenham *et al.*, 1991; Tsou *et al.*, 1998). Endogenous cannabinoids (endocannabinoids) are synthesized and released on demand from the post-synaptic cell during periods of high activity. The endocannabinoids then travel retrogradely and bind to the CB_1 receptor on the pre-synaptic cell leading to the suppression of voltage-gated Ca^{2+} channels (Alger, 2004; Twitchell *et al.*, 1997). The suppression of presynaptic Ca^{2+} channels inhibits the release of neurotransmitters from the cell, thereby causing a transient inhibition in cell signaling. In addition, CB_1 receptor activation also inhibits adenylyl cyclase, causing decreased levels of intracellular cAMP, and stimulation of potassium channels (A-type and inwardly rectifying), leading to an increased efflux of potassium (Lutz, 2004). The on-demand synthesis and release of endocannabinoids during periods of increased neuronal activity and the localization of CB_1 receptors at both inhibitory (Hajos *et al.*, 2000; Katona *et al.*, 1999) and excitatory (Katona *et al.*, 2006; Kawamura *et al.*, 2006; Marsicano and Lutz, 1999) terminals further point to the importance of the endocannabinoid system in modulating synaptic transmission in disease states such as epilepsy (Alger, 2006).

One of the mechanisms that the endocannabinoid system relies on to modulate neurotransmission is the activity dependent suppression of inhibition or excitation. Dependent on what class of terminals the CB_1 receptor is present, this regulatory mechanism is termed depolarization-induced suppression of inhibition (DSI- when GABA is involved) or excitation (DSE- when glutamate is involved). DSI is long known

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to be an endocannabinoid mediated phenomenon that occurs in CA1 and CA3 pyramidal cells and dentate gyrus granule cells in the hippocampus (Alger, 2002). DSI occurs in the hippocampus when a brief activation of the post-synaptic cell causes the on-demand synthesis and release of endocannabinoid 2-AG, which then activates the CB_1 receptors on the pre-synaptic cell (CCK-positive interneurons), briefly inhibiting their release of GABA. Thus, there is a transient suppression of the inhibitory signal to the post-synaptic cell.

The glutamatergic terminal equivalent of DSI is the depolarization induced suppression of excitation (DSE). DSE has been more challenging to study in the hippocampus compared to cerebellum. Studies have shown that DSE is more difficult to induce in hippocampal cells and it requires a longer activation of the post-synaptic cell than what is observed in the cerebellum (Ohno-Shosaku *et al.*, 2002). Reasons for this apparent discrepancy could be that there may be a greater number of receptors on the excitatory nerve terminals in the cerebellum, or better access of the released endocannabinoid to the receptors on the excitatory nerve terminals (Alger, 2002). In addition, age may also play a role in the ability to induce DSE, as studies have shown that the CB_1 agonist WIN 55,212-2 reduced field EPSPs in tissue from younger animals, while tissue from older animals failed to show an effect (Al-Hayani and Davies, 2000). Though the methods of recording were slightly different, DSE has been observed in tissue from young animals (Ohno-Shosaku *et al.*, 2002), but not in tissue from older animals (Hajos *et al.*, 2001). In general, DSE in the hippocampus is less robust than DSI, generally only showing a 30% suppression of EPSCs compared to a 50% or more suppression of IPSCs in DSI (Alger, 2002; Ohno-Shosaku *et al.*, 2002).

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DSI and DSE are important physiological functions to study in the epileptic state, as several histological studies have shown a relative upregulation of the CB_1 receptor in animal models of epilepsy(Chen *et al.*, 2003; Monory *et al.*, 2006; Wallace *et al.*, 2003). However, functional importance of these receptor changes in epilepsy (Chen *et al.*, 2007) have not been thoroughly explored. It has long been known that cannabinoids have an anti-convulsant effect in epilepsy. Studies from this laboratory have found that administration of the CB_1 agonist R(+)WIN55,212-2 (WIN) to epileptic animals or cell cultures reduced their seizure frequency (Blair *et al.*, 2006; Wallace *et al.*, 2003). It has been postulated that there is a basal endocannabinoid tone that controls excitability in the epileptic phenotype and prevents the progression to lethal SE. Indeed, our laboratory has shown that by selectively blocking the CB_1 receptors with the CB_1 antagonist N-(piperidin-1-yl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride (SR141716A), the seizure frequency of epileptic animals increased 5-fold from baseline and some epileptic animals even developed status epilepticus (Wallace *et al.*, 2003). A similar phenomenon was also observed in the hippocampal neuronal culture model of AE (Deshpande *et al.*, 2007d). These studies point to a physiologically important role of the CB_1 receptors at the excitatory synapses in controlling seizure activity. These findings are in agreement with Monory et al (Monory *et al.*, 2006) who showed that selectively deleting CB_1 receptors at glutamatergic terminals significantly increased their susceptibility to kainic-acid induced seizures but not when CB_1 receptor was absent from the GABAergic terminals. This again supports the important functional role of CB_1 receptors at excitatory synapses in controlling seizure activity.

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Immunohistochemical studies from our laboratory have confirmed that the development of epilepsy causes unique changes to the endocannabinoid system. Falenski et al (Falenski *et al.*, 2007) utilized the pilocarpine model of acquired epilepsy and found a decrease in CB_1 receptor labeling at the pyramidal cell neuropil and dentate gyrus inner molecular layers of the hippocampus. In addition, a marked increase in CB_1 receptor labeling was noted in the stratum radiatum and oriens, suggesting an increase in CB_1 receptors in areas of excitatory synapses with a decrease in CB_1 at inhibitory synapses. CB_1 receptor binding and G-protein activation were also studied to confirm a functional reorganization of the CB_1 receptors (Falenski *et al.*, 2007). These anatomical and pharmacological findings have led us to hypothesize that the relative redistribution of CB_1 receptors act as a compensatory mechanism to assist in controlling seizures in epileptic animals from transitioning into the more dangerous state of status epilepticus (SE). This would suggest that the increase in CB_1 receptors at the excitatory terminals act to dampen the excitatory signals between cells and the decrease at inhibitory terminals would allow for greater inhibition during an excitatory state. However, this hypothesis has yet to be fully explored at a physiological cellular level.

This study was initiated to explore the hypothesis that there is a physiological change in cannabinoid modulation of DSI and DSE in epilepsy. Utilizing a novel organotypic hippocampal slice culture (OHSC) model of stroke induced acquired epilepsy, we are able to study DSI and DSE in an *in vitro* system that shares many characteristics of other *in vivo* and *in vitro* models of acquired epilepsy (chapter 2) and also displays an upregulation of CB_1 receptors (chapter 3). The results from this study

provide functional physiological evidence that the CB_1 receptor upregulation may in fact play a compensatory role in the epileptic state.

B. Methods:

Organotypic hippocampal slice culture preparation

Slice cultures were prepared using the method of Stoppini et al. (Stoppini *et al.*, 1991), as previously reported by (Schanuel *et al.*, 2008). All animal use protocols are in strict accordance with the National Institute of Health guidelines for the Care and Use of Laboratory Animals and are approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Postnatal day 8 (P8) Sprague-Dawley rat pups (Harlan, Frederick, MD, USA) were deeply anesthetized with isoflurane and decapitated. The brains were removed, and hippocampi were dissected and cut into 350-µm transverse sections using a McIlwain tissue chopper (Brinkmann Instruments, Canada) and placed into Hank's balanced salt solution [HBSS – Gibco BRL (Invitrogen, Carlsbad, CA, USA)] supplemented with 0.5% sucrose and 1% penicillin-streptomycin. The middle four to six slices of each hippocampus, including part of the entorhinal cortex, were placed onto tissue culture membrane inserts (Millipore, Bedford, MA) in a 6-well tissue culture dish containing maintenance medium composed of 50% minimum essential medium, 25% horse serum and 25% HBSS, 1% penicillin-streptomycin [all from Gibco BRL (Invitrogen, Carlsbad, CA)] and supplemented with 36 mM glucose, and 25 mM Hepes (Sigma, St. Louis, MO, USA) (pH 7.2). Cultures were maintained at 34°C under room air +5% CO_2. After 1 day in culture, culture medium was replaced with

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fresh maintenance medium containing no antibiotics. Culture medium was replaced two times a week thereafter.

Glutamate injury protocol

After 21 days *in vitro* (DIV21), slice culture media was replaced by media containing 3.5mM glutamate for a period of 35 minutes (Lipski *et al.*, 2007). At the end of this period, glutamate containing media was removed and slice cultures were washed with HBSS at 34°C two times before returning to normal media. Control slices received normal media (without glutamate) during the 35 minute time period, followed by washing with HBSS twice before returning to normal media.

Intracellular recording from OHSCs

At DIV 28-33 OHSCs were excised from culture inserts and transferred to a recording chamber mounted on an upright microscope (Axioskop2, Zeiss, Germany) equipped with IR/DIC optics. Slices were perfused with aCSF (composed of: in mM)120 NaCl, 3.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1.25 NaH_2PO_4, 25.6 $NaHCO_3$, and 10 glucose, equilibrated with 95% O2-5% CO2) at a rate of 1-2 ml/min at room temperature (25°C) with a peristaltic pump (Rainin Instruments, Oakland CA). aCSF was supplemented with bicuculline (10 µM) for recording EPSCs and with CNQX (10 µM) and APV (50 µM) for recording IPSCs (Ohno-Shosaku *et al.*, 2002). Patch electrodes with a resistance of 2 to 4 MΩ were pulled on a Brown-Flaming P-80C electrode puller (Sutter Instruments, Novato, CA). For sIPSC recordings, pipettes were filled with cesium based intracellular solution containing (in mM): 100 $CsCH_3SO_3$, 40 CsCl, 3 KCl, 0.2 EGTA, 0.02 $CaCl_2$, 1 $MgCl_2$, 2 ATP, 0.3 GTP, 10 HEPES, and 5 QX-314 (lidocaine N-ethyl bromide), pH

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balanced to 7.2 with CsOH, osmolarity adjusted to 290 with sucrose. For sEPSC

recordings, pipettes were filled with a potassium gluconate based solution containing (in

mM): 120 K-gluconate, 15 KCl, 6 $MgCl_2$, 0.2 EGTA, 10 HEPES, 5 NaATP, and 5 QX-

314, pH balanced to 7.3 with KOH, osmolarity adjusted to 290 with sucrose. QX-314 is a

local anesthetic that is membrane impermeable. By adding QX-314 to the pipette

solution, voltage-gated Na^+ conductances and regenerative spiking activity are blocked,

leaving only Ca^{2+} conductances.(Pitler and Alger, 1994). CA3 pyramidal cells were

morphologically identified at 63X magnification and electrodes were manipulated to

visually patch the cell with hydraulic micromanipulators. Cells were accepted for

evaluation if series resistance was stable within 15% during experimentation. Whole cell

voltage clamp recordings were obtained from one cell per slice. Recording protocol

included holding cells at –60mV for 90 seconds, followed by a 5 second voltage step to

0mV for DSI, 10 second voltage step for DSE. This protocol was repeated 1-5 times for

each cell.

Recording of evoked currents

     Composition of pipette and extracellular solutions were similar to the solutions

used for spontaneous recordings (see above). A bipolar stimulation electrode (FHC Inc,

Bowdoin, ME) constructed from Teflon-coated tungsten wire (90 ☐m diameter) was

placed in the stratum radiatum of the CA3 area to generate eEPSCs or eIPSCs. The

placement and distance of the recording and stimulating electrodes was kept constant. To

evoke currents, a minimal stimulation pulse (5-50 ☐A) was administered from the

stimulating electrode at a rate of 0.33Hz. The depolarizing pulse used to evoke DSI and

DSE was a 5s or 10s step to 0 mV from a holding potential of −60 mV delivered through the recording electrode, respectively.

Delivery of CB₁ receptor antagonist

To investigate whether the DSI and DSE phenomena were CB_1 dependent, we utilized the CB_1 receptor antagonist N-(piperidin-1-yl)-5- (4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A). SR141716A was dissolved in DMSO. The final concentration of DMSO in the bath solution was 0.02%. OHSCs were pre-treated with SR141716A (5 M) in the culture media for 2 hours and 5 M SR141716A was perfused during recording.

Data analyses

Recordings were low-pass filtered at 2 kHz, sampled at 10 kHz and stored using the Pclamp software (version 10, Molecular Devices, Sunnyvale, CA) employing analog-digital interface Digidata 1440A (Molecular Devices). Off-line analysis of data was performed using Clampex software (version 10.2, Molecular Devices). For DSI and DSE measurements, the magnitudes of suppression are expressed as percent decrease in sIPSC or sEPSC charge transfer after the depolarizing pulse with respect to pre-pulse control period respectively. Student's t-test was utilized for analyzing the DSI/ DSE data by comparing charge transfer before and after the depolarization (Martin *et al.*, 2001). To evaluate the event frequency of spontaneous events, we evaluated the number of IPSPs or EPSPs that occurred 30 seconds before the depolarization and 20 seconds after, excluding the first 2 seconds after depolarization to allow for maximal DSI (Alger *et al.*, 1996). To evaluate DSI and DSE in spontaneous recordings, these periods were evaluated as charge

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transfer for each 5 second period such that each 5 second period was calculated as [% charge of pre-depolarization = average charge transfer per period (pA/s) / average charge transfer (pA/s) during pre-depolarization period]. For evoked currents, the amplitude of the 8 events preceding depolarization were compared to the 3 events following (Zhang and Alger, 2010). To examine suppression of inhibition or excitation, the data is represented as [% amplitude of pre-depolarization = average amplitude of 3 events after depolarization / average amplitude of 8 events before depolarization]. Statistical tests were performed using SigmaStat 2.0 and graphs were generated using SigmaPlot 11 (Systat Software Inc., San Jose, CA). All data is represented as mean ± SEM. N refers to number of OHSCs. Unless otherwise noted, Student's t-test was used to evaluate significance. A value of $p < 0.05$ is considered statistically significant.

C. Results

Increased excitability and decreased inhibition in glutamate treated slices

There were significant differences in IPSC and EPSC frequency between control and glutamate treated OHSCs. Control OHSCs showed a higher IPSC frequency and a lower EPSC frequency than the glutamate treated slices. During recording of IPSCs, control slices showed an event frequency of 2.89 ± 0.67 Hz (n=14), while glutamate treated OHSCs had a spontaneous IPSC frequency of 0.69 ± 0.12 Hz (n=6, p=0.048). In addition, the amplitude of IPSCs observed in control OHSCs (-94.03 ± 2.56 pA, n=1244 events) was significantly larger than those in glutamate treated slices (-16.56 ± 0.41 pA, n=93 events, p<0.001). In contrast, during EPSC recording, control OHSCs had a spontaneous event frequency of 0.88 ± 0.15 Hz (n=7), compared to glutamate treated

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OHSCs that displayed a spontaneous event frequency of 2.33 ± 0.41 Hz (n=8, p=0.007). The amplitude of EPSCs in glutamate treated slices (-74.19 ± 1.70 pA, n=748 events) was also significantly larger than those observed in controls (-35.93 ± 0.774 pA, n=158 events, p<0.001). This indicates a significant increase in excitatory tone in glutamate treated OHSCs, with a decrease in inhibitory tone.

DSI is similar in control and glutamate treated OHSCs during spontaneous recording

To measure DSI we measured events for 30 s before depolarization and 20s after and then expressed DSI as change in charge transfer. Representative traces are displayed in Figure 22. During spontaneous recordings of IPSCs, both control (n=16) and glutamate (n=9) treated OHSCs displayed a significant suppression in charge transfer after depolarization of the CA3 pyramidal cell. Control OHSCs displayed a significant (p< 0.05) decrease in charge transfer from the pre-depolarization period at the 5 second ($36.9 \pm 8.7\%$), 15 second ($66.0 \pm 12.4\%$), and 20 second ($64.0 \pm 10.0\%$) time points. The 10 second ($60.1 \pm 18.3\%$) time point was not significantly different from the pre-depolarization charge transfer. Glutamate treated slices showed a significant depression in sIPSC charge transfer at 5 ($18.7 \pm 6.1\%$), 10 ($31.9 \pm 10.5\%$), and 15 seconds ($46.8 \pm 15.2\%$), but sIPSC charge transfer was not significantly different from pre-depolarization by 20 seconds ($74.7 \pm 10.2\%$) (Figure 23). There were no significant differences in the amount of depression between the two groups at any of the time points suggesting that glutamate injury does not play a significant role in DSI expression.

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DSE is increased in glutamate treated OHSCs

DSE in spontaneous recordings was measured as the difference in charge transfer of spontaneous EPSCs before depolarization and after depolarization. Representative traces are displayed in Figure 24. Change was expressed as a percent of the average pre-depolarization charge transfer. When testing for DSE in control we found no significant suppression of excitation following post-synaptic cell depolarization (n=13). Normalized to pre-stimulation values, control OHSCs displayed an average sEPSC charge transfer of $87.8 \pm 9.5\%$ at 5 seconds, $92.1 \pm 9.3\%$ at 10 seconds, $122.6 \pm 11.2\%$ at 15 seconds, and $114.5 \pm 13\%$ at the 20 second time point. On the other hand, glutamate treated slices (n=12) showed significant ($p < 0.05$) suppression of sEPSCs after depolarization at both the 5 second ($50.8 \pm 8.5\%$) and 10 second ($64.5 \pm 12.0\%$) time points, but this suppression was no longer significant at 15 ($73.6 \pm 9.7\%$) or 20 ($78.6 \pm 8.7\%$) seconds after depolarization. These values were significantly different from control values at 5, 15, and 20 seconds as displayed in Figure 25.

DSI and DSE are CB$_1$ mediated

Employing a stimulating electrode to evoke EPSCs and IPSCs allowed us to further evaluate DSI and DSE in our OHSC model. Interestingly, we did not observe as much DSI in evoked potentials as we did in our spontaneous IPSC recordings. Representative traces are displayed in Figure 26. In control OHSCs, the average post-depolarization eIPSC amplitude was $78.1 \pm 4.55\%$ (n=10) of the pre-depolarization amplitude. In glutamate treated slices, we saw even less DSI, with a post-depolarization amplitude of $94 \pm 3.7\%$ (n=8) of the pre-depolarization eIPSC amplitude. These two

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values are significantly different from one another, indicating a greater amount of DSI in control OHSCs than those that had undergone glutamate treatment (p=0.019, Student's t-test). The addition of SR141716A to the culture media and perfusion solution also revealed that the DSI observed in control OHSCs is in fact CB_1 mediated. DSI was not observed in cultures that had SR141716A present in the recording solution. The average control eIPSC pre-depolarization amplitude was $106.6 \pm 5.4\%$ (n=4) of the pre-depolarization amplitude, indicating that DSI did not occur. This was significantly different from the control value that did not receive SR141716A treatment (p=0.004, t-test). Glutamate treated OHSCs that were exposed to SR141716A also did not show DSI, with a post-depolarization amplitude equal to $104.3 \pm 2.3\%$ (n=5) of the pre-depolarization amplitude. However, this is not statistically significant from the small DSI observed in glutamate treated OHSCs that did not undergo SR141716A treatment (p=0.071, Figure 27).

When observing DSE in evoked potentials, our results are similar to those observed in spontaneous EPSCs. Control OHSCs did not display DSE, with a post-depolarization amplitude of $101.1 \pm 12.6\%$ (n=5) of the pre-depolarization amplitude. This is significantly different from the DSE observed in glutamate treated OHSCs (p=0.050). Glutamate treated OHSCs display an average post-depolarization amplitude of $61.6 \pm 11.9\%$ (n=6) of the pre-depolarization amplitude, indicating a significant amount of DSE. Addition of SR141716A to the culture media and perfusion solution block this DSE, indicating that it is a CB_1 mediated effect. In control OHSCs, the addition of SR141716A lead to an amplitude of $98.1 \pm 3.3\%$ (n=4) of pre-depolarization, indicating no DSE had occurred. In glutamate treated OHSCs a $107.8 \pm 10.1\%$ (n=4) amplitude of

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pre-depolarization was observed, also indicating that SR141716A had blocked DSE. This observation was significantly different from glutamate treated slices that were not exposed to SR141716A (p=0.026), while control slices displayed no significant change (p=0.842) (Figure 28).

Figure 22- Changes in DSI following stroke-like injury in OHSCs.

Current traces from CA3 pyramidal cells from age-matched control (**A**) and from a OHSC displaying following glutamate injury (**B**). The recordings were carried out in the presence of ionotropic glutamate receptor antagonists for IPSC measurements. The sIPSCs appeared depressed shortly after a depolarizing voltage step (5 s to 0 mV from -70 mV), indicated by the square pulse above the trace, representing DSI. Note that DSI was more prominent and easier to evoke in controls.

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Control DSI

0 mV

100 pA
5 sec

0.5 sec

Glut DSI

0 mV

100 pA
5 sec

0.5 sec
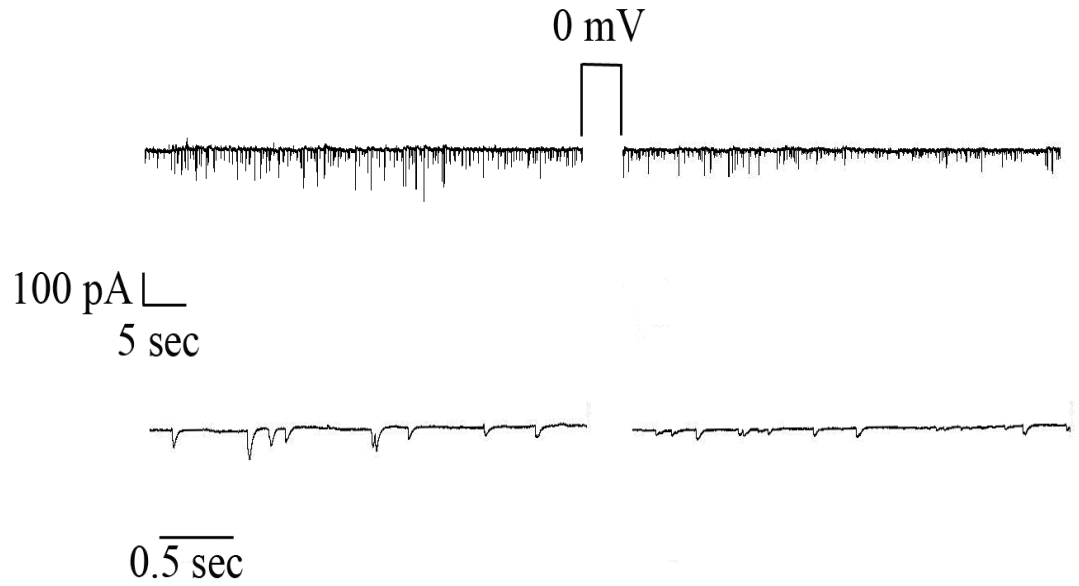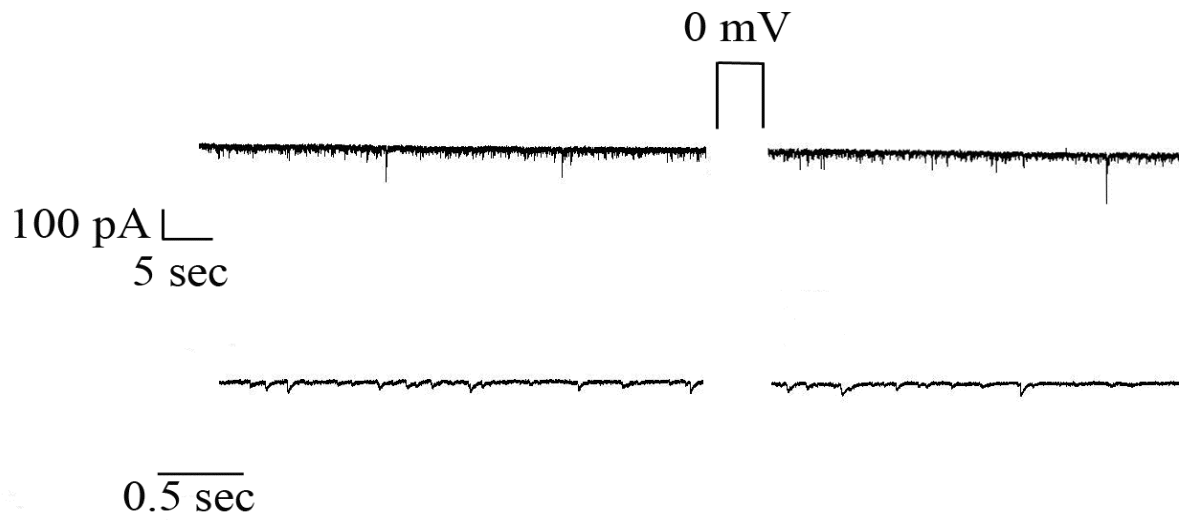
Figure 23 – Time course of DSI.

DSI was observed in both control (closed circle) and glutamate treated (open triangles) OHSCs. There was no significant difference between the two groups at any time point. Significant ($P<0.05$) DSI is observed from the -5 sec time point in both control (#) and glutamate treated (*) samples.
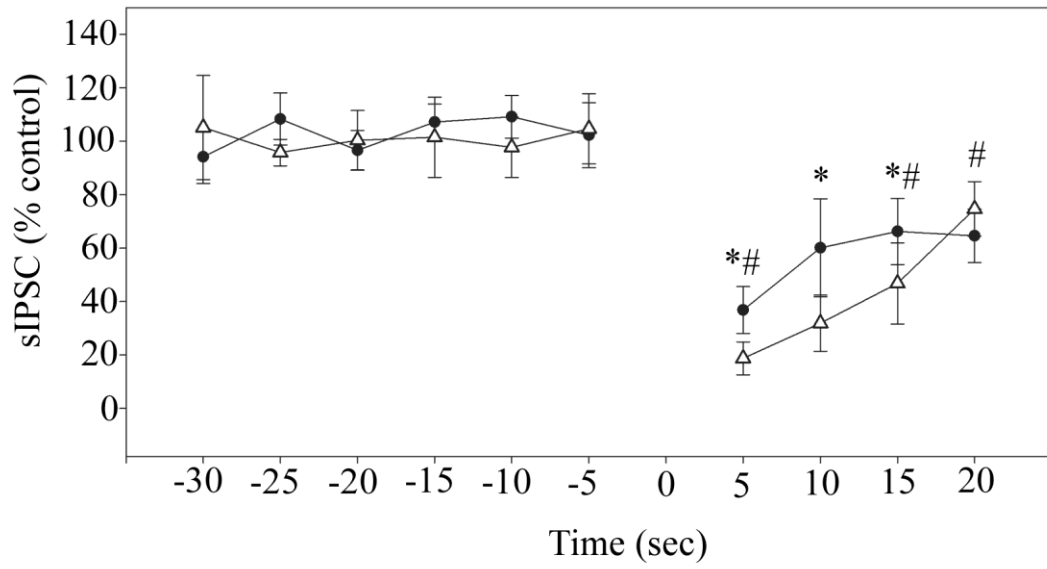
Figure 24. Changes in DSE following stroke-like injury in OHSCs.

Current traces from CA3 pyramidal cells from age-matched control (**A**) and from a slice culture displaying following glutamate injury (**B**). The recordings were carried out in the presence of GABA$_A$ receptor antagonists for EPSC measurements. The sEPSCs appeared depressed shortly after a depolarizing voltage step (10 s to 0 mV from -70 mV), indicated by the square pulse above the trace, representing DSE. Note that DSE was more prominent and easier to evoke in glutamate treated OHSCs than controls.

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Control DSE

0 mV

100 pA

5 sec

0.5 sec

Glut DSE

0 mV

100 pA

5 sec

0.5 sec
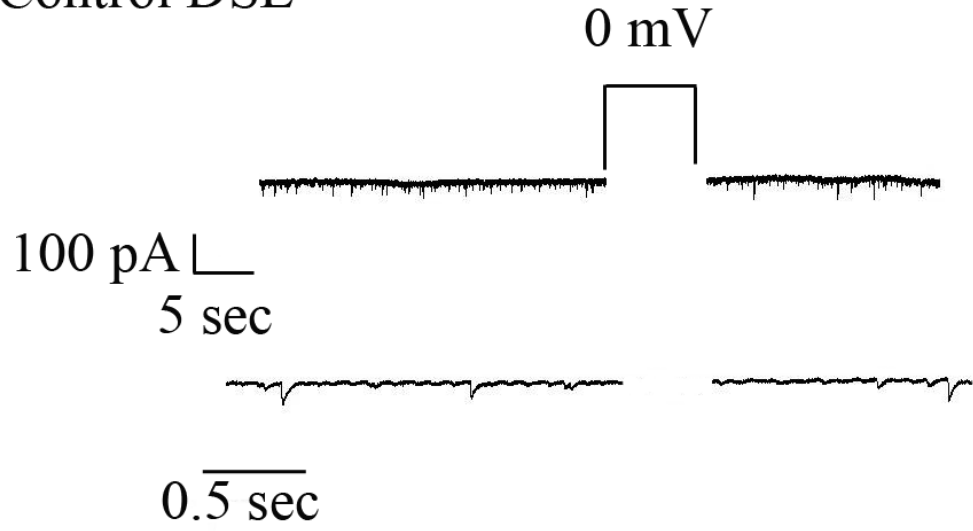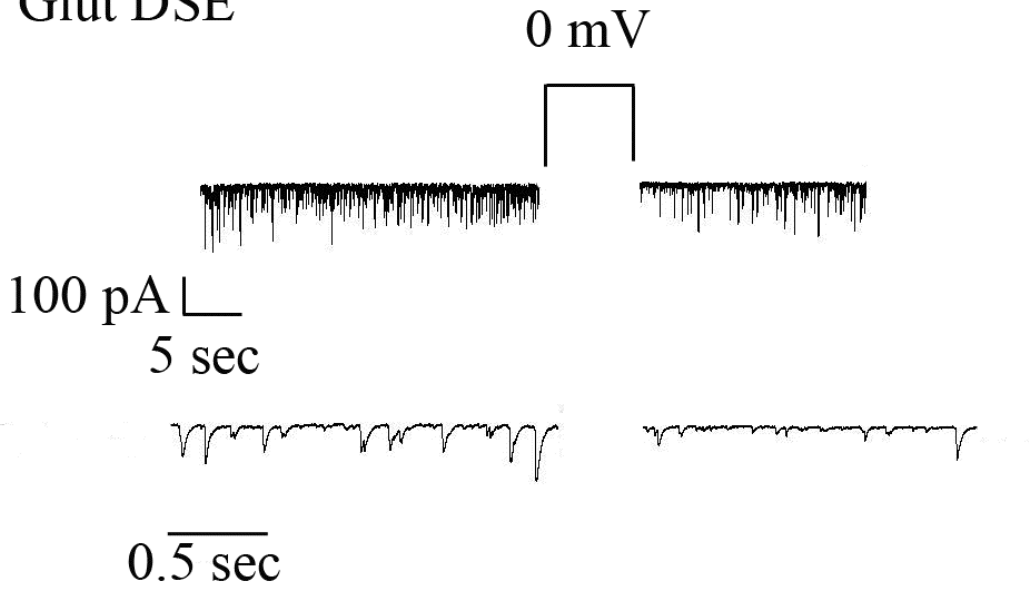
Figure 25– Time course of DSE

DSE was observed in the glutamate treated OHSCs, but not in the controls. There was a significant depression of EPSCs in glutamate slices (*) after the depolarizing pulse. This was significantly different from what was observed in controls ($). There was no significant depression in EPSCs in control OHSCs.
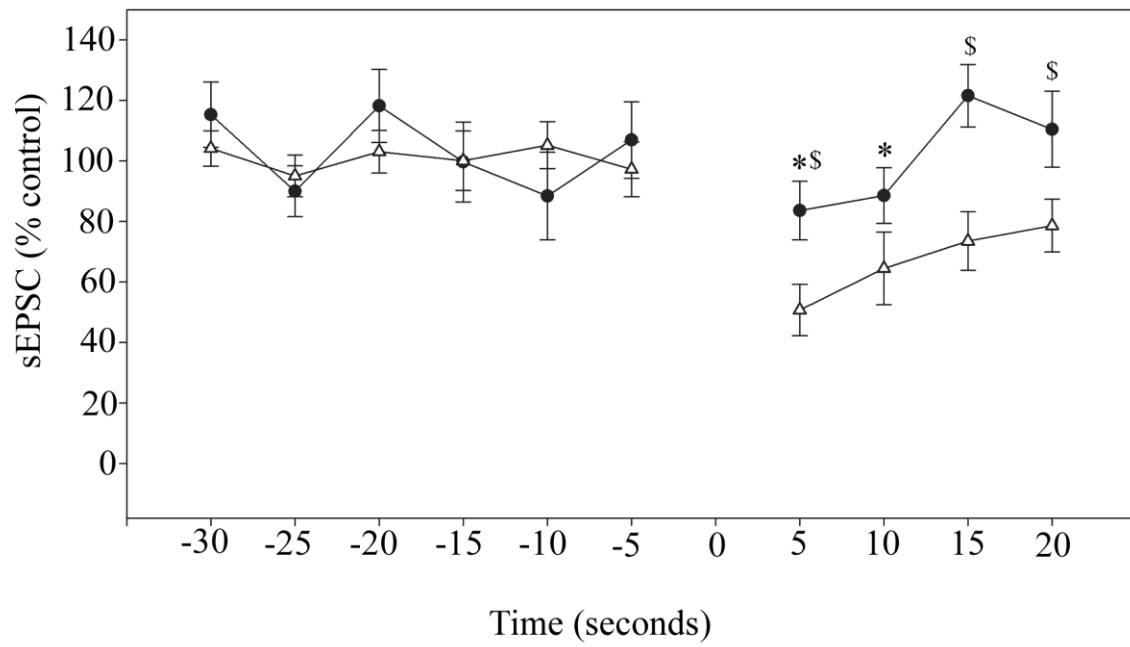
Figure 26 – Traces of evoked currents

IPSCs (a) and EPSCs (b) were evoked by placing a stimulating electrode in the stratum radiatum of the CA3 region while recording from a CA3 pyramidal cell. Currents were evoked at a rate of 0.33 Hz and a 5 sec (DSI) or 10 sec (DSE) depolarizing voltage step was applied to the pyramidal cell every 90 seconds. To measure DSI or DSE, we averaged the amplitude of the 8 evoked currents before the depolarization and compared them to the 3 currents following depolarization to calculate the suppression observed. Evoked current suppression was more apparent in controls while measuring DSI and in glutamate treated OHSCs when measuring DSE.

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Evoked DSI

Control

0 mV

Glut

0 mV

5 sec

50pA

0.5 sec

Evoked DSE

Control

0 mV

Glut

0 mV

5 sec

50pA

0.5 sec
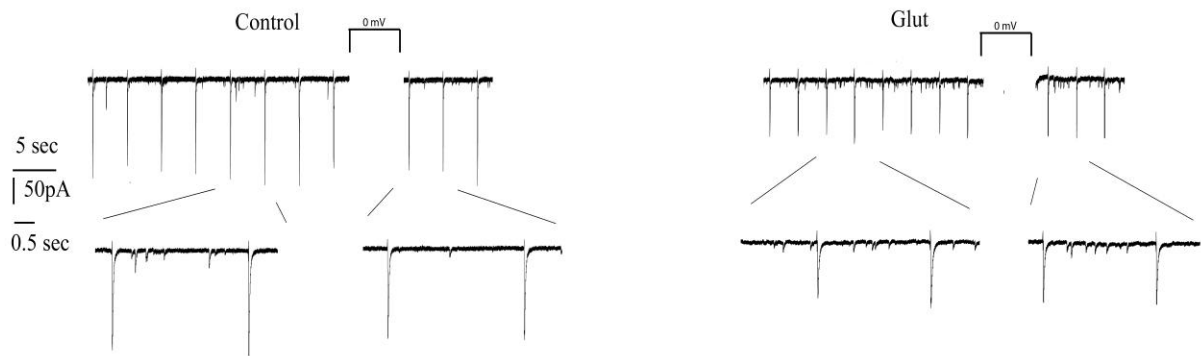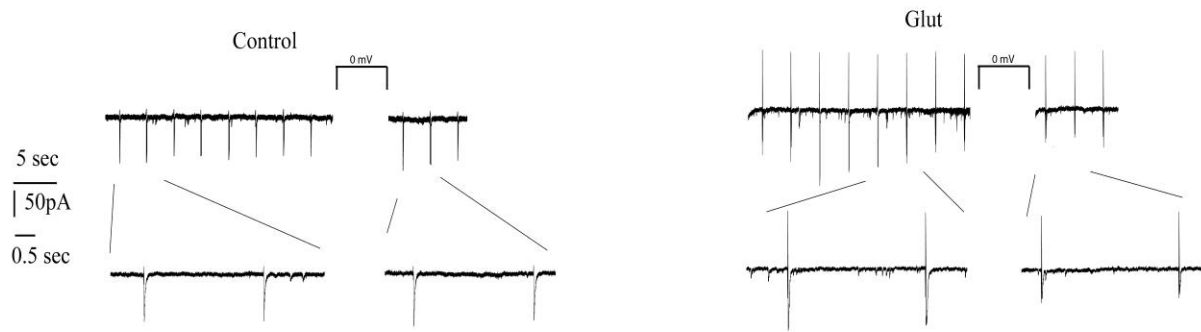
Figure 27 - eIPSCs reveal DSI in control OHSCs is CB_1 mediated.

By stimulating the slice to observe evoked IPSCs, we only observed DSI in control

OHSCs. This was significantly different from what was observed in glutamate treated

OHSCs. We also observed a significant difference in IPSC amplitude after control

OHSCs had been treated with the CB_1 receptor antagonist SR141716A (SR). This

suggests that the DSI observed in control OHSCs is CB_1 mediated.

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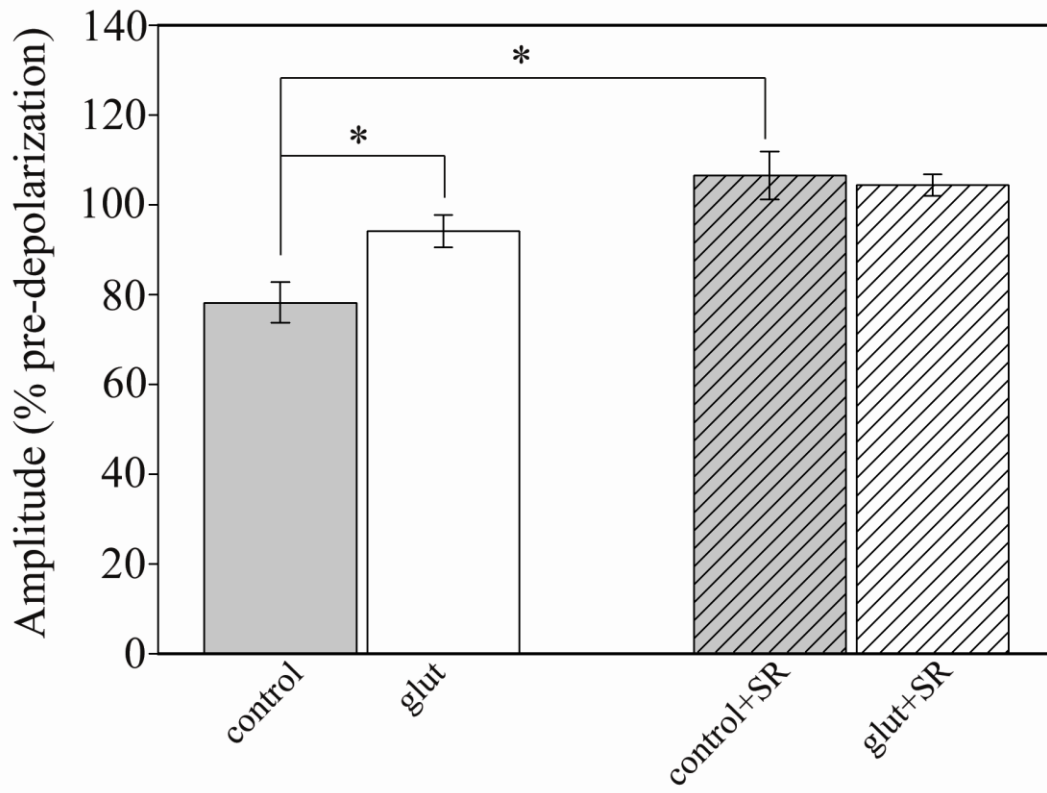
Figure 28 - eEPSCs reveal DSE in glutamate treated OHSCs is CB1 mediated.

Recording of evoked excitatory currents revealed significant DSE in glutamate treated

OHSCs, but not in controls, similar to what was observed in spontaneous recordings.

Addition of SR141716A (SR) to the recording media also suggested that this

phenomenon was CB_1 mediated.
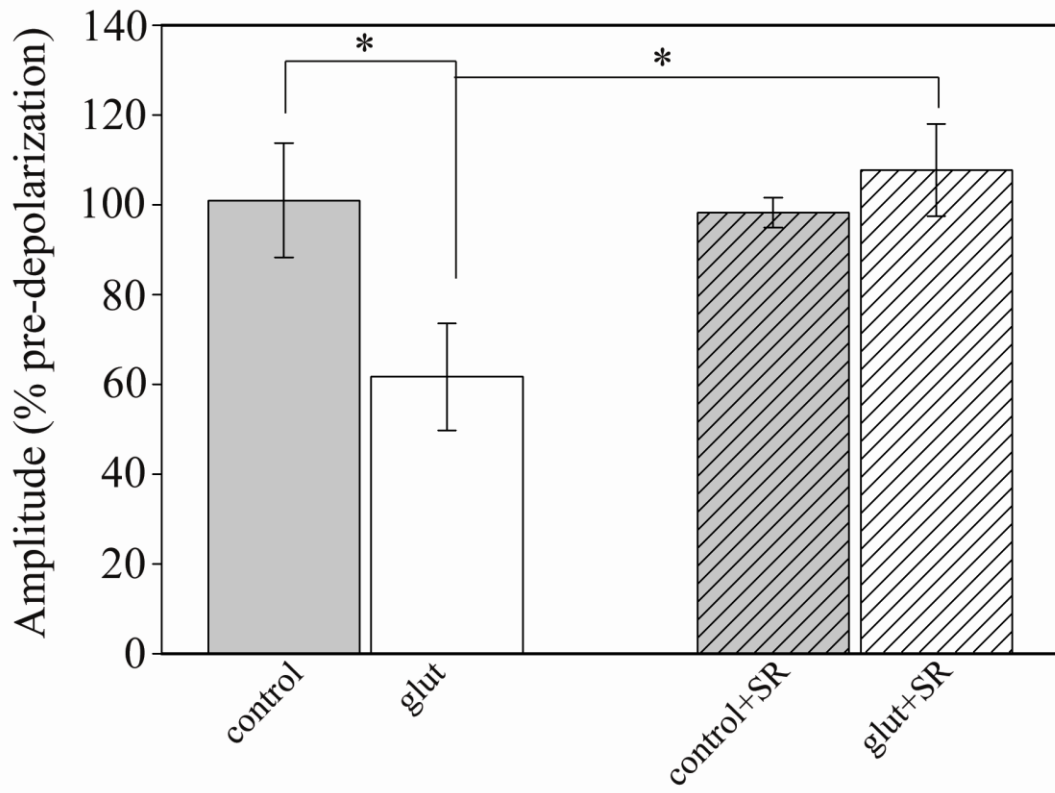
D. Discussion:

These studies were performed to investigate functional significance of cannabinoid receptor upregulation and redistribution in epilepsy. We first measured basal excitability in control and epileptic state. The results demonstrate that there is decreased inhibition and enhanced excitation in epileptic OHSCs compared to control OHSCs indicating increased excitability in epileptic condition. DSI and DSE were then measured to understand changes in activity dependent plasticity of endocannabinoid signaling. While no significant changes in DSI were observed, a significant increase in DSE was observed in epileptic OHSCs compared to control OHSCs. This suggests that following epilepsy, the CB_1 receptor redistribution on glutamatergic terminals is acting to inhibit glutamate release thereby preventing persistent excitability and its transformation into a state of SE.

These results evaluated the physiological significance of the CB_1 receptor upregulation we observed in chapter 3. In this approach we are able to further examine DSI and DSE and overall excitatory and inhibitory tone after glutamate injury. Our observation that there is relatively little DSE observed in control slices compared to glutamate injured slices is in agreement with our hypothesis that CB_1 upregulation in acquired epilepsy is a compensatory mechanism for controlling seizure activity. However, we were somewhat surprised by the conflicting results we observed while recording DSI from both spontaneous and evoked IPSCs. Our immunohistochemical data from chapter 3 showed a significant increases in CB_1 receptor staining in the stratum oriens of the CA1 region and stratum radiatum of the CA3 region (where the glutamatergic synapses are located), with no significant changes in the pyramidal cell

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regions (where the GABAergic synapses are located). The physiological data obtained in our spontaneous recordings supports the notion that these CB_1 receptor changes are functional. An increase in DSE with no change in DSI in glutamate treated slices also supports our hypothesis that the relative upregulation of CB_1 receptors after glutamate injury acts to suppress seizure activity.

However, we observed slightly different results in DSI recording with evoked currents where glutamate treated slices did not display significant DSI and controls slices saw post-depolarization amplitude of 83% of the pre-depolarization amplitude. Both control and glutamate treated OHSCs displayed a much greater depression of sIPSCs when recording spontaneous activity to 37% and 19% of pre-depolarization values, respectively. These discrepancies, however, may be inherent to the different modes of recording and reporting. In glutamate treated OHSCs, there was generally less sIPSC activity than in controls. This lack of activity may skew our spontaneous data to show a greater suppression when in fact there was just less activity overall. The DSI observed in our evoked potentials for both control and glutamate treated slices is far less than what is observed in other hippocampal studies that often observe a 50% suppression of inhibition (Alger *et al.*, 1996; Alger, 2002; Lenz and Alger, 1999). This may indicate a diminished role of the endocannabinoid system on overall GABAergic signaling in our OHSCs. It may also be that our depolarizing voltage step was too long to induce maximal DSI, as recent studies have shown greater DSI with a 250 ms step than with a 2 s step (Isokawa and Alger, 2006). A longer depolarization may shift more endocannabinoid signaling to the excitatory pathways. This phenomenon will require further future investigation.

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It has also been noted that DSI may not be the important CB_1 mediated physiological function in the control of epilepsy. Selective deletion of CB_1 receptors on GABAergic terminals failed to change susceptibility to kainic acid seizures (Monory *et al.*, 2006). In our model, it may be that DSE is the more important cannabinoid mediated effect in acquired epilepsy. In a normal hippocampus, DSE requires a longer depolarization than what may be physiologically relevant in normal tissue. However, with the prolonged depolarization that may occur in epilepsy, DSE may play a very active role in controlling seizures. Our results in both spontaneous and evoked EPSCs point to greater control of CB_1 receptor activation in inhibiting excitatory transmission in a chronic epileptic state. Thus, the increase in CB_1 receptor expression after the development of epilepsy plays a functional role in neuroprotection in this model. It has been shown that inducing DSE in hippocampal tissue is dependent on the sensitivity of the presynaptic neuron to cannabinoids as well at the duration of postsynaptic depolarization (Ohno-Shosaku *et al.*, 2002). With extended depolarization, the result of CB_1 receptor activation may be a shift from DSI to DSE. Therefore, the extended neuronal depolarization of an epileptiform discharge may cause a greater suppression of glutamate release rather than a suppression of GABA release (Wallace *et al.*, 2003).

Of course, this is not necessarily the role of the endocannabinoid system in all epilepsies. Chen et al (Chen *et al.*, 2007) have studied the CB_1 receptor in a hyperthermia model of febrile seizures. They induced febrile seizures in neonatal P3 rats and found and overall increase in CB_1 receptor expression in their brains at P10. However, paired neuron recordings revealed an increase in DSI in hyperthermia animals with no change in DSE between hyperthermia and control animals. This is in contradiction to observations

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from our study. However, age may play an important role in functional CB_1 receptor plasticity. Our study injured the tissue that had been cultured for 21 days from P8 rats, so it was roughly equivalent to neurons from a 4 week old rat (Caeser and Aertsen, 1991; Finley *et al.*, 2004; Thompson *et al.*, 2006). Interestingly, in addition to cannabinoid sensitivity, age of the animal may be an important factor in inducing DSE in healthy tissue. Several studies have shown that cannabinoids induce suppression of EPSCs occurs in young (Al-Hayani and Davies, 2000; Ameri and Simmet, 2000) or tissue cultured tissue (Sullivan, 1999), but show no effect on EPSCs in adult tissue (Stella *et al.*, 1997). This discrepancy in developmental stage at the time of injury could play a role in differential plasticity of the CB_1 receptors after injury. This may also help explain why we do not observe DSE in control tissue. The relatively small number of CB_1 receptors present at glutamatergic synapses in control tissue may not respond readily enough to display DSE in our model, whereas the upregulation of CB_1 after injury causes enough activation to display DSE as in a more juvenile state.

This study provides some important insight into the functional relationship of CB_1 receptor expression and physiological activity following the development of acquired epilepsy. Future studies of this kind are necessary in other *in vitro* and *in vivo* models to truly understand the role of the endocannabinoid system in regulating seizure activity, as they could have great clinical implications in specifically targeting receptors that control seizures.

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A. Rationale and summary of the central hypothesis

Seizure activity and epilepsy have been studied extensively in various experimental animal models using chemoconvulsants like NMDA, kainic acid, metabotropic glutamate receptor agonists and antagonists, GABA receptor antagonists or blockers (e.g. bicuculline, picrotoxin, pentylenetetrazole) and cholinergic agonists (e.g. pilocarpine), electrolytic hilar lesions or kindling, (reviewed by (Fisher, 1989)). All treatments elicit varying degrees of hippocampal cell death ranging from limited to extensive neuronal degeneration. In addition, these animal models display hippocampal structural changes with increased dentate granule cell neurogenesis (reviewed in (Parent, 2007)) and recent studies from our laboratory have also noted a significant reorganization in the cannabinoid CB_1 receptor expression in the hippocampus (Falenski *et al.*, 2007; Falenski *et al.*, 2009). Since the detailed mechanisms involved in these changes are far from known, a simplified, yet organotypic *in vitro* model for seizure activity would be helpful. The experiments performed in this dissertation have sought to establish and evaluate a new model of stroke-acquired epilepsy in organotypic hippocampal slice cultures.

Cerebral ischemia is a catastrophic event with significant morbidity and mortality. It is the leading cause of acquired epilepsy in persons 35 and older (Forsgren *et al.*, 1996)with patients who have suffered a cerebral infarct having a 17-fold greater risk of

developing recurrent seizures (Warlow *et al.*, 2003), yet little is known about the underlying basic mechanisms leading to stroke induced epilepsy. Our laboratory has previously utilized a hippocampal neuronal culture (HNC) model of stroke acquired epilepsy to study some of the molecular changes that occur in Ca^{2+} regulation in neurons following a stroke-like injury (Sun *et al.*, 2001; Sun *et al.*, 2004). Models of stroke induced epilepsy have been difficult to develop *in vivo*, but organotypic hippocampal slice cultures (OHSCs) provide and ideal substrate for developing an *in vitro* model of stroke induced AE while maintaining normal cellular connections. OHSCs have been utilized for years in various studies of stroke and seizures, though the physiological effects of a stroke-like injury have not been fully explored in this system. Slice cultures have a distinct advantage over the HNC model in that they maintain normal cellular morphology and architecture (Bahr, 1995), while allowing for easy manipulation. OHSCs have been studied extensively in oxygen-glucose deprivation and glutamate injuries to mimic the effects of ischemia. Most studies have focused on manipulating the system to prevent cell death (Lipski *et al.*, 2007; Noraberg *et al.*, 2005), without looking at the long term physiological outcomes. In addition, few studies have utilized OHSCs to produce spontaneous recurrent seizures as a model of acquired epilepsy (Bausch *et al.*, 2006; Routbort *et al.*, 1999). With this in mind, this dissertation has focused on developing a model of stroke-like injury in OHSCs to study the physiological and molecular consequences following injury.

Extensive research in animal models (Araujo *et al.*, 2010; Deshpande *et al.*, 2007b; Wallace *et al.*, 2003) along with human studies (Ludanyi *et al.*, 2008) suggest that modulation of the endocannabinoid system plays a role in epilepsy. Our laboratory has

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studied the endocannabinoid system in the rat pilocarpine model of epilepsy in great depth. Studies have found that there is a unique, functional redistribution of CB_1 receptors in the hippocampus during the development of epilepsy (Falenski *et al.*, 2007; Wallace *et al.*, 2003), with an overall increase in CB_1 receptor expression. The redistribution shown supports the hypothesis that the changes in CB_1 expression act as a compensatory mechanism to control seizures. Research also shows that the endocannabinoid system undergoes modulation in cerebral ischemia (Hillard, 2008). A model of stroke induced acquired epilepsy provides an ideal system for studying the endocannabinoid system following stroke-like injury and during epileptogenesis. OHSCs express CB_1 receptors similarly to *in vivo* tissue (Boscia *et al.*, 2008), indicating that OHSCs are an appropriate system to study CB_1 receptor changes. Based on *in vitro* and *in vivo* research in the literature and preliminary electrophysiology studies, we developed two central hypotheses for this dissertation. We hypothesized that status epilepticus and stroke like injuries cause increased neuronal excitability in organotypic hippocampal slice cultures, rendering a model of acquired epilepsy. In addition, we hypothesized that the endocannabinoid system plays a modulatory role in stroke-induced acquired epilepsy in our OHSC model. Addressing the following experimental questions in this dissertation has thoroughly tested our hypotheses.

1. Does a status epilepticus like injury induce spontaneous recurrent epileptiform discharges in our organotypic hippocampal slice cultures?

2. Does a stroke-like injury induce spontaneous recurrent epileptiform discharges in organotypic hippocampal slice cultures?

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3. Are CB_1 receptors upregulated in OHSCs following glutamate injury? What is the distribution of the CB_1 receptors that display seizure activity?

4. Is there a functional difference in CB_1 receptor activity following a stroke-like injury?

B. 4-aminopyridine exposure causes spontaneous seizure activity in OHSCs.

We have successfully cultured and maintained viable organotypic hippocampal slice cultures in our laboratory. In the first part of this thesis, we established that OHSCs in our hands would acutely respond to the K+ channel blocker 4-aminopyridine (4-AP) as previously shown in the literature (Albus *et al.*, 2008). We confirmed that 4-AP acutely caused prolonged electrographic seizures and was therefore suitable to use for a status epilepticus (SE) like injury. We therefore utilized 4-AP to induce a longer, status epilepticus like injury by including 200μM 4-AP to culture media for a 24 hour period. This led to a significant amount of cell death acutely following the injury period, but cell death was not significantly different from controls in the time periods after the injury period (5 and 14 days). The pattern of cell death appeared to mostly occur in the glutamatergic cells in the pyramidal cell and dentate granule cell layers. This follows the pattern of cell death observed in animal models of SE (Poirier *et al.*, 2000). Our findings also agree with the temporal aspect of cell death seen in other models of acquired epilepsy, wherein there is significant cell loss immediately following the injury, but not during the period of epileptogenesis (Deshpande *et al.*, 2007c; Raza *et al.*, 2004). Following prolonged treatment with 4-AP, we observed a significant increase in spontaneous seizure activity in OHSCs that underwent 4-AP treatment, with 62% of

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cultures displaying seizure activity in the CA3 region. This was significantly increased from our control slices, indicating that 24 hour treatment with 4-AP lead to an increase in spontaneous seizures 14 days later in slice cultures.

The development of this model of SE induced acquired epilepsy provides insights into the maintenance and handling of OHSCs. Our spontaneous seizure rate in this study was significantly higher than what has been reported in OHSCs treated with kainic acid in which 29% of OHSCs displayed seizures in the DG region (Bausch and McNamara, 2004), but it was not as significant as what has been observed in OHSCs treated with TTX (Bausch *et al.*, 2006) in which 89% of OHSCs displayed seizure activity following removal of chronic blockade of activity. These three different injury mechanisms provide some insight into the receptor systems that may be involved in epileptogenesis. Kainic acid acts on the kainate receptors, which play a significant role in modulating synaptic plasticity in the hippocampus (Contractor *et al.*, 2000). Prolonged agonism of this receptor did not cause the plasticity changes that resulted in a significant rate of seizures in OHSCs (Bausch and McNamara, 2004). 4-AP blocks K+ channels and though it does not act directly on NMDA receptors, 4-AP induced convulsive activity *in vivo* involves NMDA receptors, since 4-AP exposure leads to excessive release of glutamate (Fragoso-Veloz and Tapia, 1992). In our model, prolonged treatment with 4-AP induced epileptogenesis. In the final example, chronic treatment of OHSCs with TTX induces plasticity of both excitatory and inhibitory synapses. Upon removal of the blockade of action potentials, the impact of these synaptic plasticities results in spontaneous seizures (Bausch *et al.*, 2006). Of these mechanisms, the exposure to excessive glutamate may play a physiological role in many injuries known to induce epileptogenesis including

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status epilepticus and stroke.

Excessive release of glutamate is a hallmark of ischemic injury. Activation of NMDA receptors by excessive glutamate would cause an increase in intracellular Ca^{2+}, leading to activation of Ca^{2+} dependent cascades that may induce cell death or the plasticity changes of epileptogenesis. The potential role of glutamate in our OHSC model of SE acquired epilepsy requires further exploration and allows for the possibility of a stroke-like injury model of acquired epilepsy.

C. Stroke-like injury causes acquired epilepsy in OHSCs

The second set of experiments in this dissertation document a new model of epileptogenesis in OHSCs following glutamate injury. Similar to the excitotoxic injuries associated with both ischemic and anoxic stroke events, glutamate injury in this model produced a mixed population of neurons characterized by both cell survival and cell death. The pattern of cell death after glutamate injury revealed that the CA1 region was most sensitive to glutamate toxicity. This is similar to what is observed in OHSCs exposed to NMDA (Kristensen *et al.*, 2001). As in our previous excitotoxic injury in chapter 1, neurons that survived the glutamate exposure become the substrate for the development of epileptogenesis as indicated by increased excitability of the CA3 cell layer in a significant number of OHSCs. The seizure events seen in extracellular recordings expressed many characteristics of overt electrographic epileptic seizures and they responded to the anticonvulsant drugs phenobarbital and phenytoin, but not to ethosuximide. Intracellular recordings confirmed neuronal hyperexcitability in CA3 pyramidal neurons in glutamate treated OHSCs. These results demonstrated that OHSCs

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subjected to injury by glutamate exposure could be transformed into neuronal networks manifesting seizures for the life of the culture, producing an *in vitro* model of epilepsy.

Organotypic hippocampal slice cultures have been utilized in many models of excitotoxicity, including kainic acid (Routbort *et al.*, 1999), NMDA (Ring *et al.*, 2010), and oxygen glucose deprivation injuries (Lipski *et al.*, 2007). These studies have generally examined neuroprotective agents during or after an excitotoxic injury, without looking at the long term physiological consequences (Boscia *et al.*, 2006; Cho *et al.*, 2007; Lipski *et al.*, 2007; Ring *et al.*, 2010; Routbort *et al.*, 1999; Thomas *et al.*, 2005). Our model takes this a step further by characterizing the physiological changes after an excitotoxic injury with glutamate. Our model utilizes glutamate as a mode of excitotoxicity, as excessive glutamate concentration is an important aspect of the ischemic penumbra in stroke (Davalos *et al.*, 1997). Previous *in vitro* studies with a HNC model used a glutamate-induced prolonged, reversible depolarization as used in this study (Sun *et al.*, 2001; Sun *et al.*, 2004). Other models of epilepsy have implicated activation of the *N*-methyl-D-aspartate receptor (NMDAR) for epileptogenesis (Croucher *et al.*, 1988; Croucher and Bradford, 1990; DeLorenzo *et al.*, 1998; Rice and DeLorenzo, 1998; Stasheff *et al.*, 1989). Although the mechanism producing hyperexcitability in this model has not been fully delineated, it has been shown that inhibition of glutamate receptors in neurons in culture produces alterations in NMDAR subunit expression that are regulated by synaptic activity during development (Hoffmann *et al.*, 2000; Yashiro and Philpot, 2008). It is possible that alterations in NMDAR subunit expression may underlie the development of hyperexcitability in this model after over activation of glutamate receptors. Though glutamate exposure may induce changes in receptor subunit expression

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in the glutamate injury–induced epileptogenesis model, these potential changes probably occur through a separate mechanism. Further studies will delineate the receptor changes that may occur in our model.

Our model also found that there was no difference in the degree of cell death and the development of seizures. The role of cell death and the development of epilepsy requires further study as greater cell death in the excitatory neurons should lead to greater inhibitory tone (Tecoma and Choi, 1989). Further studies are needed to determine the role of selective cell death in this model and the possible roles of gap junctions, (Dudek *et al.*, 1998) ischemia-induced alterations in second-messenger systems, and gene changes (Morris *et al.*, 2000a) in mediating epileptogenesis in this model. In addition, the possible compensatory mechanisms that control hyperexcitability after an injury is another important aspect of this model that deserves further attention.

D. CB$_1$ receptors are upregulated after glutamate injury

Chapter 3 of this dissertation demonstrated that the CB$_1$ receptor undergoes changes in expression in the OHSC model of stroke acquired epilepsy. Western blot analysis found that CB$_1$ receptor expression rises to 128% of control levels following treatment with glutamate. This result supports our hypothesis that CB$_1$ receptor expression changes following an epileptogenic injury and our OHSC model has many similarities to the molecular changes observed in *in vivo* models of AE. Densitometric analysis of immunohistochemical staining of OHSCs further supported our hypothesis the the CB$_1$ receptor changes play a compensatory role in controlling hyperexcitability, as observed in the rat pilocarpine model (Falenski *et al.*, 2007; Falenski *et al.*, 2009).

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OHSCs that displayed electrographic seizures showed a specific increase in CB1 receptor staining in the stratum oriens of the CA1 and stratum radiatum of the CA3 region. This finding supports the hypothesis that the CB_1 receptor upregulation observed in Western blot analysis is specifically located at the glutamatergic terminals. CB_1 receptor distribution in OHSCs under normal conditions has been shown to be similar to what is observed in normal rats (Boscia *et al.*, 2008). Specifically, previous studies agree with images of our control OHSCs, showing the entire hippocampal slice culture decorated with a dense plexus of CB_1-immunoreactive fibers that surrounded the immunonegative principal cells, mostly located at the GABAergic terminals. The displayed upregulation further supports the hypothesis that the CB_1 receptor changes are a compensatory mechanism to control seizures. An upregulation at the glutamatergic terminals located in the stratum radiatum and stratum oriens would play a role in dampening excessive neuronal excitation.

Several studies of epilepsy have examined the importance of CB_1 receptors in controlling neuronal excitability (Araujo *et al.*, 2010; Arida *et al.*, 2005; Chen *et al.*, 2003; de Amorim Carvalho *et al.*, 2003; Ludanyi *et al.*, 2008; Monory *et al.*, 2006). These studies provide an interesting view of the role of the endocannabinoid system in epilepsy. They suggest that the upregulation of CB_1 receptors in animal models of AE might actually be a compensatory mechanism to control seizure severity after a seizure precipitating event. The studies of human tissue suggest that this compensatory mechanism does not develop in humans with intractable epilepsy. Though morphological studies can strongly support the role of CB1 receptor expression, functional studies are necessary to confirm that the upregulated receptors are indeed physiologically functional.

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In vivo studies have utilized [^{35}S] GTPγS autoradiography (Falenski *et al.*, 2007), conditional CB_1 gene deletion at glutamatergic terminals (Monory *et al.*, 2006), and the use of CB_1 antagonists (Wallace *et al.*, 2003) to assess the functionality of the CB_1 receptor reorganization following the development of epilepsy. These studies further supported the functional redistribution of the CB_1 receptors following epileptogenesis. Our OHSC model of stroke acquired epilepsy provides an additional advantage in the ability to readily assess the functionality of the observed receptor upregulation utilizing electrophysiological techniques. Functional electrophysiology experiments performed in the final chapter of this dissertation elucidate the physiological changes associated with an overall increase in CB_1 receptor expression after epileptogenic injury.

E. The CB_1 receptor upregulation has functional consequences on OHSC excitatory and inhibitory tone

The final studies of this dissertation were performed to investigate functional significance of cannabinoid receptor upregulation in epilepsy. By measuring basal excitability in control and glutamate treated OHSCs, we demonstrated that there is decreased inhibition and enhanced excitation in glutamate treated OHSCs compared to controls, indicating increased excitability in the epileptic condition. Depolarization induced suppression of inhibition (DSI) and excitation (DSE) were then measured to understand changes in activity dependent plasticity of endocannabinoid signaling. While no significant changes in DSI were observed, a significant increase in DSE was observed in glutamate treated OHSCs compared to controls. This suggests that following epileptogenesis, the CB_1 receptor redistribution on glutamatergic terminals is acting to

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inhibit glutamate release thereby preventing persistent excitability and its transformation into a state of status epilepticus.

These results evaluated the physiological significance of the CB_1 receptor upregulation we observed in chapter 3. The physiological data obtained in our spontaneous recordings supports the hypothesis that these CB_1 receptor changes are functional. An increase in DSE with no change in DSI in glutamate treated slices also supports our hypothesis that the relative upregulation of CB_1 receptors after glutamate injury is located at glutamatergic terminals and therefore acts to suppress seizure activity. Our results also support the idea that DSI is not significant in the control of epilepsy. In our model, it may be that DSE is the more important cannabinoid mediated effect in acquired epilepsy. DSE in the hippocampus has only been shown to occur with long depolarizations (Ohno-Shosaku *et al.*, 2002). This sustained depolarization may not be physiologically relevant under normal conditions, but may be of great importance during seizures. Our results in both spontaneous and evoked EPSCs point to greater control of CB_1 receptor activation in inhibiting excitatory transmission in a chronic epileptic state. In addition, studies have shown greater DSI with very short depolarizations (Lafourcade *et al.*, 2009), indicating that DSI may play a more important modulatory role in normal cell signaling without modulating pathological conditions such as epilepsy. Thus, the increase in CB_1 receptor expression after the development of epilepsy plays a functional role in neuroprotection in this model. It has been shown that inducing DSE in hippocampal tissue is dependent on the sensitivity of the presynaptic neuron to cannabinoids as well at the duration of postsynaptic depolarization (Ohno-Shosaku *et al.*, 2002). With extended depolarization, the result of CB_1 receptor activation may be a shift

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from DSI to DSE. Therefore, the extended neuronal depolarization of an epileptiform discharge may cause a greater suppression of glutamate release rather than a suppression of GABA release (Wallace *et al.*, 2003).

This study provides some important insight into the functional relationship of CB_1 receptor expression and physiological activity following the development of acquired epilepsy. Future studies of this kind are necessary in other *in vitro* and *in vivo* models to truly understand the role of the endocannabinoid system in regulating seizure activity, as this could have great clinical implications in specifically targeting receptors that control seizures.

F. Conclusion

The studies presented in this dissertation provide a powerful new model of stroke acquired epilepsy utilizing an organotypic *in vitro* system. These studies have thoroughly demonstrated the utility of organotypic hippocampal slice cultures as a system for inducing injuries that produce a model of epileptogenesis. We have demonstrated many similarities between our *in vitro* model and other *in vivo* models of acquired epilepsy. OHSCs provide several advantages over *in vivo* models in their functionality in high-throughput screening of pharmacological agents in an in-tact tissue model. We have utilized the OHSC model of stroke acquired epilepsy to further study the molecular and physiological changes of the endocannabinoid system following epileptogenesis. These studies add to the growing body of evidence that the endocannabinoid system is able to efficiently regulate presynaptic inputs onto neurons, acting as a neuromodulatory system in epilepsy. We have demonstrated that OHSCs specifically upregulate CB_1 receptors in

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the stratum radiatum and stratum oriens following a stroke-like injury with glutamate. We have also demonstrated that the CB_1 receptors on glutamatergic terminals are functional in suppressing excitatory signals to CA3 pyramidal neurons.

Findings from this study have numerous implications for persons with epilepsy. It continues to support the importance of the endocannabinoid system as an endogenous mechanism to control neuronal hyperexcitability. Indeed, animal studies have shown CB_1 receptor antagonism is detrimental in epileptic rats (Wallace *et al.*, 2003). This hypothesis was recently supported with clinical evidence utilizing the CB_1 inverse agonist Rimonabant (SR141716A). Rimonabant was marketed in Europe from 2006-2008 for the treatment of obesity. A case report of the drug chronicled a 52 year old man who had suffered two seizures as a child, but had been seizure free and without antiepileptic drugs for more than 20 years. After starting treatment with Rimonabant for weight loss, the man began suffering from nocturnal partial seizures that disappeared upon cessation of Rimonabant (Braakman *et al.*, 2009). This further supports evidence from animal models and our OHSC model of epilepsy that the distribution of CB_1 receptors in epilepsy acts to control neuronal hyperexcitability.

Results from these studies continue to support the hypothesis that development of epilepsy induces a compensatory upregulation of CB_1 receptors on glutamatergic terminals to preferentially suppress excessive neuronal excitatory signaling. This data implies that future therapeutic targets for the control of epilepsy lie in the CB_1 receptors of glutamatergic terminals. The recent development of FAAH and MAGL inhibitors provides a unique tool to study the effects of increasing concentrations of endogenously produced CB_1 agonists rather than administering exogenous agonists. However, a recent

study has found that tolerance and physical dependence develops in mice after chronic treatment with the MAGL inhibitor JZL 184, but not the FAAH inhibitor PF-3845 (Schlosburg *et al.*, 2010). Acute application of JZL 184 enhances DSE, while inhibition of FAAH has no effects on DSE(Pan *et al.*, 2009). These studies provide further evidence that 2-AG is the endocannabinoid responsible for DSI and DSE, but they raise questions of the utility of chronic treatment with MAGL inhibitors. However, these compounds have yet to be fully tested in seizure models, where we know the CB_1 receptors display a different functional distribution. A different FAAH inhibitor, AM374, has been shown to decrease seizure severity and cell damage in kainic acid induced seizures. Further evidence in animal models supports the antiepileptic effects of anandamide (Wallace *et al.*, 2002), while a recent study in humans found decreased anandamide levels in patients with temporal lobe epilepsy (Romigi *et al.*, 2010). In contrast, FAAH knockout animals have been shown to have enhanced seizures in response to kainic acid (Clement *et al.*, 2003). This conflicting data deserves greater research to delineate the role of each of the endocannabinoids in modulating seizure activity. The OHSC model of stroke-induced acquired epilepsy provides an efficient model to study these complex interactions and has the capacity to provide great insight into the utility of pharmacological manipulation of the endocannabinoid system in epilepsy.

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<u>Manuscripts:</u>

Ziobro J.M., Deshpande L.S., DeLorenzo R.J., "An organotypic hippocampal slice culture model of excitotoxic injury induced spontaneous recurrent epileptiform discharges". Brain Res. Accepted for publication.

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