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STATUS EPILEPTICUS INDUCED ALTERATIONS IN
HIPPOCAMPAL ANATOMY AND NEUROTRANSMISSION

by

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DEDICATION

I would like to dedicate this manuscript to the memory of my beautiful Grammy, Theresa M. Boileau. I am so lucky to have had such a loving, sweet and kind soul as a grandmother. Thank you for your *unending* love. Miss you forever.

This manuscript is also for my wonderful family who have always given me unconditional love and support. To my mom and dad, Brenda and Mark, for being remarkable parents and friends. Thank you for always being so incredibly supportive of the decisions I've made, for teaching me that success comes with hard work, and for always pushing me to do my best. To my little brother and sister, Danny and Melanie, thank you for being the best siblings I could have asked for. You are both amazing people and I truly value our time together and the laughs that we share. To my incredible extended family for always being so supportive- Auntie Lori, Uncle Dave, Uncle Gary, Auntie Gail, Aunt Vicki, Uncle Bob, Grandpa, Grandpa-With-The-Boots, Grandma Pam, Josh, my brother-in-law Jay, and The Adams Family *snap snap*- Ryan, Jelly, SophieAnn and Savannah. And finally to my furry friend Zeek for always putting a smile on my face after a long day. I love you all and I want to thank each one of you for making this all possible.

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"There are things known and there are things unknown, and in between are the doors of perception." ~Aldous Huxley

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ABSTRACT

Status epilepticus (SE) is a life-threatening neurologic emergency occurring when the brain is in an unrelenting state of seizure activity. Approximately 40% of people who encounter a single event of SE go on to develop epilepsy, characterized by spontaneously occurring seizures. While the exact mechanisms underlying seizure origin are not understood, at a fundamental level seizures initiate due to an imbalance between inhibitory and excitatory neurotransmission. We explored the impact of SE and the development of epilepsy on $GABA_A$ receptor mediated inhibitory neurotransmission and kainate receptor (KAR) mediated excitatory neurotransmission.

Stiripentol (STP), a positive allosteric modulator of the $GABA_A$ receptor, was found to terminate both brief and prolonged SE with the development of less pharmacoresistance than is observed with the benzodiazepine (BZD), diazepam (DZP). In addition STP, but not DZP, retained its ability to potentiate both phasic and tonic GABAergic transmission post-SE. These findings are supported by previous studies demonstrating that the actions of STP do not require the BZD-sensitive γ2-containing $GABA_A$Rs which are internalized during prolonged SE. These data demonstrate that prolonged SE significantly impacts the pharmacological profile of $GABA_A$ receptors and potential therapeutics.

KARs densely populate the hippocampal mossy fiber – CA3 pathway where they mediate a portion of excitatory neurotransmission. Significant alterations in KAR subunit expression in the dentate gyrus and CA3 regions were observed in animals at 5, 60 and 200 day post-SE. These changes were dynamic and region specific. In agreement with observed alterations in subunit expression, KAR-mediated neurotransmission was significantly reduced at mossy fiber – CA3 synapses in epileptic animals. In addition, synaptic integration by KARs during repetitive stimulation was also significantly impaired. These data demonstrate that SE significantly impacts KAR-mediated excitatory neurotransmission.

Together these studies provide new insight into the impact of SE and the development of epilepsy on both $GABA_A$ and KAR-mediated neurotransmission. The observed alterations following SE may contribute to the generation of seizures or may be compensatory mechanisms to reduce the likelihood of seizure initiation. Furthermore, these findings demonstrate the dramatic alterations observed in the diseased brain and emphasize the importance of acknowledging these differences for the development of effective therapeutics.

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LIST OF SYMBOLS

±	Plus/minus
α	Alpha
β	Beta
γ	Gamma
δ	Delta
µ	micro
Ω	Ohm
A	Amps
C°	Celsius
Ca^{2+}	Calcium
Cl^-	Chloride
CO_2	Carbon Dioxide
Hz	Hertz
K^+	Potassium
M	Molar
Mg^{2+}	Magnesium
O_2	Oxygen
n	Number of animals/samples
Na^+	Sodium
V	Volts

LIST OF ABBREVIATIONS

A/C .... Associational/commissural

aCSF .... Artificial cerebrospinal fluid

AED .... Antiepileptic drug

AHP .... Afterhyperpolarization

AMPA .... α-amino-3-hydroxy-*S*-methylisoxazole-4-propionic acid

AMPAR .... AMPA receptor

AP .... Action potential

BIC .... Bicuculline

BZD .... Benzodiazepine

CA1 .... *Cornu Ammonis* 1

CA2 .... *Cornu Ammonis* 2

CA3 .... *Cornu Ammonis* 3

Cb .... Cerebellum

CLB .... Clobazam

CNQX 6-cyano-7-nitroquinoxaline-2,3-dione

CNS .... Central nervous system

DAB .... Diaminobenzidine

$_D$-APV .... (2R)-amino-5-phosphonovaleric acid

DCG-IV .... (2S,2'R,3'R)-2-(2'3'-Dicarboxycyclopropyl) glycine

DG .... Dentate gyrus

CHAPTER 1

GENERAL INTRODUCTION

1.1 SIGNIFICANCE

Epilepsy is the fourth most common neurological disorder after migraine, stroke and Alzheimer's disease. The prevalence of the disorder is greater than that of autism spectrum disorders, cerebral palsy, multiple sclerosis and Parkinson's disease combined (Epilepsy Foundation, 1993). Despite the magnitude of the disease-awareness, understanding and treatment of epilepsy are severely limited. Epilepsy is characterized by the presence of recurring, unprovoked abnormal synchronous brain activity that results in the disruption of normal brain function (Briggs and Galanopoulou, 2011). The World Health Organization has estimated that at least 65 million people worldwide, or nearly 1% of the population, are afflicted with a type of epilepsy syndrome (England et al., 2012). Several precipitating events are linked to the development of epilepsy, including trauma, stroke and tumors (Chang and Lowenstein, 2003). However, epilepsy is considered the most challenging long-term consequence associated with status epilepticus (SE), as nearly 40% of people who endure a single event of SE develop epilepsy (Hesdorffer et al., 1998).

Status epilepticus is a neurologic emergency, characterized as a seizure that lasts longer than 5 minutes, or when two seizures occur without complete

recovery between them (Epilepsy Foundation, 1993). SE occurs with an incidence of 100,000 – 160,000 people per year in the United States (Manno, 2003), and results in death in approximately 20% of patients (Theodore et al., 1994). While SE affects people of all ages, it is considered the most common neurologic condition in childhood with an incidence of 10 – 27 per 100,000 children ranging from 1 month to 15 years of age, with the majority of events occurring in children less than four years of age (DeLorenzo et al., 1996; Hesdorffer et al., 1998; Coeytaux et al., 2000; Chin et al., 2006; Govoni et al., 2008). Both the etiology (Huff and Fountain, 2011) and duration of SE dramatically impacts the prognosis, with a longer duration of SE correlated to increased morbidity and mortality (Pellock et al., 2004).

Through a combination of anti-epileptic drugs (AEDs) and/or brain surgery, adequate control of seizures is obtained in nearly 60% of patients (Jacobs et al., 2009). However, nearly 20 – 40% of patients diagnosed with epilepsy will develop refractory status epilepticus (RSE), and will be unable to manage their seizures with available AEDs (French, 2007). Patients suffering from RSE have increased hospitalization and more frequent medical complications (Mayer et al., 2002). There are a wide variety of factors that contribute to the development of RSE, including age of onset, seizure etiology, infection, as well as genetic and environmental factors (French, 2007). In addition, in both SE-experienced patients (Grigorenko et al., 1997; Mathern et al., 1998; Loup et al., 2000; Das et al., 2012; Houser et al., 2012) and animal models of SE (Lason et al., 1997; Mikuni et al., 1999; Porter et al., 2006; Goodkin et al.,

2008; Sun et al., 2009; Rajasekaran et al., 2012), both inhibitory and excitatory receptor populations are altered, a consequence of SE that may dramatically impact the effectiveness of prescribed AEDs and contribute to RSE. Despite these changes, little is known about how these alterations in receptor populations affect receptor pharmacology and neuronal signaling in the SE-experienced brain. Understanding these alterations is crucial to being able to develop effective therapies for the treatment of status epilepticus and the progression to spontaneously occurring seizures.

At the core of status epilepticus is the inability of the inherent cellular mechanisms to terminate seizure activity (Coulter and DeLorenzo, 1999). These sustained seizure events can yield devastating consequences including becoming refractory to available AEDs and the increased risk for the development of epilepsy.

The following studies provide new evidence regarding how status epilepticus alters hippocampal inhibitory and excitatory receptor populations, and the physiological impact of these alterations on neuropharmacology and neuronal signaling.

1.2 EPILEPSY

The International League Against Epilepsy and the International Bureau of Epilepsy have defined epilepsy as a disorder of the brain characterized by an enduring predisposition to the generation of epileptic seizures. In addition neurobiological, cognitive, psychological and social consequences associated

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with the disease have been identified. In accordance with a variety of diagnostic tests, the diagnosis of epilepsy requires the occurrence of at least one epileptic seizure, defined as a transient occurrence due to abnormal, excessive or synchronous brain activity (Fisher et al., 2005). Epilepsy is a heterogeneous disease with no obvious geographic, racial or social boundaries. Despite its diversity, epilepsy tends to be more frequent within the African American population, slightly more common in men than women, and is more commonly diagnosed in infants and adults over 65 years of age (Theodore et al., 2006). Epilepsy is believed to affect nearly 65 million people worldwide, and may affect as many as 2.8 million Americans (England et al., 2012). In the United States the total annual hospital charges billed for the treatment of epilepsy totaled nearly $3 billion (Vivas et al., 2012). However the annual economic burden of the disease, including lost employment, wages and productivity, has been estimated at $17.6 billion. In addition to the economic burden, the diagnosis of epilepsy carries with it an increased risk for the development of social stigma and severe comorbidities.

1.2.1 EPILEPSY STIGMA

The earliest written description of seizure disorders dates back to nearly 2000 B.C. in the region known as Mesopotamia, while the first medical documentation of the disease was crafted by Hippocrates in 400 B.C. (Magiorkinis et al., 2010). Despite the vast amount of time in which people have known about epilepsy the lack of understanding and knowledge about the

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disease led people to believe that seizures were the result of demonic possession, sin and even lunacy. Despite the efforts of modern scientific and medical research to cast light on the disease many of the original social stigmas continue to impact epilepsy patients, caregivers and family members. In addition, patients who feel they experience the stigma often report increased levels of depression and anxiety, reduced self-esteem, and an overall decrease in quality of life (Jacoby and Austin, 2007). In addition to the social stigma associated with the disease, people suffering from epilepsy have difficulty obtaining driver's licenses, proper medical insurance, and face limited job opportunities as careers in teaching, medicine, and police or fire enforcement may be restricted (Jacoby and Austin, 2007). In order to reduce the stigma associated with epilepsy the scientific community must join efforts with the millions of people suffering from seizure disorders and begin to raise public awareness and knowledge about the disease.

1.2.2 COMORBIDITIES OF SEIZURE DISORDERS

While people suffering from epilepsy continue to face the stigma associated with their disease they are also at risk for a myriad of moderate to severe psychiatric, cognitive and behavioral comorbidities. For decades it was believed that these comorbid conditions were side-effects of the seizures, however more recent data suggest that some conditions may precede the seizures. In addition, control of seizures through available AEDs may not alleviate the symptoms associated with the comorbid conditions. Comorbidities

associated with seizure disorders require adequate attention as these conditions may be more severe and debilitating than the seizures themselves. Alterations in comorbidities are also observed during the progression of epilepsy and have been correlated with age of onset, seizure frequency and duration of seizures (Jacobs et al., 2009).

Psychiatric and cognitive comorbidities are commonly observed in patients suffering from seizure disorders. Diagnosis of psychiatric comorbidities including depression, anxiety, mood disorders and attention-deficit hyperactivity disorder are increased in epileptic patients (Lin et al., 2012). Of these conditions depression is the most common with some studies reporting upwards of 55% of patients suffering from depressive like symptoms (Jackson and Turkington, 2005). Depression is not a uniform comorbidity in epilepsy, as it appears to be more common in complex partial (temporal lobe) seizures, than in generalized seizure disorders (Jackson and Turkington, 2005). In addition to depressive symptoms, suicide is also increased from 1.4% in the non-epileptic population to nearly 5% in epileptic patients (Jackson and Turkington, 2005).

As observed with psychiatric comorbidities, the impact of seizures on cognition appears to be related to seizure etiology, frequency, duration and severity (Saling et al., 1993; Jones-Gotman, 2000; Helmstaedter and Kurthen, 2001; Jokeit and Ebner, 2002; Aldenkamp and Arends, 2004; Dodrill, 2004). In addition cognitive impairments are more prevalent in patients suffering from temporal (van Rijckevorsel, 2006) and frontal lobe epilepsies (Hernandez et al., 2003). Commonly reported symptoms include impairments in memory, attention

and a general mental slowness (van Rijckevorsel, 2006). More recently, it has become evident that impaired cognitive abilities may precede the development of epilepsy, as studies have demonstrated academic underachievement in children (Austin et al., 2001; Dunn et al., 2002; Oostrom et al., 2003; Hesdorffer et al., 2004; Berg et al., 2005) as well as adults (Hesdorffer et al., 2000; Hesdorffer et al., 2006) prior to seizure onset. Furthermore, commonly prescribed AEDs may initiate comorbid side-effects or exacerbate existing symptoms, as they have been shown to negatively impact cognition, as well as increase depression, anxiety and nervousness (Bootsma et al., 2006; Meador, 2006; Schmitz, 2006; Gomer et al., 2007).

1.2.3 SEIZURE CLASSIFICATION

In April of 1964, led by Henri Gastaut, several European branches of the International League Against Epilepsy met to develop an international classification of epileptic seizures. This initial system has been continuously amended and improved as knowledge and understanding of epilepsy and differences in seizure types emerged. In 1981 a detailed version of the classification system, which included seizure video and electroencephalogram (EEG) recordings, was approved by the General Assembly of the International League Against Epilepsy and has become the hallmark for seizure classification by clinicians.

As approved in 1981, seizures can be classified into two broad categories, partial (also referred to as focal) and generalized seizures. Partial seizures

account for approximately 60% of new cases of epilepsy (Hauser et al., 1991), and generally only impact a specific region of the brain. Depending upon the brain region affected symptoms may impact motor, cognitive, sensory, or autonomic abilities. Partial seizures are further classified depending upon alterations in consciousness. Consciousness is not altered in simple partial seizures; however it is lost during complex partial seizures, also referred to as temporal lobe or psychomotor seizures. These types of seizures occur in about 35% of patients, who have no memory of the event and usually report feelings of confusion and tiredness following the seizure event (Kammerman and Wasserman, 2001). Given that partial seizures occur in a specific region of the brain, they do have the ability to spread throughout the brain, a term referred to as secondary generalization.

The second seizure category is referred to as generalized seizures (primary generalized) and account for approximately 40% of epilepsy cases (Hauser et al., 1991). Unlike partial seizures, which are focal in nature, generalized seizures affect the entire brain, specifically the motor cortex and involve a loss of consciousness and impaired memory of the event. Six types of generalized seizures have been described. However, the most common is the grand-mal (tonic-clonic) seizure, which accounts for about 25% of all generalized seizures. Other types of generalized seizures include petit-mal (absence), myoclonic, clonic, tonic, and atonic. Each of the six types of generalized seizures is accompanied by distinct physical characteristics and movements by the patient (Kammerman and Wasserman, 2001).

Both partial and generalized seizures are considered self-limiting, as they will terminate within seconds to minutes of onset. However a separate category of seizures exists, those referred to as continuous seizures or SE. Similar to self-limiting seizures, SE can also be divided into two broad categories; general convulsive SE (GCSE) and non-convulsive SE (NCSE). GCSE is the most common form of SE, associated with higher morbidity and mortality and characterized by continuous, rhythmic movements. In contrast, NCSE manifests as confusion, psychosis without overt alterations in motor activity. Patients with GCSE or NCSE will demonstrate bilateral impairments on EEGs, however due to the lack of physical manifestations in NCSE an EEG is required for this diagnosis. Figure 1.1 serves as a modified diagram illustrating the different seizure categories discussed.

The current project will focus on general convulsive status epilepticus and the progression to spontaneously occurring seizures.

1.3 STATUS EPILEPTICUS

Status epilepticus (SE) is classically defined as the occurrence of two or more seizures without recovery of consciousness or 30 minutes of continuous seizure activity. However, due to the seriousness of SE more recent literature has reduced this time to merely 5 minutes of seizure activity (Huff and Fountain, 2011). Unlike more classic seizure disorders, SE represents the inability of inherent cellular mechanism to terminate the seizure (Coulter and DeLorenzo, 1999), and thus the seizure persists. This sustained seizure activity suggests that
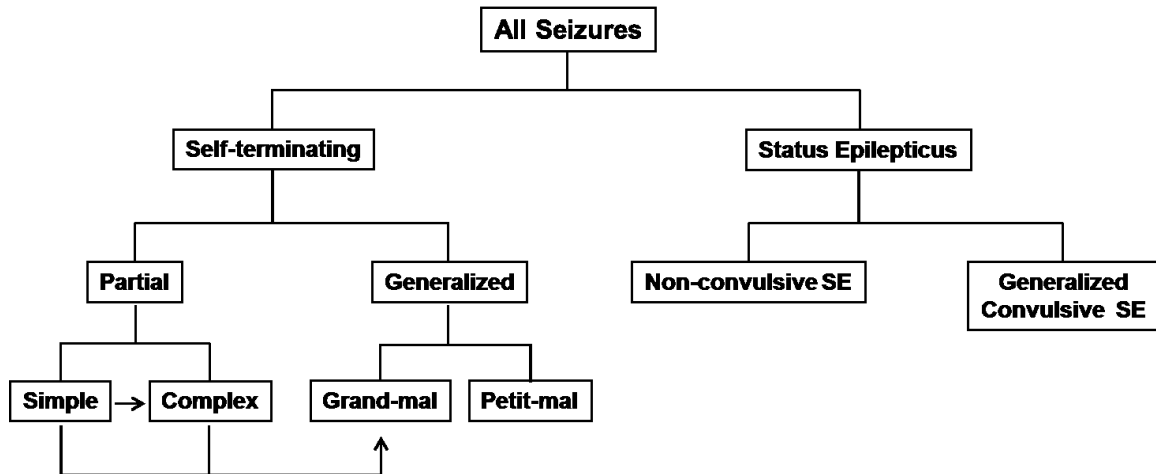
Figure 1.1. Seizure classification. Two broad categories of seizures exist, those that are self-terminating and those that are sustained, referred to as status epilepticus (SE). Self-terminating seizures can be further classified into partial and generalized seizures. Partial seizures are localized to a particular brain region whereas generalized seizures can affect the entire cortex. These categories are further identified based upon behavioral consequences including loss of consciousness. Partial seizures can spread to more severe generalized seizures. Sustained seizures, SE, can be subdivided into non-convulsive SE (NCSE) and generalized convulsive SE (GCSE). Additional types of SE exist but are not easily classified into NCSE or GCSE categories.

SE is composed of both an activation and a maintenance phase (Mazarati et al., 1998). The proposed phases of SE are likely regulated by the several different receptor populations which can mediate excitatory and inhibitory neurotransmission.

In the United States there are approximately 150,000 cases of SE each year (Chapman et al., 2001), of which at least 50% are diagnosed as the more severe GCSE. SE is associated with nearly a 20% mortality rate, a figure even higher in cases of RSE (Aminoff and Simon, 1980; Towne et al., 1994). In 20 – 40% of patients a single SE event eventually leads to the development and diagnosis of epilepsy (Hesdorffer et al., 1998; Sankar et al., 2000). While 50% of SE cases occur in patients with no known seizure disorder, a previous diagnosis of epilepsy does significantly increase the risk of SE (Hesdorffer et al., 1998), with approximately 15% of epilepsy patients experiencing an episode of SE during their lifetime (Trinka et al., 2012). While risk factors for the development of SE are limited it is acknowledged that one factor is age, as approximately 40% of SE patients are under 2 years of age and the greatest incidence of SE occurs in adults over 60 years of age (Shinnar et al., 1997). Additional risk factors include possible genetic factors and lower socioeconomic standing.

There are two general categories of underlying causes of SE, acute and chronic. Acute etiology includes an imbalance in electrolytes, drug toxicity, infection of the central nervous system (CNS), cerebral trauma, etc., while more chronic etiologies include a pre-existing seizure disorder, alcoholism, cerebral tumors or lack of AED compliance (Trinka et al., 2012). Understanding the

possible origin for the development of SE greatly influences potential treatment protocols for the patient.

1.3.1 SEIZURE MECHANISMS

At a basic level seizures initiate due to abnormal neuronal firing or imbalance within the inhibitory and excitatory systems of the brain. Due to the highly interconnected network of the brain, epileptiform activity in one area or within one population of neurons can easily spread to neighboring areas and may eventually alter transmission throughout the entire cerebrum. The majority of seizures are considered self-limiting, in that within seconds to minutes the seizure will terminate without the need for intervention. However when a seizure does not self-terminate it may continue indefinitely, a neurologic emergency referred to as SE. The exact mechanism by which seizures terminate is not clear. It is postulated that mechanisms involved in seizure termination occur at the level of a single neuron or within a network of neurons, and that in some individuals these mechanisms may simply fail (Lado and Moshe, 2008).

One of the most critical mechanisms involved in regulation of neuronal firing is proper regulation of the afterhyperpolarization (AHP) which occurs at the level of the single neuron. Action potential invasion into a neuron produces an increase in intracellular Ca^{2+} which activates voltage-gated K^+ channels that allow for K^+ to flow out of the cell. This efflux of K^+ leaves the intracellular space more negatively charged and the cell hyperpolarizes, which prevents further excitability (Alger and Nicoll, 1980; Timofeev et al., 2004). Network regulation of

seizures includes depletion of synaptic glutamate which serves to limit burst activity (Staley et al., 1998), and acidification of both the intracellular and extracellular spaces due to the increase in CO_2 levels during prolonged seizure activity (Chesler and Kaila, 1992). Network synchronization, a hallmark of seizures, is partly regulated by interneuronal gap junctions (Mancilla et al., 2007). The acidification during a seizure also results in the decoupling of these gap junctions, which serves to limit neuronal discharge and network synchronization (de Curtis et al., 1998). There are many different mechanisms that serve to limit seizure activity, any number of which could be altered resulting in prolonged status epilepticus.

1.3.2 TREATMENT OF REFRACTORY STATUS EPILEPTICUS

A major component to successful termination of SE is the ability of the patient to receive rapid treatment. It has been demonstrated that treatment even prior to arrival at a hospital is critical (Pellock et al., 2004). In a study investigating seizure treatment it was determined that over half of patients who were not successfully treated prior to a hospital were twice as likely to require intensive care (Alldredge et al., 2001). Fast and effective treatment of SE is so essential that standard treatment algorithms have been developed that indicate the hierarchy of available drugs (Pellock et al., 2004). In agreement with clinical studies indicating the need for pre-hospital treatment of SE, treatment algorithms begin with conventional antiepileptic drugs (AEDs) including benzodiazepines

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(BZDs) and advance to the use of barbiturates and even anesthesia in extreme cases.

BZDs, including diazepam, are considered the first line of treatment of SE. To achieve the highest rate of seizure termination BZDs should be administered within minutes of seizure onset. One rationale for immediate treatment is that while BZDs are considered the safest of the available AEDs they have been shown to lose effectiveness during the progression of SE (Walton and Treiman, 1988; Kapur and Macdonald, 1997; Jones et al., 2002; Grosenbaugh and Mott, 2013). Patients who develop pharmacoresistance to BZDs and other medications may develop RSE. Despite adequate treatment with available AEDs nearly 45% of patients with SE will develop RSE, which brings with it an increased risk for a variety of neuropathological consequences including death in nearly 25% of cases (Mayer et al., 2002; Holtkamp et al., 2005; Rossetti et al., 2005). In cases of RSE when available AEDs are ineffective patients are often put into medically induced comas through exposure to a variety of anesthetics (Knake et al., 2009).

Chapter 3 will explore the use of stiripentol, a novel AED, for the treatment of BZD-refractory status epilepticus.

1.4 THE HIPPOCAMPUS

The term 'hippocampus', derived from the Greek word for seahorse, was first coined by anatomist Giulio Cesare Aranzi in 1587. Investigation into this newly discovered structure continued and by the mid 1900's it was evident that

the early neuroanatomists such as Camillo Golgi, Karl Schaffer, and Santiago Ramon y Cajal, where incredibly accurate in their initial description and depiction of this region. In fact a rendering of the hippocampus by Ramon y Cajal in his book *Histologie du Systeme Nerveux* (1911) is widely used today. Despite a rapidly growing understanding of the anatomy and structure of the hippocampus the function of this region was not as clear. This changed in 1957 with the patient H.M. who underwent a bilateral mesial temporal lobectomy in which his hippocampus, among other limbic structures, was removed in an attempt to cure his epilepsy. The studies and observations of H.M. by William Scoville and Brenda Milner (Scoville and Milner, 1957) concluded that while the surgery was successful in reducing the seizures, the patient was left with global amnesia. This was the first time a direct link between memory and the hippocampus had been detected as prior to this event the hippocampus was believed to be involved in the limbic 'emotional' circuitry of the brain.

The connection between the hippocampus and memory is nearly universally agreed upon, despite discrepancies regarding its precise role. However, it is generally accepted that the hippocampus is critical for both declarative memory and spatial memory (Burgess et al., 2002). Declarative, or explicit, memory is thought to include both episodic memory which is the capacity to remember personal experiences, as well as semantic memory which involves factual knowledge related to meanings and general concepts. As the name would indicate, spatial memory involves encoding information regarding one's spatial orientation and environment. Consistent with the understanding of the

hippocampus's role in spatial memory is the presence of hippocampal 'place cells' that encode an animal's location independent of its orientation (O'Keefe, 1976; Wilson and McNaughton, 1993; Muller et al., 1994). In addition to encoding specific types of information the hippocampus is also involved in memory retrieval. While research in this area is complicated it is believed that damage to the hippocampus, including lesions, sclerosis or atrophy, results in the inability to form new memories (anterograde amnesia), as well as difficulty recalling events just prior to the injury (temporally graded retrograde amnesia) (Squire, 1992; Hodges, 1994; Squire and Alvarez, 1995).

The importance and central role for the hippocampus in learning and memory is without question. In accordance, hippocampal atrophy or sclerosis is a hallmark of many diseases linked to severe cognitive impairment, including Alzheimer's disease (Jack et al., 1999; Wang et al., 2006), schizophrenia (Heckers, 2001), and as is the focus of this document, epilepsy (Chang and Lowenstein, 2003). The ability to develop therapeutics for the treatment of these diseases and their severe comorbidities (i.e. cognitive impairment) likely lies within our understanding of the hippocampus. As this region is highly organized and relatively encapsulated its anatomical structure and circuitry in the healthy brain has been well characterized.

1.5 HIPPOCAMPAL ANATOMY AND CIRCUITRY

The hippocampal formation lies deep within the mesial temporal lobes and is just one of several brain structures located within the limbic system. The

hippocampal formation contains several brain structures including the presubiculum, parasubiculum, entorhinal cortex (EC), subiculum, dentate gyrus (DG), and hippocampus proper. The hippocampus proper is composed of three distinct cellular subdivisions; CA1, CA2 and CA3, where 'CA' comes from the term *cornu ammonis* or Ammon's horn. Most commonly, and throughout this document, the term hippocampus refers collectively to the dentate gyrus and to areas CA1, CA2, and CA3.

A unique feature of the hippocampus is the highly organized and unidirectional flow of information. This feature is apparent in what is referred to as the 'trisynaptic circuit'. The EC is considered the first step in this circuit as the axons from cells in the EC project into the dentate gyrus through the perforant path. The axons of the principal cells within the dentate gyrus are called mossy fibers which then connect the dentate gyrus to area CA3. In turn, the CA3 pyramidal cells serve as the major source of input into area CA1 through the Schaffer collaterals. Finally CA1 projections exit the hippocampus as they enter the subiculum (Figure 1.2). This trisynaptic pathway emphasizes the concept that the major source of input into the hippocampus comes from within the hippocampus itself.

1.5.1 DENTATE GYRUS ANATOMY AND CIRCUITRY

The dentate gyrus (DG) is the first hippocampal structure to receive input directly from the entorhinal cortex and is classically comprised of three distinct layers. The most superficial layer, located closest to the hippocampal fissure, is
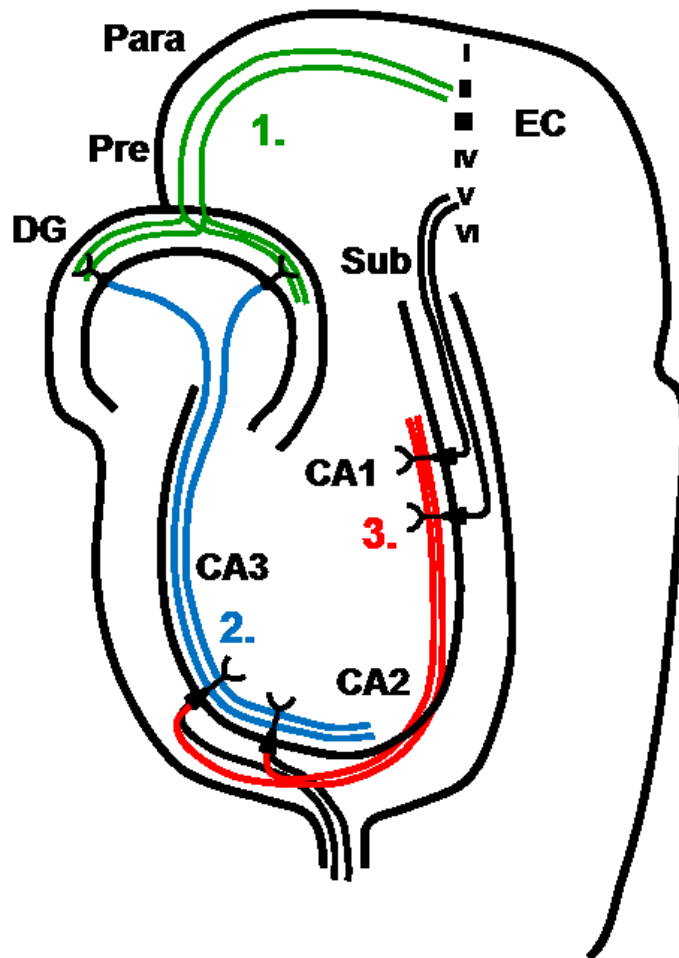
Figure 1.2. Hippocampal organization and the trisynaptic pathway.
Transmission from layer II of the entorhinal cortex (EC) enters the dentate gyrus
(DG) through the perforant pathway (*1. green*). Signaling continues from the DG
into area CA3 via the mossy fiber pathway (*2. blue*). From area CA3 signaling
persists into area CA1 (*3. red*) before exiting the hippocampus through the
subiculum (Sub) and terminating in layer V of the EC. In addition, layer III of the
EC can directly synapse in area CA1, creating the temporoammonic pathway,
and the perforant pathway can terminate directly in area CA3 (not shown).

This study will explore the impact of SE on transmission from the perforant
pathway into the DG as well as alterations in the mossy fiber – CA3 pathway.

the molecular layer, *stratum moleculare*. This layer is subsequently divided into the inner and outer-layers, each receiving slightly different inputs from the EC. The principal cells of the DG are termed granule cells and form a very dense cellular layer, *stratum granulosum*. The cell layer located between regions CA1 and CA3 is termed the suprapyramidal blade, while the layer opposite of this, which extends beneath area CA3 is the infrapyramidal blade. The U-shaped structure of these blades encompasses the polymorphic layer, commonly referred to as the hilus, which serves as the third layer of the dentate gyrus.

Dentate granule cells (DGCs) are formed within the ventricular germinative layer of the hippocampus, located near area CA1. During embryonic development newly generated DGCs can migrate directly to *stratum granulosum*, or into a second germinative layer located in the hilus prior to entering *stratum granulosum*. Later during postnatal development and during the process of neurogenesis, newly generated DGCs enter the granule cell layer through the hilar germinative layer. The morphology of granule cells is highly conserved, despite differences in how they may enter the cell body layer and which blade of the DG they are located along. Granule cells display spiny dendrites that project toward the superficial portion of the molecular layer, with the dendrites terminating near the hippocampal fissure. The majority of granule cell axons, termed mossy fibers, project to area CA3, while axon collaterals innervate mossy cells located in the hilus. Mossy cells reside in the hilar region of the DG, and have axons that project to both the ipsilateral and contralateral inner molecular layer (associational/commissural pathways). In addition, the proximal dendrites of

the mossy cells are covered in large complex spines called thorny excrescences that serve as the termination site for the mossy fibers. These neuroanatomical projections within the dentate gyrus are illustrated in Figure 1.3.

1.5.2 CA3 ANATOMY AND CIRCUITRY

Similar to the dentate gyrus, areas CA1 – 3 are comprised of principal cells and are composed of several distinct cellular layers. While the principal cells of the DG are called granule cells, in areas CA1 – 3 they are referred to as pyramidal cells (i.e. CA3 pyramidal cells). In each of these regions pyramidal cells are very tightly packed to form the pyramidal cell layer, *stratum pyramidale* (SP). Located deep to the pyramidal cell layer is *stratum oriens* (SO), which contains several types of interneurons. SO also contains the CA3 – CA3 associational fibers, which provide input from CA3 neurons to other CA3 neurons in the same hemisphere, as well as a portion of the Schaffer collaterals that connect CA3 and area CA1. Directly above the CA3 pyramidal cell layer is *stratum lucidum* (SL), which is composed of mossy fibers originating in the DG. *Stratum lucidum*, meaning "clear layer" is appropriately named, as the unmyelinated mossy fibers and lack of cells within this layer contribute to its translucent appearance. Superficial to SL is another distinct cellular layer referred to as *stratum radiatum* (SR). In areas CA2 and CA1, SR is located directly above the pyramidal cell layers, as CA3 is the only region to contain *stratum lucidum*. Similar to SO, SR also contains interneurons, CA3 – CA3 associational fibers and a portion of the Schaffer collaterals entering
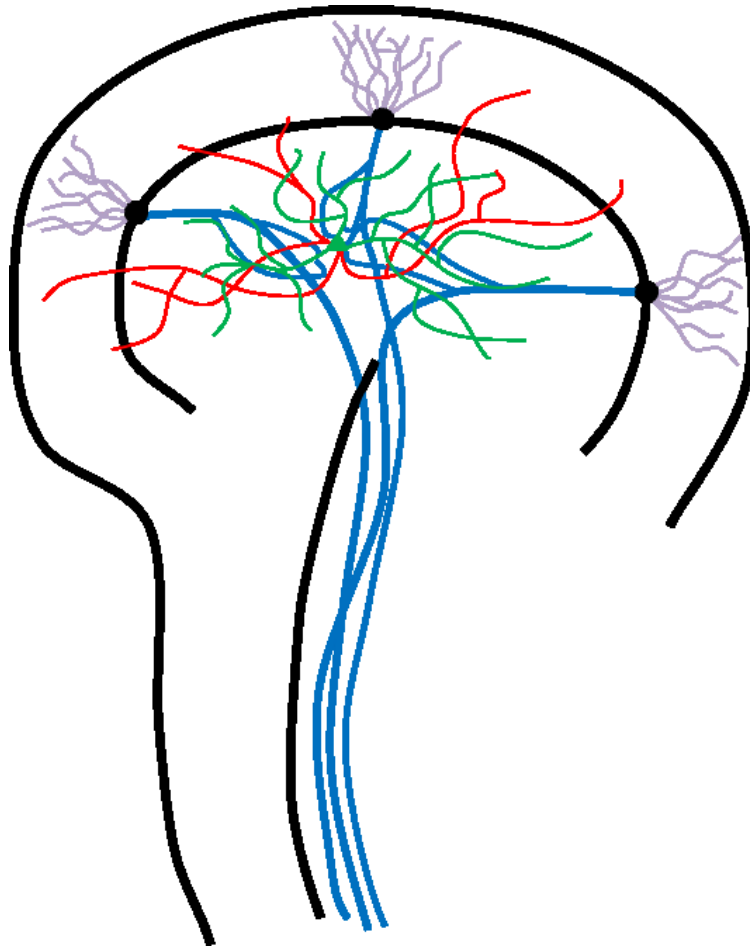
Figure 1.3. Anatomical projections of the dentate gyrus. Dentate granule cells (*black circles*) reside in the dentate granule cell layer and have dendrites that extend into the molecular layer (*purple*). The axons of granule cells, termed mossy fibers (*blue*) travel through the hilus and terminate in area CA3 *stratum lucidum*. Mossy fibers can also synapse on hilar mossy cell dendrites (*green*), which extend throughout the hilar region. The axons of mossy cells (*red*) terminate in both the ipsilateral and contralateral inner molecular layer.

area CA1. The most superficial layer of the hippocampus is *stratum lacunosum-molleculare* (SLM), which is the thin layer in which fibers from the entorhinal cortex terminate and serves as the home for several different types of interneurons.

Pyramidal cells, like DGCs, originate in the ventricular germinative layer of the hippocampus and migrate into their respective regions during embryonic development. Mature pyramidal cells located in area CA3 (as well as CA2 and CA1) are composed of a cell body which lies within the pyramidal cell layer, basal dendrites that extend into *stratum oriens* and apical dendrites that extend to *stratum lacunosum-moleculare*, near the hippocampal fissure. The axons of these cells bifurcate and often send a branch of the axon back towards the soma of the neuron. In area CA3 these axon collaterals project to areas CA1 – CA3 of both the ipsilateral and contralateral hemispheres, referred to as the associational and commissural pathways, respectively. In addition to these abundant projections, a smaller number of CA3 axon collaterals also terminate within the hilar region of the DG. Together, this extensive network of excitatory CA3 projections is believed to make up approximately 80% of all intrahippocampal synapses (Amaral and Witter, 1989). These complex neuroanatomical projections through area CA3 are depicted in Figure 1.4.

1.5.3 MOSSY FIBER PATHWAY

Projections stemming from area CA3 pyramidal cells overwhelmingly contribute to the intrahippocampal network. In order for this to occur it is essential
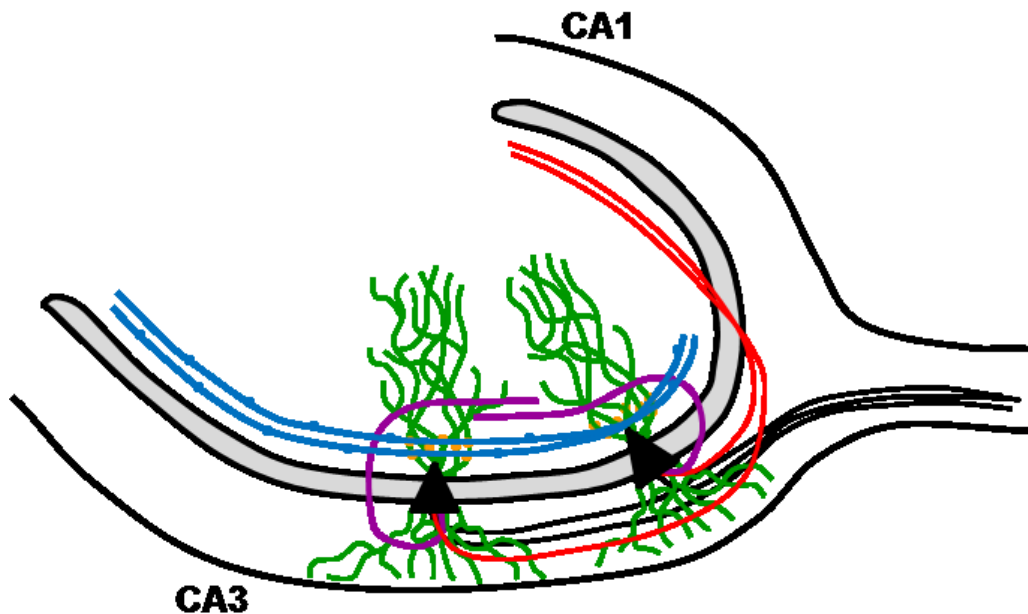
Figure 1.4. Anatomical projections of area CA3. CA3 pyramidal cells (*black triangles*) reside in the pyramidal cell layer. The apical dendrites of these pyramidal cells extend into *stratum lacunosum-moleculare* while the basal dendrites extend into *stratum oriens (green)*. Collateralized axons of CA3 pyramidal cells extend through *stratum oriens* before terminating in area CA1 creating the Schaffer collaterals (*red*). Axons also give rise to associational (*purple*) and commissural (*black*) projections between the ipsilateral and contralateral hippocampi, respectively. Mossy fibers, comprised of large *en passant* terminals (*blue fibers and circles*), originate from dentate granule cells and synapse on the large thorny excrescences located on the apical dendrite of CA3 pyramidal cells (*orange circles*).

that the dentate gyrus effectively communicate with area CA3. The manner in which this occurs is through the mossy fiber pathway, first documented in the late 1800s (Golgi, 1886; Sala, 1891). In rats, each of the approximately one million granule cells (approximately 15 million in humans) produces a single mossy fiber that travels into area CA3 through *stratum lucidum*. Prior to entering area CA3 each mossy fiber gives rise to roughly 7 collaterals that synapse onto hilar mossy cells which mediate both inhibitory and excitatory transmission. A distinct characteristic of mossy fibers is that unlike any other hippocampal principal cell, granule cells have more than one type of terminal along their axons (Ramón y Cajal, 1911). The first type is the large terminals that occur along the length of the axon and so were given the name 'en passant' meaning 'in passing'. Each mossy fiber is believed to contain approximately 15 of these large (3 – 8 µM in diameter) en passant terminals, also referred to as mossy fiber expansions. Each expansion forms highly complex and intertwined connections with the thorny excrescences located on hilar mossy cells and the proximal dendrites of CA3 pyramidal cells. Furthermore, a single mossy fiber expansion can make as many as 37 synaptic contacts with a single dendrite from a CA3 pyramidal cell. However, typically these large expansions do not contact two different spines on a single dendrite and thus do not contact two different pyramidal cells. In addition to the large en passant terminals that synapse onto mossy cells and CA3 pyramidal cells, mossy fibers also contain smaller en passant terminals and filopodial extensions that innervate GABAergic interneurons (Acsady et al., 1998).

The expansiveness and intricacy of the mossy fiber pathway allows for a single granule cell to communicate with up to 15 CA3 pyramidal cells, and with a single CA3 pyramidal cell receiving input from nearly 72 dentate granule cells. Furthermore, as DGCs are continuously generated through the process of neurogenesis, each newly formed granule cell also generates a newly formed mossy fiber. Thus the mossy fiber pathway would be highly plastic and undergoing constant reorganization. The unique ability for the dentate gyrus and CA3 to communicate via the mossy fiber pathway has drawn considerable attention in an attempt to understand its role in hippocampal synaptic transmission.

1.6 MOSSY FIBER SYNAPTIC PLASTICITY

Synaptic transmission at the mossy fiber – CA3 synapse is predominately regulated by the excitatory neurotransmitter glutamate. Glutamate is contained in vesicles located within the presynaptic terminal which upon release activate postsynaptic glutamate receptors such as NMDA, AMPA and/or kainate receptors. These synapses have the ability to modify their strength and efficacy through an activity-dependent manner, a phenomenon referred to as synaptic plasticity. Synaptic plasticity is a hallmark of the mossy fiber – CA3 synapse and is believed to be essential in the ability to form memories, a function of the hippocampus as a whole. Activity at these synapses can either enhance or depress synaptic transmission, alterations can persist for milliseconds to minutes (short-term plasticity) or may continue for hours or days and in some cases may

become permanent (long-term plasticity). Two components of short-term plasticity include paired pulse facilitation and frequency facilitation. The strength of this form of plasticity is a distinguishing characteristic of the mossy fiber – CA3 synapse and will be discussed in detail.

1.6.1 SHORT-TERM PLASTICITY

The process of neurotransmission is initiated when an action potential (AP) invades the presynaptic terminal resulting in its depolarization. This depolarization allows for voltage-gated Ca^{2+} channels to open and Ca^{2+} to enter the presynaptic terminal. The increase in the internal Ca^{2+} concentration initiates a process in which presynaptic vesicles fuse with the membrane and release their contents (i.e. glutamate) into the synapse to active post-synaptic receptor populations. If two APs occur close in time (milliseconds), the second AP may result in enhanced transmitter release, due to the build-up of residual Ca^{2+} in the presynaptic terminal, a phenomenon referred to as paired-pulse facilitation (PPF) (Katz and Miledi, 1968; Zucker, 1989). The ratio of PPF, or paired pulse depression (PPD) is inversely related to the presynaptic release probability (Dobrunz and Stevens, 1997). A synapse that demonstrates PPF would be suggestive of a low basal release probability in that a second stimulus is required to increase both internal Ca^{2+} stores and subsequent transmitter release. The mossy fiber – CA3 synapse has a low probability of transmitter release (Jonas et al., 1993; Lawrence et al., 2004), and as would be expected demonstrates PPF. However, PPF at this synapse is 2 – 3x greater than what is observed at other

synapses in the brain (Salin et al., 1996).

A second form of short-term plasticity is frequency facilitation, in which low stimulus frequencies enhance synaptic transmission and synaptic strength. Increasing the frequency of stimulation at the mossy fiber – CA3 synapse from a basal 0.05Hz to 0.2Hz stimulation dramatically increases the amplitude of the post-synaptic event, while the parameters do not impact the associational/commissural (A/C) input into CA3 (Salin et al., 1996). In addition, the maximal facilitation at the CA3 – A/C pathway was about 125%, whereas the mossy fiber synapse potentiation was nearly 600% (Salin et al., 1996). Similar to the high level of PPF, the mossy fiber – CA3 synapse also demonstrates dramatically enhanced frequency facilitation compared to other synapses.

The characteristic low basal transmitter release probability is likely responsible for the high level of both PPF and frequency facilitation that is observed at the mossy fiber – CA3 synapse in comparison to other synapses. This dramatic difference is one reason in which this synapse has been extensively studied. For synaptic integration to occur DGCs and mossy fibers need to fire at similar frequencies. However, DGCs fire at very low frequencies (Jung and McNaughton, 1993). The broad frequency in which mossy fibers can function allows them to fire in a manner in which they can process information in the relevant range of granule cells. This strengthens the network activity between DGCs and mossy fibers and promotes effective neurotransmission (Salin et al., 1996).

1.7 ALTERATIONS IN HIPPOCAMPAL CIRCUITRY IN EPILEPSY

The relationship between seizures and damage to the hippocampus has been known since the early 1800s. In patients suffering from seizure disorders researchers noted neuronal loss and a hardening of the mesial temporal lobe, the area encompassing the hippocampus (Bouchet and Cazauvieilh, 1825). This hardened, or sclerotic, hippocampus is observed in 50 – 70% of patients and includes sizeable cell loss within areas CA1 and CA3 of the hippocampus (Margerison and Corsellis, 1966). In addition to the dramatic cell loss in those regions, a smaller amount of cell loss has been detected in the dentate gyrus, hilus and presubiculum (Corsellis and Bruton, 1983; Mathern et al., 1997). Within the last two decades, the abnormal growth of mossy fiber axons into the supragranular region of the dentate gyrus, as opposed to area CA3, has also been identified (Tauck and Nadler, 1985; Represa et al., 1987; Sutula et al., 1989; Houser et al., 1990; Babb et al., 1991; Sutula and Dudek, 2007). The aberrant formation of these axons is referred to as 'mossy fiber sprouting' and is now considered a hallmark of seizure disorders. In addition, these newly sprouted mossy fibers form functional synapses believed to create a recurrent excitatory network within the hippocampus (Sutula et al., 1988; Cronin et al., 1992; Wuarin and Dudek, 1996).

Several different animal models of epilepsy exist, each producing distinct anatomical alterations. However, the lithium-pilocarpine and pilocarpine models used in the proposed set of experiments remain the most relevant models to the human disease (Curia et al., 2008). The mechanism of SE induction by

pilocarpine is through activation of the cholinergic system, as pilocarpine is a muscarinic agonist and atropine, a muscarinic antagonist, blocks pilocarpine-induced SE (Clifford et al., 1987). However, once induced it is believed that the continuation and maintenance of SE is through a glutamatergic mechanism (Nagao et al., 1996; Smolders et al., 1997). As has been observed during the course of the current experiments a high dose of pilocarpine (390 mg/kg) does not always result in production of SE. To enhance the actions of pilocarpine, lithium chloride can be administered nearly 24 hours prior to pilocarpine treatment. In addition to increasing the effectiveness of pilocarpine, pre-administration of lithium chloride also significantly reduces the amount of pilocarpine needed to induce SE (Honchar et al., 1983; Clifford et al., 1987; Morrisett et al., 1987).

The prolonged SE that follows administration of pilocarpine (with or without administration of lithium chloride) brings with it an increased risk of mortality but it also initiates a severe and widespread cellular loss that persists for up to months following the event and eventually leads to tremendous neurodegeneration within the hippocampus and surrounding limbic regions. (Turski et al., 1983; Turski et al., 1984; Leite et al., 1990; Cavalheiro, 1995). In the present study, by 60 days post induction of SE, animals demonstrated nearly a 20% loss of both CA1 and CA3 pyramidal cells and a nearly 40% loss of hilar interneurons, while dentate granule cells remained essentially unaltered (Figure 1.5).
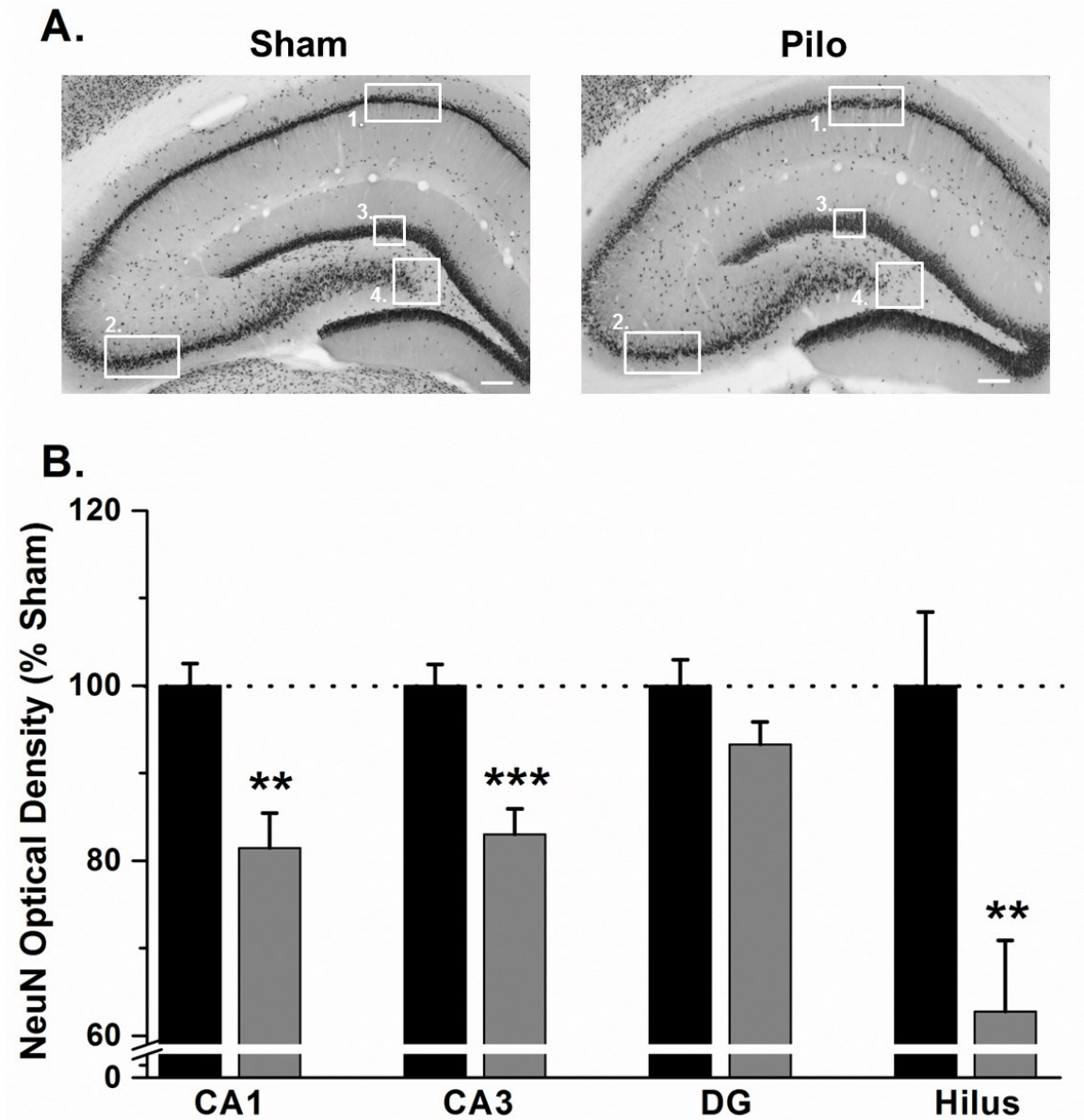
***Figure 1.5. Neuronal cell loss in animals 60d post-SE.* A.** Representative images of NeuN labeling from sham (*left*) and pilo-treated (*right*) animals 60 days post treatment. **B.** Optical density measurements from NeuN labeling in four hippocampal regions. *1.* CA1: 81.5 ± 4.0% of control, *n* = 7 – 9 *2.* CA3: 83.0 ± 2.9% of control, *n* = 7 – 9 *3.* DG: 93.3 ± 2.5% of control, *n* = 8 – 9 *4.* Hilus: 62.7 ± 8.1% of control, *n* = 6 – 7. **p < 0.01; ***p < 0.001, Scale bar, 200 μM.

In addition to cell specific neuronal loss, the pilocarpine model also produces similar mossy fiber sprouting that is observed in patients with seizure disorders (Figure 1.6) (Turski et al., 1983; Turski et al., 1984; Leite et al., 1990; Cavalheiro, 1995; Sutula et al., 1998; Buckmaster and Dudek, 1999). The pilocarpine model is also associated with increased levels of astrocytes (Figure 1.7) (Garzillo and Mello, 2002; Binder and Steinhauser, 2006), a characteristic not as widely noted in epilepsy patients. Overall the anatomical alterations observed within the hippocampus of both humans and animals demonstrate the dramatic impact that seizures have on the network organization and functionality of the hippocampus.

The pilocarpine model provides an excellent animal model for studying status epilepticus and the development of epilepsy, however there are some disparities that should be acknowledged. One important consideration is the age in which the event occurs. As stated previously, SE is most common in children and precipitating injuries are also more common in childhood (Mathern et al., 2002). However the anatomical and physiological alterations associated with SE are difficult to reproduce in animals younger than about 21 days of age (Curia et al., 2008). Additionally, human epilepsy is often associated with more brief and focal seizures. These patients demonstrate limited, asymmetric brain damage and likely appear healthy in other neurological aspects. However the pilocarpine model of SE is composed of a prolonged generalized status epilepticus event. Animals exhibit more widespread, bilateral brain damage, both cognitive and behavioral alterations and frequent spontaneous seizures (Sloviter, 2005). A final
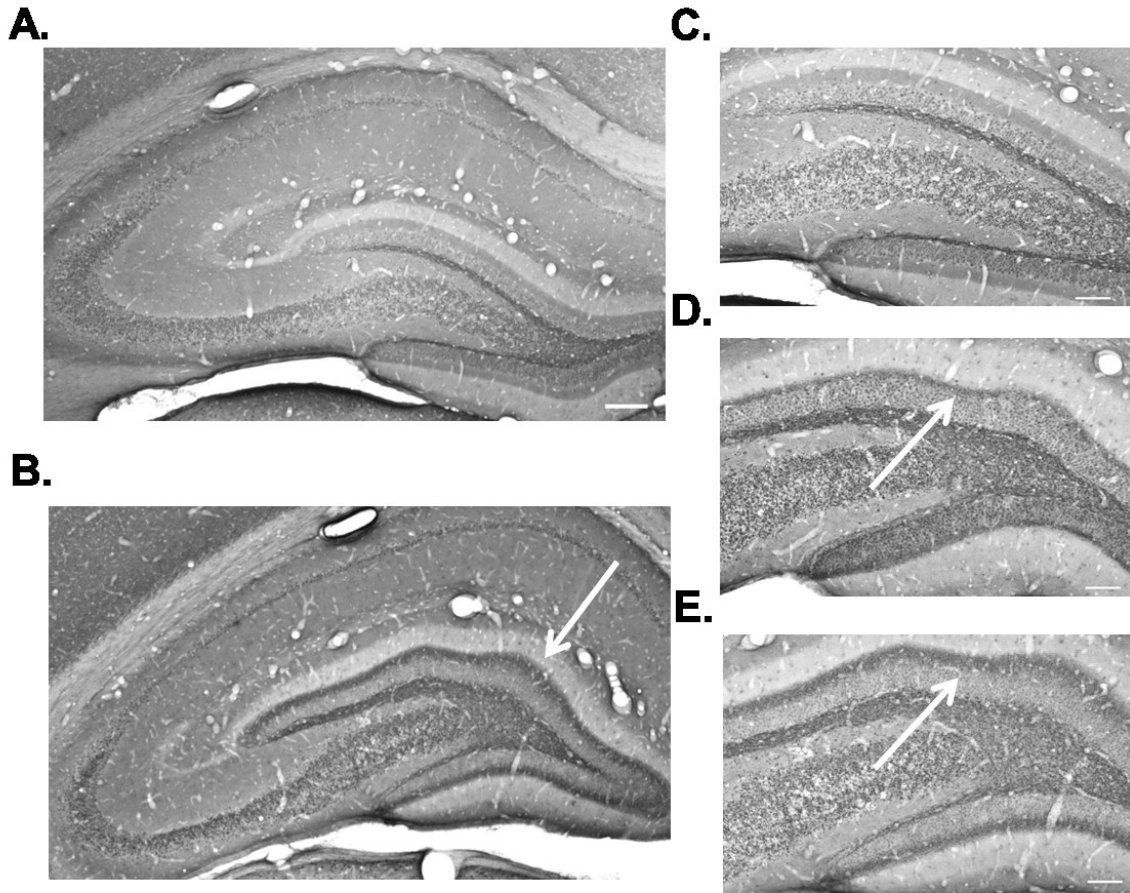
Figure 1.6. ZnT-3 labeling of mossy fiber sprouting in animals 60 and 200d post-SE. **A., C.** ZnT-3 labeling in a sham-treated animal at the 200 day time point, demonstrates robust labeling throughout mossy fibers and the hilar region. In animals 60d **(D.)** and 200d post-SE **(B, E.)** robust ZnT-3 labeling is observed within the inner molecular of the dentate gyrus (*white arrows*) indicative of aberrant mossy fiber sprouting. Scale bars, *A, B*, 200 µM; *C – D*, 100 µM.
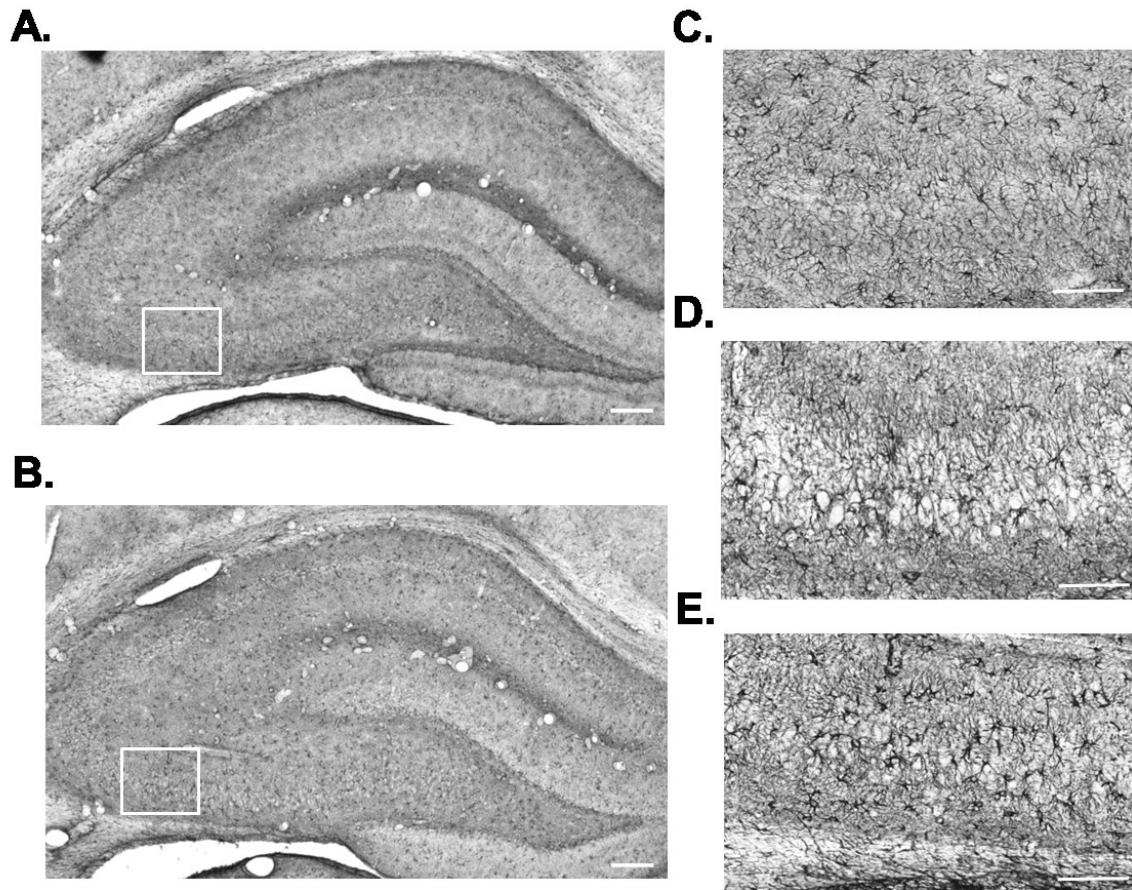
Figure 1.7. Increase in GFAP labeling of astrocytes in animals 60 and 200 days post-SE. A., C. Minimal GFAP labeling in the hippocampus and area CA3 in a sham-treated animal at the 200 day time point. In animals 60d **(D.)** and 200d post-SE **(B, E.)** robust GFAP labeling is apparent within area CA3. GFAP expression was significantly increased in area CA1, CA3 and DG at 5d, 60d and 200d post-SE. Scale bars, *A, B,* 200 µM; *C – D*, 100 µM.

consideration is the evidence that the site of spontaneous seizure generation in pilocarpine-treated animals is not the same as epilepsy patients (Mello and Covolan, 1996; Harvey and Sloviter, 2005; Sloviter et al., 2007). Differences in the site of seizure initiation may be due to differential neuronal damage or basic hippocampal circuitry. Despite the differences between the pilocarpine model and human epilepsy, this model is widely used and yields reproducible alterations sufficient for studying the epileptic condition.

1.8 GABA

γ-Aminobutyric acid (GABA) was originally synthesized in 1883, however it was not until 1950 that it was identified to be an integral part of the mammalian central nervous system (Petroff, 2002). The brain and spinal cord contain an abundance of GABA, whereas trivial amounts are found in peripheral tissue such as the liver, spleen and heart. GABA is synthesized by the alpha-decarboxylation of glutamate by the enzyme L-glutamic acid decarboxylase (GAD) and its cofactor pyridoxal phosphate (Petroff, 2002). GABA is an inhibitory neurotransmitter responsible for mediating the preponderance of inhibition within the brain. Three distinct forms of the GABA receptor exist; $GABA_A$ receptors which mediate fast synaptic neurotransmission (Sieghart and Sperk, 2002; Rudolph and Mohler, 2004), $GABA_B$ receptors responsible for mediating slow metabotropic actions (Couve et al., 2000; Bettler and Tiao, 2006), and $GABA_C$ receptors which mediate fast synaptic current in the retina (Bormann and Feigenspan, 1995).

1.8.1 GABA$_A$ RECEPTORS

GABA$_A$ receptors (GABA$_A$Rs) are members of the ligand-gated ion-channel superfamily, which also includes nicotinic acetylcholine receptors, 5-hydroxytryptamine 3 receptors and glycine receptors (Unwin, 1989; Barnard et al., 1998). GABA$_A$Rs are heteropentamers with each available subunit containing a large extracellular amino-terminal domain, four transmembrane domains (TM), a large intracellular domain between TM3 and TM4, and a smaller extracellular carboxy-terminal domain. The large amino-terminus serves as the binding site for GABA as well as other compounds such as benzodiazepines, and the large intracellular loop provides a site for receptor modulation via protein interactions and post-translational modifications (Jacob et al., 2008). At this time 18 different GABA$_A$R subunits have been identified and divided into seven different subunit classes, some with additional members: α (1 – 6), β (1 – 3), γ (1 – 3), δ, ε (1 – 3), θ, and π.

While many different subunit combinations are possible, the majority of functional GABA$_A$Rs are comprised of two α subunits, two β subunits and one γ subunit; although the γ subunit can be replaced by the δ, ε, θ, or π subunit (McKernan and Whiting, 1996; Rudolph and Mohler, 2004). The large amino-terminus of the subunit regulates the assembly of particular subunits. Unassembled or misfolded proteins, as well as homomeric subunits are degraded in the endoplasmic reticulum (ER) (Gorrie et al., 1997; Bedford et al., 2001). Receptors that survive the ER are trafficked to the Golgi apparatus where they are packaged into vesicles, transported and inserted into the plasma

membrane. Several factors aid in the translocation of receptors out of the ER, including GABA$_A$ receptor-associated protein (GABARAP) which interacts with the intracellular domain of the γ subunit (Wang et al., 1999), and *N*-ethylmaleimide-sensitive factor (NSF) a protein involved in intracellular vesicle fusion (Kittler et al., 2001; Zhao et al., 2007). Once inserted into the plasma membrane the β (1 – 3) and /or γ2 subunit of the GABA$_A$R directly bind to a particular subunit of a clathrin-adaptor protein 2 (AP2) complex. This interaction initiates the clathrin-dependent endocytosis, the major mechanism by which receptors are internalized and recycled (Kittler et al., 2000).

GABA$_A$ receptor subunits are ubiquitously expressed throughout the brain, however the one focus of this document is on GABA$_A$Rs within the dentate gyrus. Within this region of the adult hippocampus GABA$_A$R subunits α1, α2, α4, β3, γ2, and δ are most highly expressed, followed by the β1subunit, α3, α5, β2 and γ3 subunits are weakly expressed, and nearly undetectable levels of γ1 subunit are observed (Sperk et al., 1997). Expression of these subunits is also developmentally regulated not only within the dentate gyrus but also throughout the hippocampus and other brain regions (Laurie et al., 1992). GABA$_A$R subunits are targeted to different regions along the plasma membrane where they impart distinct physiological and pharmacological properties, including agonist affinity.

1.8.2 FUNCTION OF HIPPOCAMPAL GABA$_A$ RECEPTORS

Upon invasion of an action potential, presynaptic GABA is released in high concentrations into the synaptic cleft where it binds to the postsynaptic GABA$_A$

receptor. Binding of the ligand allows for negatively charged Cl^- ions to flow through the channel pore and into the cell. The increase in Cl^- ions allows for the inside of the cell to become more negatively charged and the cell hyperpolarizes. This hyperpolarization increases the threshold for an action potential and ultimately reduces neurotransmission.

As previously stated the majority of inhibitory neurotransmission is mediated by the $GABA_A$ receptor, and the particular subunit composition of the receptor imparts distinct characteristics. Synaptically located $GABA_A$Rs mediate fast phasic inhibition (Rudolph and Mohler, 2004), whereas extrasynaptic receptors mediate tonic inhibition (Brunig et al., 2002). In addition to influencing the localization of the receptor the subunit composition dramatically influences receptor pharmacology. $GABA_A$Rs are modulated by a variety of compounds including benzodiazepines, barbiturates and neurosteroids. The BZD sensitivity of the $GABA_A$R has been well studied, particularly in relation to seizure disorders, and will be discussed in detail in Chapter 3. Generally, seizures may result from a reduction in $GABA_A$R-mediated inhibition in the brain (Jacob et al., 2008), thus understanding the precise manner in which these receptors regulate neurotransmission is essential.

1.8.3 PHASIC INHIBITION

The presynaptic release of GABA yields high, millimolar concentrations within the synaptic cleft (Mody et al., 1994) which binds to the synaptically located $GABA_A$Rs and produces an inhibitory post-synaptic current (IPSC).

37

IPSCs regulate the rapid and precise, and near-synchronous, phasic inhibition.

Studies have demonstrated that the high concentration of GABA within the cleft

dissipates, either through re-uptake or diffusion, with a time constant of 100 –

500 µs (Overstreet et al., 2000; Mozrzymas et al., 2003; Mozrzymas, 2004). As

opposed to mass quantities, GABA can also be released by a single synaptic

vesicle. This spontaneously occurring event, results in a miniature inhibitory post-

synaptic current (mIPSC). Phasic inhibition is typically mediated by $GABA_ARs$

composed of α1, 2, 3 or 5 subunits together with β and γ subunits (Rudolph and

Mohler, 2004). Most notable for synaptically located $GABA_ARs$ is the presence of

the γ2 subunit, which is essential for the synaptic clustering of $GABA_ARs$ (Essrich

et al., 1998).

1.8.4 TONIC INHIBITION

In addition to the well-characterized rapid phasic inhibition, it is recognized

that ionotropic receptors may also mediate a slower form of transmission (Mody,

2001), including a tonic activation of the receptor. As opposed to synaptically

located $GABA_ARs$ that interact with millimolar concentrations of GABA, the

extrasynaptic receptors must have a higher affinity for GABA, as the

concentration in the extracellular space is likely within the micromolar range

(Nyitrai et al., 2006). In addition to their high affinity, extrasynaptic receptors must

demonstrate little desensitization (Mtchedlishvili and Kapur, 2006; Glykys and

Mody, 2007), a process by which receptor channels close despite the continued

presence of agonist bound to the receptor. The tonic activation of extrasynaptic

GABA$_A$Rs would be significantly reduced if the receptors became desensitized to the sustained presence of extrasynaptic GABA. Tonic inhibition is generally mediated by GABA$_A$Rs composed of α4 or 6 subunits, together with β and δ subunits (Barnard et al., 1998). Most notable for extrasynaptic receptors is the presence of the δ subunit, which is exclusively located in the extrasynaptic membrane (Nusser et al., 1998; Wei et al., 2003).

1.9 GABA$_A$ RECEPTORS IN EPILEPSY

At a fundamental level seizures result from an imbalance between the excitatory and inhibitory transmission within the brain. While several glutamatergic receptors, including kainate receptors which will be discussed later, regulate excitatory neurotransmission, the GABA$_A$ receptor is responsible for regulating the majority of the inhibitory tone in the brain. Studies have demonstrated that both the trafficking of GABA$_A$ receptors as well as their subunit composition is altered in animal models and patients with seizure disorders such as status epilepticus and temporal lobe epilepsy (for review see, Jacob et al., 2008).

One pharmacological consequence of SE is alterations in the surface expression of GABA$_A$ receptor subunits. Subsequently these alterations impact receptor pharmacology and even the network physiology. It has been shown that during the progression of SE the surface expression of the γ2 and β2/3 GABA$_A$ receptor is significantly reduced, while other receptor subunits such as the extrasynaptic δ subunit remain constant (Goodkin et al., 2008). This finding

supported the pharmacological observation that SE patients and animal models of SE develop pharmacoresistance to BZDs (Walton and Treiman, 1988; Kapur and Macdonald, 1997; Jones et al., 2002; Grosenbaugh and Mott, 2013). BZDs, such as diazepam, bind to the $GABA_AR$ at a specific allosteric site, located at the interface of α1, α2, α3, or α5 and the γ2 subunit (Sigel, 2002). Activation of the BZD-binding site results in a prolonging or increase in $GABA_AR$-mediated synaptic current (Otis and Mody, 1992). The sensitivity of the $GABA_AR$ to BZDs is conferred by the presence of the γ1 - 3 subunit (Sieghart and Sperk, 2002), which is significantly reduced during SE, and thus explains reduced efficacy in patients. In addition, alterations in pharmacological changes in the subunit expression can also impact the network physiology of the brain. Given that extrasynaptic δ-containing $GABA_AR$ subunits remain functional during SE, the increase in extracellular GABA during prolonged SE (Naylor et al., 2005), is able to produce an increase in the overall tonic inhibitory tone of the network (Naylor et al., 2005; Grosenbaugh and Mott, 2013). As $GABA_A$ receptors mediate the majority of the inhibitory neurotransmission within the cerebrum, it is critical to understand how SE alters their subunit expression and the subsequent alterations on receptor pharmacology and physiology.

1.10 GLUTAMATE RECEPTORS

Two classes of receptors, ionotropic and metabotropic, are responsible for mediating excitatory neurotransmission within the CNS. Ionotropic receptors, commonly referred to ligand-gated ion channels, are responsible for mediating

the fast neurotransmission that occurs at the synapse. Specific binding of a ligand or neurotransmitter to the receptor results in a conformational shift allowing for the flow of ions (i.e. Na^+, Ca^{2+}, K^+, Cl^-) through the channel. Three distinct classes of excitatory ionotropic receptors have been established- α-amino-3-hydroxy-*S*-methylisoxazole-4-propionic acid (AMPA), *N*-methyl-D-aspartate (NMDA), and kainate (KA). In contrast to ionotropic receptors are metabotropic receptors which activate a second-messenger system, following binding of a ligand or neurotransmitter. Unlike ionotropic receptors which always mediate excitatory neurotransmission, the second-messenger system associated with metabotropic glutamate receptors (mGluRs) allows them to regulate either inhibitory or excitatory neurotransmission. Eight different mGluRs (mGluR$_{1-8}$) have been identified and classified into three distinct groups (Group 1 – III). Of particular interest is the ability of kainate receptors (KARs) to mediate both ionotropic and metabotropic transmission, however the focus of the proposed experiments will be on the ionotropic action of KARs.

1.10.1 KAINATE RECEPTOR ASSEMBLY AND LOCALIZATION

Kainate receptors (KARs) were identified as a separate class of ionotropic glutamate receptors based on their sensitivity to the agonist kainate (kainic acid), a naturally occurring compound found in seaweed. Studies conducted throughout the early 1990's concluded the presence of five distinct kainate receptor subunits, GluK1 – 5 (Hollmann and Heinemann, 1994), which form functional tetramers. Each of the available KAR subunits contains a large extracellular amino-terminal

domain, involved in subunit assembly, followed by the extracellular ligand binding domain. The membrane region is composed of three transmembrane domains (TM1, TM3, TM4) and a re-entrant P-loop (TM2), which does not fully span the membrane. The M4 domain gives way to the intracellular carboxy-terminus of the subunit (Mayer, 2006).

GluK1 – 5 subunits are divided into two families based on their sequence homology, functionality and agonist affinity. GluK1 – 3 subunits (Bettler et al., 1990; Egebjerg et al., 1991; Bettler et al., 1992; Sommer et al., 1992) and GluK4 – 5 subunits (Werner et al., 1991; Herb et al., 1992; Sakimura et al., 1992) are each about 70% identical within their family, but share only a 40% identity with each other. In addition to differences in the sequence homology of the two groups, GluK1 – 3 subunits can form functional homomeric receptors (Egebjerg et al., 1991; Sommer et al., 1992; Schiffer et al., 1997), while GluK4 – 5 subunits must be co-expressed with GluK1 – 3 (Werner et al., 1991; Herb et al., 1992). Binding assays from recombinant KARs have demonstrated that GluK4 – 5 subunits have significantly higher agonist affinity than do the GluK1 – 3 subunits (Monaghan and Cotman, 1982; Monaghan et al., 1989). In addition to the differences between GluK1 – 3 and GluK4 – 5 subunits, GluK1 and GluK2 are also subject to mRNA editing that alters Ca^{2+} permeability and sensitivity to intracellular polyamines (Sommer et al., 1991; Rosenthal and Seeburg, 2012). Furthermore, two membrane proteins that interact with KAR subunits were identified, Neuropilin and Tolloid-like 1 and Neuropilin and Tolloid-like 2 (Neto1 and Neto2, respectively) (Zhang et al., 2009; Straub et al., 2011b; Tang et al.,

2011). These proteins have been shown to dramatically alter the basic properties of KARs (Copits et al., 2011; Straub et al., 2011a; Fisher and Mott, 2012, 2013).

KARs composed of GluK2/GluK5 subunits are believed to be the most prominent within the brain (Petralia et al., 1994), thus the majority of studies investigating KAR assembly and trafficking have focused on these two subunits. One check-point involved in proper receptor assembly is the occupancy of the ligand-binding site. Recombinant studies have demonstrated that mutations that reduce agonist affinity results in the subunits being retained within the ER and not inserted into the plasma membrane (Mah et al., 2005; Gill et al., 2009; Fisher and Housley, 2013). For homomeric GluK2 receptors, glutamate binding promotes proper folding resulting in a conformational change that is essential for assembly and trafficking of the receptor (Gill et al., 2009). The GluK5 subunit was found to contain several ER retention motifs that prevented surface expression unless this subunit was bound to a GluK1 – 3 subunit (Gallyas et al., 2003; Ren et al., 2003), a process that promotes formation of heteromeric receptors. Furthermore it was recently determined that agonist binding to the GluK5 subunit alone was sufficient to promote the formation of heteromeric GluK2/5 subunits (Fisher and Housley, 2013). Together these studies demonstrate the complexity of receptor assembly and trafficking as well as represent a key role for both the GluK2 and GluK5 subunits.

Early studies have conclusively demonstrated that mRNA for a portion of kainate receptor subunits is expressed throughout the brain and spinal cord (Wisden and Seeburg, 1993; Bahn et al., 1994; Bettler and Mulle, 1995). Similar

results were noted in the hippocampus, in which mRNA for all of the KAR subunits was also observed, however the expression within the hippocampus varies between regions and cell types. Furthermore, the mossy fiber – CA3 synapse was the first hippocampal region shown to utilize kainate receptors (Castillo et al., 1997; Vignes and Collingridge, 1997). In this region the CA3 pyramidal cells, as well as dentate granule cells, show elevated mRNA levels for GluK2, GluK4 and GluK5. GluK3 mRNA appears to be restricted to dentate granule cells, while interneurons predominately express GluK1, in addition to GluK2 – 3 (Bahn et al., 1994; Bureau et al., 1999; Paternain et al., 2000; Darstein et al., 2003).

Variability in subunit localization, differences in the biophysical properties of the subunits and regulation by Neto1 and Neto2 make it difficult to study KARs *in vivo*. Furthermore, these differences become more apparent when attempting to use pharmacological agents to activate or block the receptor, as each subunit may behave differently to agonists and antagonists. As a result of this variability the role of KARs in regulating excitatory neurotransmission, specifically within the diseased brain, is not fully understood.

1.11 FUNCTION OF HIPPOCAMPAL KAINATE RECEPTORS

Kainate receptors are ubiquitously expressed in the brain and hippocampus (Wisden and Seeburg, 1993), and are heavily expressed throughout the mossy fiber – CA3 pathway. Specifically, KARs have been localized on postsynaptic CA3 pyramidal cells, on the presynaptic terminals of

the mossy fibers and on the mossy fiber axons extending from dentate granule cells. At these sites KARs regulate specific forms of excitatory neurotransmission, and given the relationship between the CA3 region and the generation of seizures (Lothman et al., 1991; Ben-Ari and Cossart, 2000), KARs pose an interesting avenue to explore.

1.11.1 POSTSYNAPTIC KAINATE RECEPTORS

Throughout the brain the majority of glutamatergic transmission is mediated by postsynaptic AMPA and NDMA receptors, while excitatory transmission mediated by KARs is limited to only a few synapses in the brain (Cossart et al., 1998; Frerking et al., 1998; Li and Rogawski, 1998; Kidd and Isaac, 1999; Li et al., 1999; Bureau et al., 2000). KARs located on postsynaptic CA3 cells regulate transmission originating from presynaptic mossy fiber terminals, but not inputs from the A/C fibers (Castillo et al., 1997; Vignes and Collingridge, 1997). At the mossy fiber – CA3 synapse postsynaptic KARs are activated by synaptically released glutamate which generates an excitatory postsynaptic current (EPSC) (Cossart et al., 2002). The KAR-mediated EPSC has two characteristic features; a small amplitude, approximately 10% of the total peak observed for AMPA receptors, and significantly slower deactivation kinetics (Castillo et al., 1997; Vignes and Collingridge, 1997; Frerking et al., 1998; Kidd and Isaac, 1999).

As stated previously, the mossy fiber – CA3 synapse is likely comprised of receptors composed of GluK2, GluK4 and GluK5 subunits, which would heavily

impact agonist affinity as well as the decay kinetics of the response. Heteromeric receptors, presumed to be more common, containing the GluK4 or GluK5 subunit would have significantly higher affinity for glutamate than would homomeric GluK2 receptors (Monaghan and Cotman, 1982; Monaghan et al., 1989). In addition, the GluK5 subunit confers a slower decay than does the GluK4 subunit (Contractor et al., 2003). While many studies suggest that GluK2/5-containing receptors are the principal KAR at the mossy fiber – CA3 synapse, it remains possible that the chief receptor at this synapse is in fact comprised of the GluK2/4 subunit combination. However lack of selective and reliable KAR antagonists have limited the understanding of precisely which KAR subunits regulate the majority of transmission at this synapse.

Relative to the much larger AMPA receptor-mediated currents, the characteristic slow decay and small amplitude of the KAR-mediated EPSC may have little impact on excitatory transmission. However, the slow decay allows for KAR-mediated EPSCs to develop a large depolarization envelope at frequencies as low at 3Hz (Frerking and Ohliger-Frerking, 2002). In addition, the summation produced by KARs has a more global and consistent impact on membrane activity than do AMPA receptors which produce substantial variability (Frerking and Ohliger-Frerking, 2002). Collectively, while the postsynaptic KAR-mediated EPSC may be significantly smaller than that observed by the AMPA receptors, the significant levels of depolarization and temporal summation that are observed suggest that KARs may play a significant role in regulating CA3 network activity.

1.11.2 PRESYNAPTIC KAINATE RECEPTORS

Initial studies exploring KAR subunit localization suggested that the majority of subunits may actually be restricted to the presynaptic mossy fiber, as opposed to the postsynaptic CA3 cell (Agrawal and Evans, 1986; Represa et al., 1987). In support of the heavily presynaptic localization of KARs was the finding that selective destruction of the mossy fibers results in a dramatic decrease in high affinity [^{3}H] kainate binding, whereas it remains chiefly intact following destruction of the CA3 pyramidal cells (Represa et al., 1987). It is now well known and accepted that presynaptic KARs regulate neurotransmission, and in fact do so in a bidirectional manner, at both excitatory and inhibitory synapses (Lerma, 2003). Furthermore, presynaptic KARs are heavily implicated in the short-term synaptic plasticity which was discussed in section 1.6.1.

Presynaptic regulation of glutamate release has been extensively studied at the mossy fiber – CA3 synapse where KARs modulate release in a bimodal manner. Several studies have confirmed that low nanomolar concentrations of kainate facilitate glutamate release, while larger concentrations depress release (Contractor et al., 2000; Lauri et al., 2001; Schmitz et al., 2001a). In addition to regulating transmitter release there is substantial evidence that presynaptic KARs also act as autoreceptors that sense synaptically released glutamate and subsequently increase glutamate release (Contractor et al., 2001; Lauri et al., 2001; Schmitz et al., 2001b).

The exact mechanism by which presynaptic KARs regulate transmitter release is unknown. However, similar to the postsynaptic KARs, quantal release

of glutamate is also sufficient to activate presynaptic KARs (Contractor et al., 2001; Schmitz et al., 2001b), which increases release from internal Ca^{2+} stores and promotes subsequent transmitter release (Lauri et al., 2003; Scott et al., 2008). However, other studies have proposed that released glutamate depolarizes the presynaptic terminal to facilitate release (Kamiya and Ozawa, 2000; Schmitz et al., 2000; Schmitz et al., 2001b; Schmitz et al., 2001a; Schmitz et al., 2003). Regardless of its action, presynaptic KARs are heavily involved in short-term synaptic plasticity, predominately in the frequency-dependent facilitation that is a hallmark of the mossy fiber – CA3 synapse. Despite the lack of availability of specific KAR subunit antagonists, the use of some antagonists coupled with genetically modified mice have demonstrated that presynaptic mossy fibers are likely composed predominately of GluK2 (Contractor et al., 2001; Schmitz et al., 2003; Breustedt and Schmitz, 2004) and GluK3 (Pinheiro et al., 2007) subunits.

1.11.3 MOSSY FIBER KAINATE RECEPTORS

In addition to their localization on postsynaptic CA3 pyramidal cells and presynaptic mossy fiber terminals, KARs are also located along mossy fiber axons where they regulate axonal excitability (Kamiya and Ozawa, 2000; Schmitz et al., 2000; Mott et al., 2008). Similar to the biphasic actions on transmitter release, low agonist concentrations facilitate axon excitability while higher concentrations suppress excitability (Kamiya and Ozawa, 2000; Schmitz et al., 2000; Mott et al., 2008). KARs located along the mossy fiber axons are

sensitive to both synaptically released glutamate as well as glutamate located in the extracellular space (Contractor et al., 2003; Schmitz et al., 2003). It is believed that KARs located on mossy fiber axons are predominately composed of GluK2, GluK4 and GluK5 subunits (Wisden and Seeburg, 1993). In a GluK4 knockout mouse it was observed that the amount of axon excitability normally produced by low nanomolar concentrations of kainate was significantly reduced (Catches et al., 2012), suggestive of a role for GluK4-containing KARs in regulating mossy fiber axon excitability. Alterations in KAR sensitivity and regulation of mossy fiber axon excitability would affect neuronal transmission and communication between the dentate gyrus and area CA3, as well as alter synaptic plasticity at the mossy fiber – CA3 synapse.

1.12 KAINATE RECEPTORS IN EPILEPSY

Kainate receptors have prominently been implicated in seizure disorders [for review see, (Ben-Ari and Cossart, 2000; Vincent and Mulle, 2009; Contractor et al., 2011; Lerma and Marques, 2013)], but have also been linked to a variety of diseases (Lerma and Marques, 2013). In particular, alterations in KAR subunit expression have been observed in psychiatric conditions including anxiety, depression and schizophrenia (Lerma and Marques, 2013), all disorders with increased prevalence in epilepsy (Jacobs et al., 2009). Systemic administration of kainate has been used for decades as a reliable animal model of epilepsy and produces similar pathologies as seen with human epilepsy (Nadler, 1981; Ben-Ari, 1985). Following administration of kainate, animals develop limbic seizures

followed by a latent period of epileptogenesis and finally the development of persistent spontaneously recurring seizures (Ben-Ari, 1985; Leite et al., 1990; Cavalheiro et al., 1991). In addition GluK1-selective antagonists block pilocarpine-induced seizures (Smolders et al., 2002), and genetic knockout of the GluK2 subunit significantly reduces seizure susceptibility (Mulle et al., 1998).

As described earlier, the aberrant sprouting of mossy fiber axons in the DG has become an anatomical hallmark of seizures in both human and animal models. This newly formed circuit predominately regulates excitatory glutamate transmission (Buckmaster et al., 2002), and it was recently determined that newly formed KARs partly regulate this transmission (Epsztein et al., 2005). KARs have also been linked to the excitotoxicity that occurs in epilepsy (Vincent and Mulle, 2009). Specifically, KARs are densely located in area CA3, a cellular region that is highly susceptible to excitotoxicity (Nadler et al., 1978). Furthermore, KARs have also been linked to pyramidal cells in layer III of the medial entorhinal cortex, a region that rapidly degenerates due to excitotoxic cell death following administration of kainate (Du et al., 1995; West et al., 2007).

Alterations in hippocampal KAR subunit expression have also been examined in patients with seizure disorders (Grigorenko et al., 1997; Mathern et al., 1998; Li et al., 2010; Das et al., 2012). Despite a true consensus, possibly due to the type of seizure disorder, duration of the disease and therapeutics, these studies clearly demonstrate that KAR subunit expression is altered in the epileptic brain. Animal models have also demonstrated dramatic changes in KAR subunits, findings that persist despite differences in the seizure model utilized

(Lason et al., 1997; Tandon et al., 2002; Zhang et al., 2004; Ullal et al., 2005; Porter et al., 2006). However, while a minimal number of studies have addressed these alterations even fewer have addressed changes in KAR subunit expression that occur during the progression of the disease (Ullal et al., 2005). Furthermore, the majority of these studies examined alterations in KAR mRNA expression within the hippocampus as a whole. Given that KAR subunit localization is not uniform within the hippocampus, there is reason to believe that alterations in the epileptic brain would be region specific and that protein levels would provide more relevant information than mRNA. Despite the evidence linking KARs to epilepsy, currently there are no antiepileptic drugs that target KARs. This may be partially due to the limited understanding of KAR-mediated expression and physiology in the epileptic brain, two areas examined in the current document.

Chapters 4 – 6 will explore alterations in KAR subunit expression and the functional impact of those changes on mossy fiber – CA3 neurotransmission and hippocampal axon excitability.

1.13 SIGNIFICANCE REVISITED

Status epilepticus is a neurologic emergency characterized by the inability of inherent cellular mechanisms to terminate seizure activity. The occurrence of a single SE event leads to the development of epilepsy in upwards of 40% of patients, making the development of epilepsy the most challenging long-term consequence associated with SE. Currently available AEDs are successful in treating SE in approximately 60% of patients. However this leaves a large portion

of the patient population suffering from uncontrolled SE, thus increasing their risk for the development of epilepsy and associated comorbidities. This project undoubtedly demonstrates that the ability to develop safe and effective therapeutics for the treatment of SE and seizure disorders lies within the understanding that a single SE event can drastically alter the brain.

The overarching hypothesis of this project is that *status epilepticus and the development of epilepsy produce striking alterations in GABA$_A$ and kainate receptor populations. Furthermore, these alterations significantly impact receptor pharmacology and neurotransmission in the SE-experienced brain*.

CHAPTER 2

GENERAL METHODS

2.1 ANIMAL MODEL OF STATUS EPILEPTICUS

The ability to understand the epileptic brain and to develop therapeutics is dependent upon the use of animal models. These models must as closely as possible replicate the distinct anatomical and physiological alterations seen in the human disease including sclerosis and cell loss. When exploring potential models several features are identified that are similar to the human pathology. Some of these features include the appropriate seizure phenotype (convulsive/non-convulsive), similar neuropathological consequences, chronic hyperexcitability and appropriate responsiveness to AEDs. Commonly used animal models fall into five distinct categories; electrical, chemical, thermal, *in vitro*, and refractory (Reddy and Kuruba, 2013).

The current study will utilize a chemical model involving administration of pilocarpine and lithium-pilocarpine to initiate status epilepticus followed by a period of epileptogenesis and eventually the development of spontaneously recurring seizures. As discussed in section 1.7 both of these models very closely replicate the hippocampal injury associated with human epilepsy.

2.1.1 ANIMAL CARE AND USE

All animal care and use procedures were carried out in accordance with protocols written under the guidance of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of South Carolina. Male Sprague-Dawley rats were purchased from Harlan, and housed at 1 – 3 rats per cage with *ad libitum* access to food and water. Rats were housed in a climate controlled facility with a light/dark cycle of 12/12 hours.

2.2 LITHIUM PILOCARPINE MODEL OF STATUS EPILEPTICUS

Young male Sprague-Dawley rats (15 – 23 days; Harlan, Indianapolis, IN) received intraperitoneal (i.p.) injections of lithium chloride (127 mg/kg) followed 15 – 20 hours later by scopolamine methyl bromide (2 mg/kg). Status epilepticus was then induced 15 – 30 minutes later by injection with pilocarpine hydrochloride (60 mg/kg). As pilocarpine is a muscarinic agonist, scopolamine is pre-administered in order to block peripheral muscarinic receptors and ensure selectivity of pilocarpine. Following pilocarpine injections, rats were observed continuously for the occurrence of behavioral seizures and were scored based upon the Racine scale (Racine, 1972) (Table 2.1). Diazepam (DZP, 0.3 – 100 mg/kg) or stiripentol (STP, 10 – 1000 mg/kg) was administered either at the onset of the first stage 3 seizure (brief SE) or 45 minutes after the first stage 3 seizure (prolonged SE; pSE). This experimental protocol of the lithium pilocarpine (Li-Pilo) model was used in Chapter 3 when exploring the actions of

STP and DZP on behavioral SE and GABAergic neurotransmission.

Table 2.1. Racine scale of epileptic seizures

Score	Observed Motor Behavior
0	Normal behavior (exploring, walking, grooming, etc.)
1	Immobility, staring 'curled up' posture
2	Automatisms (Chewing, head bobbing, twitching, wet-dog shakes)
3	Forelimb/hindlimb myoclonic jerking, head tremor
4	Rearing and whole-body clonus
5	Rearing and falling over; loss of posture
6	Tonic-clonus seizures with tonic forelimb flexion/extension, whole-body clonus
7	Wild-running with bouncing; death

2.3 PILOCARPINE MODEL OF STATUS EPILEPTICUS

Adult male Sprague-Dawley rats (37 – 47 days; Harlan, Indianapolis, IN) received intraperitoneal (i.p.) injections of scopolamine methyl bromide and terbutaline hemisulfate (2 mg/kg) followed 30 – 45 min by pilocarpine hydrochloride (390 mg/kg). In addition to administration of scopolamine to block peripheral muscarinic receptors, animals also received terbutaline which acts as a bronchodilator to prevent respiratory depression associated with prolonged SE. Following pilocarpine injection, rats were observed continuously for the occurrence of behavioral seizures and were scored on the Racine scale (Racine, 1972) (Table 2.1). Approximately 75% of rats experienced a seizure score of 5

on the Racine scale. Animals that did not exhibit SE after 45 mins were given an additional 'booster' of pilocarpine (200 – 300 mg/kg). Nearly 90% of animals receiving a pilocarpine 'booster' experienced sufficient SE. After 2 hours of status epilepticus, DZP (25 mg/kg) was administered intraperitoneally. This group of animals served as the 'pilo' group. In order to limit physical distress and any peripheral effects of pilocarpine animals received additional doses of the scopolamine/terbutaline cocktail approximately one-hour into SE as well as upon termination of SE with DZP.   A separate group of animals was treated identically, but received saline (390 mg/kg) instead of pilocarpine hydrochloride. This group of animals served as the 'sham' group. Approximately 1 – 2 hours after seizures were terminated animals received a subcutaneous dose of the scopolamine/terbutaline cocktail (2 mg/kg), lactated Ringer's solution (2 mL), and rat chow that had been softened and sweetened in high sucrose water. Animals that did not enter SE were excluded from the study.

All animals were housed overnight in the animal facility and checked as early as possible on the following day to determine if they had successfully recovered from treatment (normal posture, movement and activity levels). Animals still experiencing side-effects from treatment (lethargy, immobility) were given additional subcutaneous injections of lactated Ringer's solution and the scopolamine/terbutaline cocktail and placed on a heating pad. For animals who endured the first night post-treatment the survival rate was nearly 100%.   This experimental protocol was used in Chapters 4 – 6 to explore alterations in

kainate receptor subunit expression and kainate receptor-mediated electrophysiological alterations in the epileptic brain.

Video monitoring of animals a minimum of 30 days post-treatment confirmed the appearance of spontaneously occurring seizures in pilocarpine treated animals. In addition, by approximately 60 days post-SE animals began to demonstrate neuronal cell loss and the appearance of mossy fiber sprouting.

2.4 DETERMINATION OF BRAIN-PLASMA STP CONCENTRATION

Brain and plasma concentrations of STP were determined in four separate groups of male Sprague-Dawley rats: 1) naïve juvenile (20 – 27 days old), 2) prolonged SE-experienced juvenile (20 – 21 days old), 3) naïve adult (62 – 63 days old) and 4) prolonged SE-experienced adult (61 days old). Fifteen minutes following i.p. administration of 300 mg/kg STP, naïve and prolonged SE rats were anesthetized and decapitated in order to collect brain and blood samples. Trunk blood was collected in 6 mL sodium heparin lined BD vacutainers and centrifuged for 10 min at 2000 rpm at 4°C to collect blood plasma. Brains were removed and rapidly frozen on dry ice. Brain tissue and plasma were stored at -80°C until use.

Brain and plasma concentrations of STP were measured by high-performance liquid chromatography (HPLC) with ultraviolet detection. For plasma analysis 100 µl of internal standard (2 µg of AB1191 per mL of 50/50 methanol/H_2O) and 50 µL of 50/50 methanol/H_2O with 2 mL of tert-Butyl methyl ether were added to a 50 µL plasma sample. Following agitation for 15 min,

decantation and freezing in a dry ice/acetone bath, the organic layer was transferred and evaporated to dryness under nitrogen at 45°C.

For brain tissue analysis, 100 µL of internal standard (50 µg of AB1191 per mL of 50/50 methanol/H_2O) and 50 µL of 50/50 methanol/H_2O with 2 mL of H_2O were added to the weighed brain sample and mixed. A 0.5 mL volume of homogenate was extracted with 3 mL of pentane. Following agitation for 15 min, decantation and freezing in a dry ice/acetone bath, the organic later was transferred and evaporated to dryness under nitrogen at 45°C.

Residues from plasma and brain samples were reconstituted with 200 µL of mobile phase (acetonitrile/H_2O; 60/40) and used for HPLC analysis. UV detection was accomplished at 264 nm with a limit of sensitivity of 0.5 µg/mL for plasma and 0.45 µg/g or 0.35 µg/g in juvenile and adult brain respectively. The concentration levels of STP in plasma (µg/mL) and brain (µg/g) were expressed as total drug concentration. The coefficients of variations for intra- and inter-assays were 0.2 – 13% and 3 – 14% respectively.

2.5 INTRODUCTION TO BRAIN SLICE ELECTROPHYSIOLOGY

Neuronal function is based on the ability of cells to integrate and propagate electrical signals such as action potentials. Electrophysiology is the study of this electrical activity within the brain and provides detailed information on the functionality of individual neurons and networks of neurons. In the 1950's Henry McIlwain developed the brain slice preparation and demonstrated the ability of brain slices to retain their metabolic activity as well as their cellular

membrane potentials (Mc et al., 1951; McIlwain and Buddle, 1953; Li and Mc, 1957; Yamamoto and McIlwain, 1966). These studies set the foundation for brain slice electrophysiology by demonstrating that neurons could be kept alive in an artificial setting while their electrical properties were explored.

In vitro brain slice electrophysiology can be conducted on many, if not all, brain regions. However successful experiments are dependent upon quality slices which are defined by the ease and reliability in which the tissue is prepared, the visibility of structural landmarks and the extent in which fibers and synaptic fields are observed within the slice (Lynch and Schubert, 1980). In regards to these properties, one structure in particular lends itself to the technique of brain slice electrophysiology, the hippocampus. The hippocampus is a region that can be easily removed from the brain without the need for a microscope. In addition once sectioned, little to no magnification is needed to visualize its unique structure composed of pyramidal and granule cell layers. Furthermore, the laminar organization of the hippocampus allows for properly sectioned slices to contain cell bodies, dendrites and axons which often extend throughout the slice (Lynch and Schubert, 1980). Together these properties of the hippocampus increase the ease of experiments as well as provide the ability to study individual intrinsic cell properties (whole-cell electrophysiology), as well as extracellular network activity (field-potential electrophysiology). The following sections will detail the concept and methods used for both whole-cell and field-potential electrophysiology in the rat hippocampus.

2.6 WHOLE-CELL ELECTROPHYSIOLOGY

Patch clamp electrophysiology is an intracellular recording technique that allows for the study of ion flow through a single channel and was first demonstrated in 1976 by Erwin Neher and Bert Sakmann (Neher and Sakmann, 1976), who in 1991 won the Nobel Prize in Physiology or Medicine. Several different configurations of this technique exist, one of which is whole-cell recording. To acquire a whole-cell configuration a glass electrode is placed on the surface of a cell and mild suction is then applied to rupture the membrane, allowing for a diffusional exchange of the contents of the cell cytoplasm and the glass pipette (Figure 2.1). The whole-cell configuration produces a convenient method to directly apply substances into the cell in order to study the electrical currents and properties from the entire cell.

Whole-cell electrophysiology is used to study the movement of electrically charged ions, commonly through voltage-gated or ligand-gated channels. The $GABA_A$ receptor is selectively permeable to negatively charged Cl^- ions. When the receptor is open, Cl^- ions flow into the cell creating a more negative intracellular environment. This hyperpolarization of the cell brings it further away from the threshold of firing an action potential (~-40mV) and thus reduces neuronal firing. The postsynaptic current produced by activation of GABA receptors is referred to as an inhibitory postsynaptic current (IPSC). In contrast, glutamatergic receptors such as kainate or AMPA are permeable to Na^+ and K^+ ions. When these receptors are open, the concentration gradient maintained by a Na^+/K^+-ATPase pump, allows for Na^+ to enter the cell creating a more positive
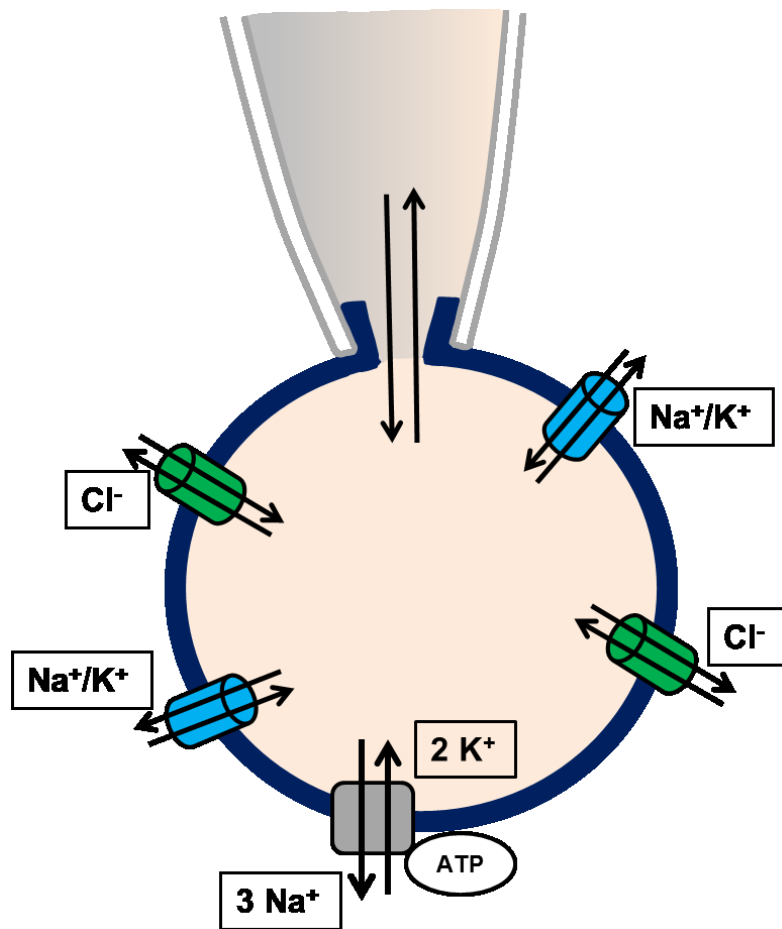
Figure 2.1. Schematic of whole-cell recording configuration. One of several configurations of patch-clamp electrophysiology. A glass electrode filled with experiment-specific internal solution is applied to the surface of a cell. Slight suction is applied to rupture the cell membrane allowing the contents of the electrode and the cell to easily diffuse. Schematics of receptor complexes, including Cl^-, Na^+ and K^+ are expressed on the surface of the cell. In addition the Na^+/K^+-ATPase pump is essential to maintain concentration gradients.

intracellular environment. If this depolarization of the cell is sufficient it will fire an action potential. Thus activation of these glutamatergic receptors, in contrast to GABAergic receptors, generally increase neuronal firing, and produce an excitatory postsynaptic current (EPSC).

2.6.1 EXPERIMENTAL PROTOCOL FOR WHOLE-CELL RECORDINGS

<u>Chapter 3-</u> Transverse 300 μm thick slices from isoflurane-anesthetized animals were cut using a vibratome (Leica VT1000S, Nussloch, Germany). Brain slices were prepared in cold (4°C), oxygenated (95% O_2 / 5% CO_2) sucrose-based 'cutting' artificial cerebrospinal fluid (aCSF) that contained (in mM): 248 sucrose, 2 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 0.5 $CaCl_2$ and 5 $MgS0_4$ (350 mOsm). Slices were incubated for approximately one hour at room temperature in oxygenated (95% O_2 / 5% CO_2) aCSF containing (in mM): 125 NaCl, 2.7 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 0.5 $CaCl_2$ and 5 $MgSO_4$, 0.02 D-APV and 1 kynurenic acid (pH 7.4; 305-312 mOsm).

Currents were recorded from dentate granule cells located in the infra-pyramidal blade of the dentate gyrus, visually identified with infrared-differential interference contrast optics. Currents were recorded using a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA) and filtered at 1 kHz. Currents were digitized using a Digidata 1440A A-D board (Molecular Devices, Sunnyvale, CA) and analyzed using pClamp10 software. $GABA_A$ IPSCs were evoked by stimulation in *stratum moleculare* of the dentate gyrus near the recording electrode. Stimuli were 0.1ms, cathodal, monophasic, rectangular constant

current pulses (10 – 100 µA) delivered through monopolar, platinum-iridium stimulating electrodes (FHC Inc., Bowdoin, ME). IPSCs and miniature excitatory post-synaptic currents (mEPSCs) were recorded using borosilicate glass electrodes (resistance 5 – 8 MΩ) filled with an internal solution containing (in mM): 130 D-Gluconic Acid, 130 CsOH, 7CsCl, 10 HEPES, 3 QX-314, 2 MgATP, 0.3 Na_2GTP, yielding a chloride reversal potential of -42 mV. Miniature inhibitory post-synaptic currents (mIPSCs) and tonic currents were recorded using an internal solution containing (in mM): 140 CsCl, 10 HEPES, 3 QX-314, 4 MgATP, 0.3 Na_2GTP, yielding a chloride reversal potential of 0 mV. Voltage-clamp recordings were made at a holding potential of -20 mV for evoked IPSCs, -60mV for tonic currents and mIPSCs, and -70mV for mEPSCs. Input and series resistance were monitored throughout the experiment and recordings in which either changed significantly were discarded.

Miniature IPSCs (mIPSCs) were pharmacologically isolated using the glutamate receptor antagonists, D-2-amino-5-phosphopentanoic acid (D-APV, 50µM), or 5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate (MK-801, 10 µM) and 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX, 50 µM), plus the addition of tetrodotoxin (TTX, 1 µM) for mIPSCs and tonic current. Bicuculline methochloride (Bic, 20 µM) was added at the end of each experiment to confirm that the recorded IPSCs were mediated by $GABA_A$ receptors. mEPSCs were isolated using 30 µM bicuculline methochloride and 1 µM TTX. Drugs were perfused for a minimum of 20 minutes in order to obtain a stable baseline.

Chapter 5- Animals were deeply anesthetized with isoflurane and transcardially perfused with a sucrose-based aCSF fluid containing (in mM): 248 sucrose, 2 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 2.4 pyruvate, and 1.3 ascorbic acid (345 – 351 mOsm) for approximately 2 min. The brain was then rapidly removed and transverse 300 µM thick slices were cut using a vibratome (Leica VT1000S, Nussloch, Germany). The same sucrose-based solution was also used to prepare brain slices, with the addition of (in mM): 0.02 $_D$-APV and 1 kynurenic acid, and exclusion of pyruvate and ascorbic acid (pH 7.4; 305 – 312 mOsm). Slices were incubated for approximately one hour in room temperature oxygenated (95% O_2 / 5% CO_2) NaCl-based aCSF solution containing (in mM):125 NaCl, 2.8 KCl, 1.0 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 0.5 $CaCl_2$ and 5 $MgSO_4$, 0.02 $_D$-APV and 1 kynurenic acid (pH 7.4; 300 – 310 mOsm).

Currents were recorded from dentate granule cells visually identified with infrared-differential interference contrast optics. Recordings were performed using a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA) and filtered at 1 kHz. Currents were digitized using a Digidata 1440A A-D board (Molecular Devices, Sunnyvale, CA) and analyzed using pClamp10 software.

EPSCs were recorded using borosilicate glass electrodes (resistance 4 – 6 MΩ) filled with an internal solution containing (in mM): 130 D-Gluconic Acid, 130 CsOH, 5 CsCl, 10 HEPES, 5 CsBAPTA, 2 $MgCl_2$, 0.5 $CaCl_2$, 2 MgATP, 0.3 NaGTP. Voltage-clamp recordings were made at a holding potential of -70mV with input and series resistances monitored throughout the experiment. Currents were evoked with borosilicate glass pipettes (resistance 2 – 3 MΩ) filled with 10

µM domoate dissolved in external recording solution. Every 60 seconds, a 50 ms puff was applied approximately 70 µM from the dentate granule cell body using a Pneumatic PicoPump (PV800, World Precision Instruments, Sarasota, FL).

EPSCs were pharmacologically isolated in an antagonist cocktail containing bicuculline methochloride (Bic, 15 µM), [(2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](phenylmethyl)phosphinic acid hydrochloride (CGP55845, 1 µM), MK-801 (10 µM), plus the addition of tetrodotoxin (TTX, 1 µM). KAR-mediated currents were isolated using 1-(4-Aminophenyl)-3-methylcarbamyl-4-methyl-3,4,-dihydro-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride (GYKI 53655, 50 µM) remaining current was blocked by application of CNQX (100 µM).

2.7 FIELD POTENTIAL ELECTROPHYSIOLOGY

In contrast to whole-cell electrophysiology which occurs intracellularly, field-potential electrophysiology is performed extracellularly. Once the tissue is prepared a stimulating electrode is placed in the tissue in order to sufficiently active a population of neurons. In contrast to recording from a single neuron, field potential electrophysiology allows the researcher to record the activity of a population of neurons by placing the recording electrode within a cell body layer, or within the dendrites or axons of the neuronal population. Activation of these neurons results in a summation of the extracellular potentials creating either a field inhibitory postsynaptic potential (fIPSP) or a field excitatory postsynaptic potential (fEPSP).

Due to the fact that these recordings are extracellular, the placement of the recording electrode, relative to the stimulating electrode is crucial in order to properly interpret of the data.   Relative to the electrode, neuronal activation can create what is denoted as an extracellular sink or source. The flow of positively charged ions into a cell or dendrite leaves the extracellular space more negative, creating an extracellular sink. In order to maintain electroneutrality the sink is balanced by an extracellular source comprised of an efflux of positively charged ions into the extracellular space. Field potential recordings in the mossy fiber – CA3 synapse are at particular risk for improper electrode placement, as the CA3 region contains mossy fibers input as well as the associational/commissural fibers. Pure stimulation of the mossy fibers, elicited through activation of dentate granule cells, would evoke an extracellular sink in area CA3 *stratum lucidum*. However, if the placement of the recording electrode was not precise an extracellular source evoked from the CA3 A/C fibers would contaminant the response. The overall response would be a fEPSP comprised of both a downward sink and upward source. Thus in experiments involving the mossy fiber – CA3 synapse steps were taken to ensure pure mossy fiber stimulation and recording.

2.7.1 EXPERIMENTAL PROTOCOL FOR FIELD POTENTIAL RECORDINGS

Animals were deeply anesthetized with isoflurane and transcardially perfused with a sucrose-based aCSF solution containing (in mM): 248 sucrose, 2 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 2.4 pyruvate, and 1.3 ascorbic acid

(345 – 351 mOsm) for approximately 2 min. The brain was then rapidly removed and transverse 500 µM thick slices were cut using a vibratome (Leica VT1000S, Nussloch, Germany). The same sucrose-based solution was also used to prepare brain slices, with the addition of (in mM): 0.02 $_D$-APV and 1 kynurenic acid, and exclusion of pyruvate and ascorbic acid (pH 7.4; 305 – 312 mOsm). Slices were prepared in cold (4°C), and incubated for 30 min in heated (37°C), oxygenated (95% O_2 / 5% CO_2) sucrose-based aCSF. After 30 min incubation in heated sucrose-based aCSF slices were gradually transitioned to a room-temperature oxygenated (95% O_2 / 5% CO_2) NaCl-based solution containing (in mM): 125 NaCl, 2.8 KCl, 1.0 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 0.5 $CaCl_2$ and 5 $MgSO_4$, 0.02 $_D$-APV and 1 kynurenic acid (pH 7.4; 301 – 307 mOsm).

Following a minimum of 30 min incubation slices were placed in a submerged chamber that was continuously perfused with the warm, oxygenated (31 – 34 °C; 95% O_2 / 5% CO_2) NaCl-based solution containing (in mM): 125 NaCl, 2.7 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 0.5 $CaCl_2$ and 5 $MgSO_4$ (pH 7.4; 301 – 307 mOsm). Stimuli were 0.1 ms, cathodal, monophasic, rectangular constant current pulses delivered through monopolar, platinum-iridium stimulating electrodes (FHC Inc., Bowdoin, ME) referenced to a bath ground. fEPSPs and antidromic spikes were recorded through a borosilicate glass electrode (resistance 2 – 3 MΩ) filled with external recording aCSF. Two separate stimuli paradigms were used, 1) Mossy fiber – CA3 fEPSPs were evoked in the dentate gyrus granule cell layer every 30 sec and recorded in CA3 *stratum lucidum*. Mossy fiber fEPSPs were characterized by their large paired

pulse facilitation (Salin et al., 1996) and inhibition by the group II-selective metabotropic glutamate receptor (mGluR) agonist (2S,2'R,3'R)-2-(2'3'-Dicarboxycyclopropyl) glycine (DCG-IV, 1 µM) (Kamiya et al., 1996). 2) Recurrent mossy fiber fEPSPs in the dentate gyrus were evoked in the inner one-third of the molecular layer every 60 sec and recorded in the granule cell layer (Epsztein et al., 2005). Stimuli recorded in both CA3 and dentate gyrus were evoked at 50% of the maximal response determined though an input-output (I/O) curve. Antidromic population spikes were evoked in two distinct pathways. 1) Mossy fibers were stimulated in CA3 *stratum lucidum* and the response was recorded in the dentate granule cell layer. 2) CA3 axons were stimulated in *stratum radiatum* and the response was recorded in the CA3 pyramidal cell layer. Figure 2.2 depicts each of the four experimental paradigms utilized in Chapters 5 and 6.

Both antidromic spikes and fEPSPs were pharmacologically isolated in an antagonist cocktail containing bicuculline methochloride (Bic, 20 µM) and MK-801 (10 µM). AMPA receptor mediated transmission was blocked by application of GYKI53655 and remaining KAR-mediated transmission was blocked by application of (*S*)-1-(2-Amino-2carboxyethyl)-3-(2-carboxythiphene-3-yl-methyl)-5-methylprimidine-2,4-dione (UBP310, 3 µM) or (*S*)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxy-5-phenylthiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione (ACET, 300 nM) and residual transmission was blocked by application of CNQX (100 µM).
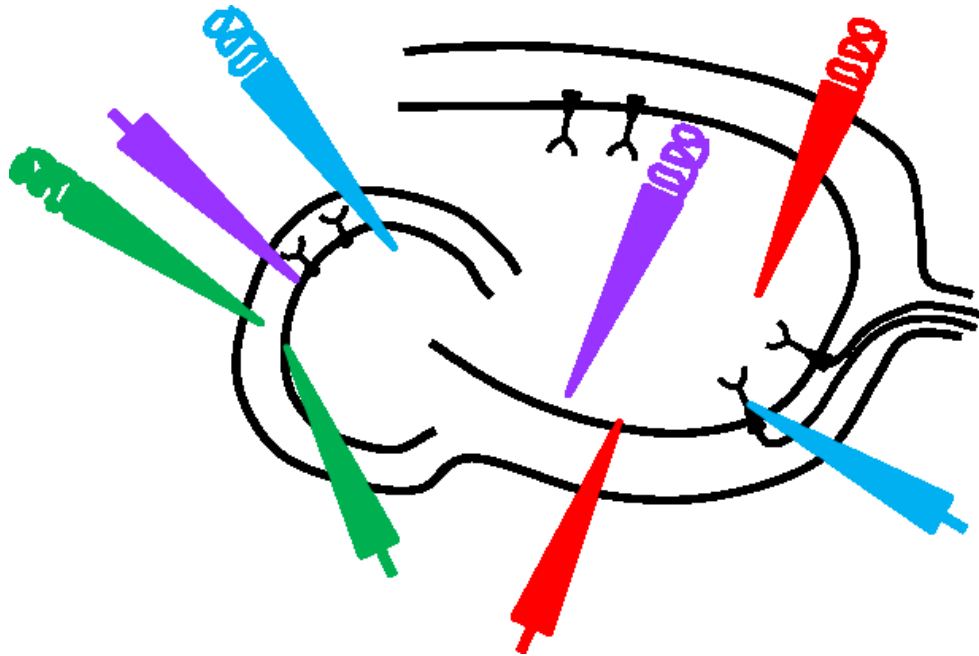
Figure 2.2. Experimental paradigms for field potential electrophysiology. *Blue electrodes*: MF – CA3 fEPSPs were elicited by stimulation of the mossy fibers and recorded in CA3 *stratum lucidum*. To limit activation of the recurrent A/C pathway stimulation of the mossy fibers originated near the dentate granule cell layer. *Green electrodes*: Recurrent mossy fiber collaterals were activated by stimulation of the inner one-third of the molecular layer and recorded in the dentate granule cell layer. *Purple electrodes:* The mossy fiber antidromic spike was elicited through stimulation of the mossy fibers in CA3 *stratum lucidum* and recorded in the dentate granule cell layer. *Red electrodes*: CA3 antidromic spikes were produced by stimulation of the CA3 axons located in *stratum radiatum* and recorded in the CA3 pyramidal cell layer.

2.8 WESTERN BLOT ANALYSIS

The technique of western blotting (i.e. protein blotting, immunoblotting) was first developed during the late 1970's and has become a widely used tool to determine the presence and abundance of particular proteins (MacPhee, 2010). Western blotting begins with successful extraction and isolation of a particular lysate followed by separation onto a polyacrylamide gel through electrophoresis. The proteins are then transferred onto a membrane and probed with antisera to detect the presence of particular proteins. Determining the proper antisera can require several preliminary runs as both polyclonal and monoclonal antisera are available, each bringing its own advantages and disadvantages. However, a chief difference between the antisera is their specificity for an individual protein of interest. Once the appropriate antisera is bound to the protein, its relative expression can be determined through processes such as enhanced chemiluminescence (ECL). The peroxidase conjugated secondary antisera serves to oxidize the luminol substrate within the ECL to emit light which is then captured onto film and measured using densitometry. In general, western blotting is a widely used and acceptable technique to determine relative expression and abundance of proteins. The following sections describe in detail the western blotting protocol utilized in Chapter 4 to determine differences in kainate receptor subunit abundance within specific brain regions following pilocarpine-induced status epilepticus.

2.8.1 MEMBRANE PREPARATION

Animals were anesthetized, the brain removed, and immediately placed on dry ice. Brains were cut at 100 µM on a freezing microtome (Microm, Waldorf, Germany). Punches from prefrontal cortex, amygdala and striatum were collected, while hippocampi were removed and micro-dissected into dentate gyrus (DG), CA1 and CA3 regions. Microdissected regions collected from the hippocampus are outlined in Figure 2.3. All tissue collections were immediately placed in ice-cold homogenization buffer (0.32 M sucrose, 2mM EDTA, 2mM EGTA, 20mM HEPES, pH 7.6, containing 1:100 protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). The samples were then homogenized and centrifuged for 10 min at 500 x g at 4ºC to remove the nuclear fraction. The supernatant containing the total membrane fraction was centrifuged at 31,000 x g for 30 min at 4ºC. The resulting pellet, crude plasma membrane, was re-suspended in phosphate buffered saline (PBS) and stored at -80ºC prior to use. Protein concentrations were determined by using the Bradford method utilizing BSA (Bovine Serum Albumin, Thermo Scientific) as a standard. Linear regression analysis was performed in Microsoft Excel to extrapolate the unknown protein concentration.

2.8.2 SDS-PAGE AND ANTIBODY DEVELOPMENT

Aliquots of 2 – 10 µg of crude plasma membrane proteins were denatured by diluting them in a 1:1 ratio with BioRad Laemmli Sample Buffer with 5% β-
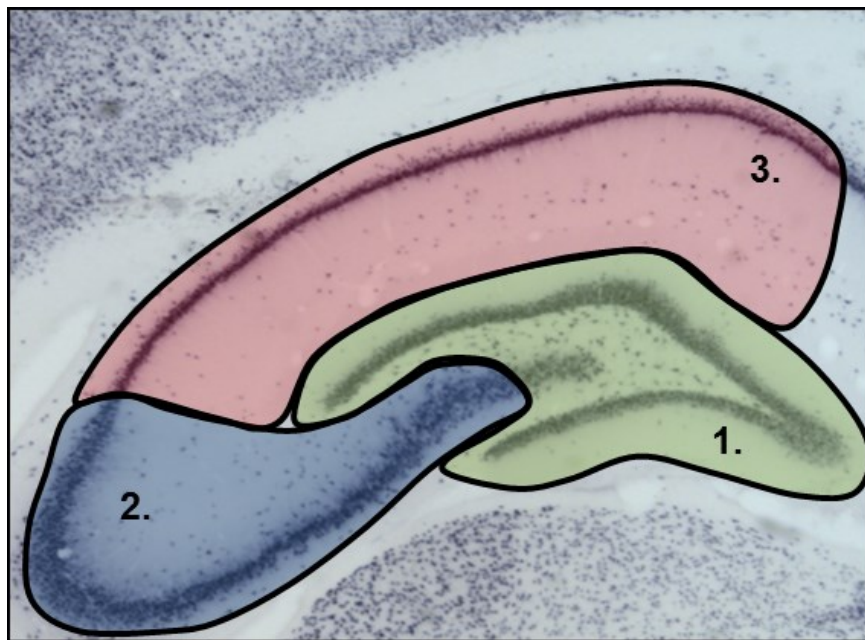
Figure 2.3. Microdissected hippocampal regions for western blot analysis. Hippocampal sections 100 µM thick were microdissected into **Green, 1.** Dentate gyrus **Blue, 2.** CA3 and **Red, 3.** CA1 regions prior to homogenization and processing for western blot analysis. This process allowed for increased resolution of region specific alterations in KAR subunit expression following pilocarpine-induced SE.

mercaptoethanol. Samples were then heated at 95ºC for 5 min and loaded into pre-cast polyacrylamide gels (4 – 15%). Running buffer (in mM: 25 Tris, 240 Glycine, 0.1% SDS) was added to the appropriate tank, BioRad mini PROTEAN-Tetra or BioRad Criterion, and run using the BioRad Power Pack HC for at least 30 min at 175 – 200 V. Following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein separation, proteins were transferred to polyvinylidene fluoride (PVDF) membranes using the BioRad Trans-Blot SD Semi-Dry Transfer Cell. Protein transfer was completed using the BioRad Power Pack HC for 30 min at 25V. Transfer buffer contained (in mM): 25 Tris, 192 Glycine, and 20% methanol. All equipment was acquired from Bio-Rad Laboratories, Inc.

Following transfer, PVDF membranes were blocked in tris-buffered saline (TBS) plus 5% non-fat dry milk for 60 min at room temperature. PVDF membranes were incubated with primary antisera (in TBS/5% non-fat dry milk) overnight at 4ºC with gentle shaking. Primary antibodies are listed in Table 2.2. Normalization for protein loading was performed using a mouse monoclonal primary antibody selective for β-actin. Following overnight incubation membranes were washed with TBS plus 0.05% Tween 20 (TBS-T) and incubated in peroxidase-labeled, species-specific secondary antibodies. PVDF membranes were washed with TBS-T and developed using enhanced chemiluminescence reagents (ECL, Pierce) as described by the manufacturer. Images were captured on Kodak BioMax XAR film and optical densitometric analysis was performed using ImageJ 1.47v (National Institutes of Health, USA).

Table 2.2. Primary antibodies for western bot analysis

Ligand	Manufacturer; Catalog Number	Primary Concentration
GluK2/3	Millipore; 04-921	1:8,000
GluK4	SantaCruz; sc8916	1:200
GluK5	Millipore; 06-315	1:8,000
GFAP	Cell Signaling; 3670	1:8,000
β-Actin	Sigma; A5441	1:50,000

2.9 IMMUNOHISTOCHEMISTRY

The art of immunohistochemistry (i.e. immunostaining) was first demonstrated in the mid 1930's, and the first study was published in the mid 1940's (Matos et al., 2010). Since this time the technique has evolved into a commonly used method used to determine the specific expression patterns of proteins, enzymes or peptides within cells and tissue samples. In order to generate reproducible and reliable immunohistochemical data, several preliminarily experiments may be required to determine the appropriate type of tissue fixation, sectioning, storage, antisera and analysis. Following tissue fixation, commonly utilizing paraformaldehyde, tissue may be immediately sectioned and stored for several days while antibody detection is performed. Other commonly used techniques involve cryoprotecting the tissue which allows for more long-term storage while analysis is conducted. In addition, antigen retrieval steps may be performed which serve to uncover antigenic sites which may have been shielded during fixation or through handling of the tissue. One must also consider the ability of the antisera to permeate the tissue and/or cell membrane. Various concentrations and types of detergents, such as Triton-X-

100, can be utilized to increase the permeability and penetration of the antisera. In general, the protein of interest and the available antisera will often dictate the types of fixation, sectioning and storage of the tissue samples. The use of immunohistochemistry in determining protein localization and expression patterns is widely used and simply requires that the protocol be amended to the particular protein of interest. The following sections describe in detail the immunohistochemical protocol used in Chapter 4 to determine alterations in kainate receptor subunit expression within the hippocampus following pilocarpine-induced status epilepticus.

2.9.1 PERFUSION AND TISSUE FIXATION

Rats were deeply anesthetized using the anesthetic isoflurane. Once anesthetized, a lateral incision to the lower abdomen was made which extended upwards to expose the chest cavity and the heart. A hemostatic clamp was placed on the descending vena cava to prevent perfusate flow to the lower extremities. A 20 x ½ gauge needle attached to the perfusion pump was inserted through the apex of the left ventricle and held firmly in place with a hemostat. Once inserted, the right atrium was clipped to allow perfusate flow. Ice cold, oxygenated (95% O_2 / 5% CO_2) 0.05M PBS (pH 7.4) was perfused at approximately 60mL/min for 4 – 5 min, followed by the ice cold fixative (0.1M phosphate buffer / 4% paraformaldehyde), for approximately 20 min. Whole brains were removed and blocked to remove olfactory lobes and cerebellum. The

remaining portion of the brain was post-fixed at 4ºC for 3 hours in tissue fixative and then transferred to 0.1M phosphate buffer (PB) until sectioned.

Brains were sectioned at 50 μM-thick coronal sections on a vibratome (Leica VT1000S, Nussloch, Germany). Sectioning was performed in 0.1M PB and sections were stored at 4 ºC in 0.1M PB and 0.1% sodium azide to inhibit bacterial growth until used for immunohistochemical processing.

2.9.2 IMMUNOPEROXIDASE

Free floating tissue sections were transferred from 0.1M PB/0.1% sodium azide to TBS (0.08M Tris Base, 0.42M Tris Hydrochloride, 1.54M NaCl) for approximately 40 minutes (4 x 10 min). Tissue was then exposed to an antigen retrieval step in which sections were placed for 5 min in a Na$^+$-Citrate buffer (10 mM Na-Citrate) heated to 85ºC. Following the 5 min incubation sections were cooled at room temperature for 5 – 10 min and returned to TBS solution for approximately 40 min (4 x 10 min) to remove excess Na$^+$-Citrate buffer. Antigen retrieval was performed prior to labeling with GluK2/3, GluK4 and GluK5 labeling only. Tissue was then treated with a methanolic peroxide (90 mL TBS, 10 mL 100% methanol, 2 mL 30% hydrogen peroxide) rinse for 15 min at room temperature. This step was performed to reduce endogenous peroxidase in the tissue which aids in reducing background exposure during development. Following the methanolic peroxide step, tissue was then rinsed for 30 min (3 x 10 min) in TBS, and then blocked for 1 hour in solution containing TBS, 3 – 5% donkey serum, and 0.1 – 0.2% Triton-X-100 (TBS+). The concentration of

donkey serum and Triton-X-100 is dependent upon the primary antibody; see Table 2.3 for further detail. Once blocking was complete the tissue was transferred to TBS+ containing the primary antibody of interest at the appropriate concentration. Tissue was gently rocked overnight at room temperature. Following incubation with the primary antibody, tissue was rinsed for 30 min in TBS (3 x 10 min), and incubated for 1.5 hour at room temperature in the appropriate biotinylated secondary antibody in TBS+ (1:1,000, Jackson ImmunoResearch). Tissue was then rinsed again for 30 min in TBS (3 x 10 min) and incubated for 1 hour in TBS containing 0.1 – 0.2% Triton-X-100 and horseradish peroxidase-conjugated streptavidin (1:1600, Jackson ImmunoResearch). Following the tertiary incubating tissue was rinsed for 50 min in TBS (5 x 10 min) and developed using nickel ammonium sulfate/cobalt chloride intensified diaminobenzidine (DAB, Sigma, St. Louis, MO; 0.025% DAB, 50% TBS, 50% Nickel-Cobalt). Reaction was initiated by the addition of 0.3% hydrogen peroxide. Development time was determined on one slice from a sham-treated animal and the appropriate development time was used for both sham and pilocarpine-treated tissue. Development times were dependent upon the primary antibody in use.

2.9.3 SLIDE PREPARATION AND ANALYSIS

Free floating tissue sections were mounted on slides with a 0.15% gelatin solution and allowed to dry overnight. Once dry, tissue went through a serial dehydration step with increasing concentrations of ethanol (50%, 70%, 95%,

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100%, 100%; 3 min/bath) followed by two, 10 min incubations in Xylene. Slides were immediately cover-slipped using cover glass and DPX mounting solution (Sigma, St. Louis, MO) and allowed several days to dry prior to analysis and long-term storage. Analysis of immunohistochemical data was performed on MCID Elite 7.0 analysis system (Imaging Research, Inc., St. Catharines, Canada).

Table 2.3. Primary antibodies for immunohistochemical processing

Primary Antibody	Manufacturer; Catalog Number	Primary Concentration	[Donkey Serum]	[Triton-X-100]
GluK2/3	Chemicon; AB5683	1:1,000	5%	1%
GluK4	SantaCruz; sc8916	1:300	5%	1%
GluK5	Millipore; 06-315	1:1,000	5%	1%
GFAP	Cell Signaling; 3670	1:1,000	3%	2%
ZnT-3	Synaptic Systems; 197-002	1:300	3%	2%
NeuN	Millipore; MAB377	1:2,000	3%	2%

2.10 STATISTICAL ANALYSIS

Electrophysiological data was analyzed using pClamp10 (Molecular Devices, Sunnyvale, CA), Origin7.5 (OriginLab, Northampton, MA), GraphPad Prism (v. 4.00, GraphPad Software, San Diego, CA) and Mini Analysis 6.0 (Synaptosoft, Inc., Decatur, GA). Statistical significance was determined using one-way analysis of variance (ANOVA) with post-hoc Bonferroni tests or the

Student's *t*-test, with $\alpha < 0.05$. Values are depicted as the mean ± standard error of the mean (SEM).

Data collected from western blot analysis and immunohistochemistry was analyzed using MCID ELITE 7.0 (Imaging Research, Inc., St. Catharine's, Canada) or ImageJ 1.47V (National Institute of Health, USA). Optical density measurements were obtained using a standard curve. Data depicting changes in KAR subunit expression were obtained by averaging data collected from a minimum of two separate western blots.

CHAPTER 3

Effects of Stiripentol on Benzodiazepine- Refractory Status Epilepticus[1]

3.1 Introduction

Benzodiazepines (BZDs) are the first-line therapy for the treatment of status epilepticus (SE) and reduce seizure activity by enhancing $GABA_A$ receptor- mediated inhibition. However, clinical studies have revealed that nearly 35% of patients suffering from SE are refractory to BZDs (Mayer et al., 2002). While many factors could contribute to this pharmacoresistance, clinical and animal studies have demonstrated that the likelihood of a patient responding to BZDs decreases as the duration of SE increases. Sensitivity to BZDs requires the presence of γ2-containing $GABA_A$ receptors (Mayer et al., 2002; Sieghart and Sperk, 2002). However, following the onset of SE, these BZD sensitive, γ2-containing receptors are internalized (Goodkin et al., 2008). This internalization reduces the number BZD-sensitive receptors on the membrane surface, and thus during prolonged SE the efficacy of BZDs is significantly reduced. While BZD-sensitive receptors are internalized during SE, other populations of BZD-insensitive $GABA_A$ receptors, such as those containing α4 and δ subunits, remain

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[1]Stiripentol is anticonvulsant by potentiating GABAergic transmission in a model of benzodiazepine-refractory status epilepticus. Grosenbaugh, D.K., and Mott, D.D. 2013. *Neuropharmacology*. 67: 136-143.
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functional (Goodkin et al., 2008).

Stiripentol (STP, Diacomit) is an anticonvulsant drug approved for use in Europe for the treatment of severe myoclonic epilepsy in infancy (Dravet syndrome). Typically, STP is co-administered with other anti-epileptic drugs (AEDs) as it has been shown to increase their efficacy through inhibition of several hepatic cytochrome P450 enzymes (Tran et al., 1997). However, several animal models have demonstrated that STP may provide anticonvulsant effects when administered alone via enhancement of GABAergic inhibition (Poisson et al., 1984; Quilichini et al., 2006). In support of this finding, Fisher (2009) reported that STP is a positive allosteric modulator of $GABA_A$ receptors containing any of the α, β or γ-subunits, with greatest activity at α3 or δ-containing receptors.

The subunit selectivity profile of STP suggests its ability to potentiate both phasic and tonic GABAergic inhibition. In addition, STP may retain this ability to potentiate GABAergic inhibition during prolonged SE when γ2-containing, BZD-sensitive, $GABA_A$ receptors have been internalized. The goal of the current project was to determine the ability of STP to terminate SE in BZD-resistant rats, and to understand the mechanism by which STP may be anticonvulsant.

3.2 HYPOTHESIS

STP has been previously demonstrated to potentiate $GABA_A$ receptors composed of any of the α, β, or δ-subunits. Given that during status epilepticus $GABA_A$ receptors containing α4 and δ subunits remain functional, we

hypothesize that STP will remain effective during prolonged SE when seizures have become resistant to benzodiazepines.

3.3 MATERIAL AND METHODS

3.3.1 BEHAVIORAL EXPERIMENTS

Male Sprague-Dawley rats (15 – 24, and 57 – 63 days old) received intraperitoneal (i.p.) injections of lithium-chloride (LiCl, 127 mg/kg) followed 15 – 20h later by scopolamine methyl bromide (2 mg/kg). Status epilepticus was then induced after 15 – 30 min by injection with pilocarpine (60 mg/kg). Diazepam (DZP; 0.3 – 100 mg/kg) or stiripentol (STP; 10 – 1000 mg/kg) was administered either at the onset of stage 3 seizures (brief SE) to assess its effect on brief behavioral convulsions or 45 min after stage 3 seizure onset (prolonged SE; pSE) to assess its effect during prolonged seizures. Behavioral seizure scores were recorded throughout the course of SE. Seizure scores reported in this study correspond to scores 15 min following administration of STP or DZP.

3.3.2 DETERMINATION OF STP CONCENTRATIONS

Four separate groups of male Sprague-Dawley rats: 1) naïve juvenile (20 – 27 days old), 2) prolonged SE-experienced juvenile (20 – 21 days old), 3) naïve adult (62 – 63 days old) and 4) prolonged SE-experienced adult (61 days old), were used to determine brain and plasma concentrations of STP. Fifteen minutes following i.p. administration of 300 mg/kg STP, naïve and prolonged SE rats were

anesthetized and decapitated in order to collect brain and blood samples. A detailed description is provided in Chapter 2.4.

3.3.3 WHOLE-CELL RECORDINGS

Transverse (300 µM thick) hippocampal slices were prepared from 5 – 65 day old rats as described in Chapter 2.6. Rats were either naïve or had experienced prolonged SE, in which slices were prepared 45 min after the first stage 3 seizure. Animals were selected for slice experiments only if they had an average seizure score between 3 and 4 during the prolonged SE.

GABA IPSCs were evoked by stimulation in *stratum moleculare* of the dentate gyrus near the recording electrode. IPSCs were pharmacologically isolated using the glutamate receptor antagonist D-APV (50 µM), or MK-801 (10 µM), and CNQX (50 µM), plus the addition of tetrodotoxin (TTX, 1 µM) for miniature IPSCs (mIPSCs) and tonic current. Bicuculline methochloride (20 µM) was added at the end of each experiment to confirm that the recorded IPSCSs were mediated by GABAA receptors. Miniature EPSCs (mEPSCs) were isolated using 30 µM bicuculline methochloride and 1 µM TTX. Drugs were perfused for a minimum of 20 min in order to obtain a stable baseline.

Stiripentol was a generous gift from Biocodex. Scopolamine methyl bromide, pilocarpine, kynurenic acid, diazepam, clobazam and all salts were purchased from Sigma Chemical Company (St. Louis, MO). D -APV, MK-801, CNQX, flumazenil, tetrodotoxin and bicuculline methochloride were purchased from Ascent Scientific (Princeton, NJ). For behavioral experiments all drugs were

made fresh on the day of the experiment and dissolved in 0.9% saline, except for STP which was dissolved in 20% propylene glycol and 80% ethanol. All drugs were administered via i.p. injection. For electrophysiological experiments drugs were bath applied in the perfusion medium. Drugs were diluted from frozen stocks in water except STP, DZP and clobazam which were diluted from frozen stocks in dimethyl sulfoxide (DMSO). The final concentration of DMSO in working solutions was less than 0.3%.

3.4 RESULTS

3.4.1 STP TERMINATION OF BRIEF BEHAVIORAL SE IS AGE DEPENDENT

Behavioral convulsions began approximately 3 min after injection of pilocarpine. Behavioral seizures progressed through stages, as previously described in Table 2.1. Stage 3 behavioral convulsions, characterized by forelimb clonus without rearing and falling began approximately 20 min after administration of pilocarpine. Treatment with stiripentol at the start of stage 3 seizures was effective in reducing seizures at doses above 10 mg/kg with a dose of 1000 mg/kg terminating seizures in all tested animals (n = 4). In contrast, treatment with vehicle alone (20% propylene glycol/80% ethanol) did not reduce seizures. Dose-response analysis was performed on young rats (15 – 23 days old) by administration of STP (10 – 1000 mg/kg) at the onset of stage 3 seizures and yielded an ED_{50} of 100 (58.1 – 171.8) mg/kg; Figure 3.1A. To determine the age-dependence of this effect, STP was also tested in adult animals (61 – 63 days old). In these animals the ED_{50} for STP was shifted approximately 4 fold to
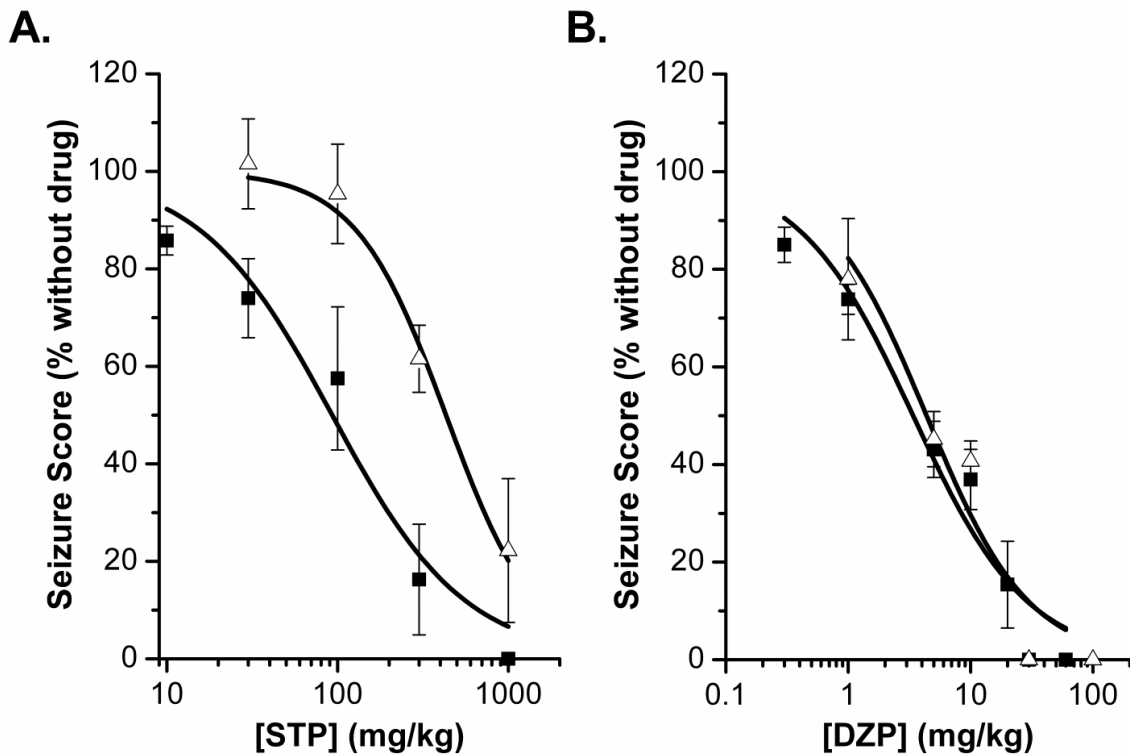
Figure 3.1. STP and DZP both terminate brief behavioral convulsions. The anticonvulsant effect of STP and DZP on brief SE was compared in young (15 – 23 days old) and adult (59 – 63 days old) male rats. Seizure scores in the presence of STP or DZP were expressed as a percentage of the seizure score at the same time point from animals that did not receive drug. **A.** STP dose-dependently reduced seizure score in young (*closed squares, n* = 5 – 10) and adult animals (*open triangles, n* = 5 – 9). The anticonvulsant effect of STP in adult animals was shifted significantly to the right. **B.** DZP dose-dependently reduced seizure score in young (*closed squares, n* = 4 – 19), and adult animals (*open triangles, n* = 3 – 10). DZP was similarly effective in both age groups.

the right (ED_{50} = 377.6 (256.4 − 555.9) mg/kg; Figure 3.1A). The confidence intervals of the ED_{50} did not overlap, indicating a real difference in the STP potency with maturation, as previously reported (Cao et al., 2012). In comparison, the 95% confidence intervals for DZP in both young (21 − 23 days old; ED_{50} = 3.1 (2.2 − 4.2) mg/kg; Figure 3.1B) and adult (59 − 63 days old; ED_{50} = 4.2 (2.2 − 7.8) mg/kg; Figure 3.1B) rats did overlap, indicating no real difference in the ED_{50} for DZP during maturation. These findings indicate that, like DZP, STP is able to terminate brief SE in the Li-Pilo model.

3.4.2 PHARMACORESISTANCE DEVELOPS TO DZP BUT NOT STP DURING PROLONGED SE

As with status epilepticus in humans, seizures in the Li-Pilo model become pharmacoresistant to DZP during prolonged SE (Walton and Treiman, 1988; Kapur and Macdonald, 1997; Jones et al., 2002). In agreement, we found that in young (15 − 23 days old) animals the ED_{50} for DZP increased about 14 fold when it was administered 45 min after the first stage 3 behavioral seizure (ED_{50} = 3.1 (2.2 − 4.2) mg/kg after brief SE; ED_{50} = 43.1 (33.0 − 56.0) mg/kg after prolonged SE; Figure 3.2A,B). In contrast, we observed no real difference in the ED_{50} for STP during prolonged SE (ED_{50} = 100 (58.1 − 171.8) mg/kg after brief SE; ED_{50} = 216.3 (136.8 − 342.0) mg/kg after prolonged SE; Figure 3.2A,B). Similarly, in adult (57 − 63 days old) animals the ED_{50} for DZP increased 11 fold after prolonged SE (ED_{50} = 4.2 (2.2 − 7.8) mg/kg after brief SE; ED_{50} = 47.2 (33.2 − 67.1) mg/kg after prolonged SE; Figure 3.2C,D), while the ED_{50} for STP was not
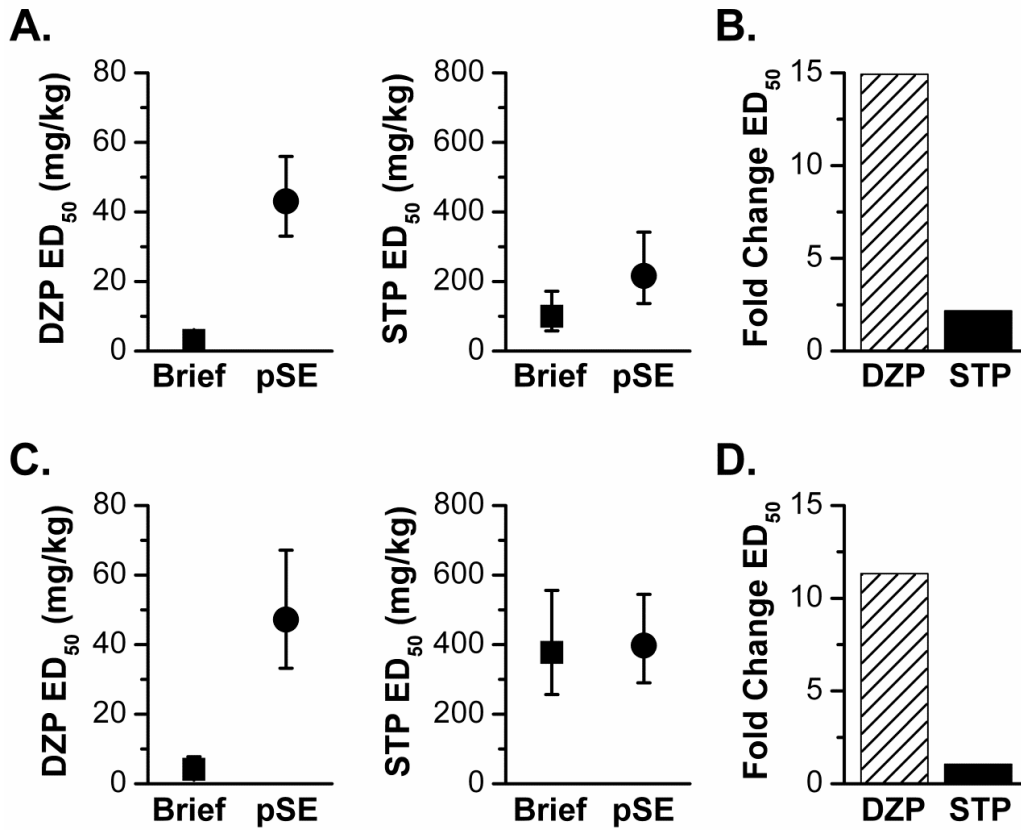
Figure 3.2. Less pharmacoresistance develops to STP than to DZP following prolonged SE. SE was induced in young (15 – 23 days old, top panels) and adult (57 – 63 days old, bottom panels) male rats. DZP or STP was administered at different doses at the onset of the first stage 3 behavioral convulsion (*closed squares*) or 45 min following the first stage 3 convulsion (*closed circle*) and the dose response curve measured. ED_{50} values for anticonvulsant effect of DZP (left panel, n = 3 – 19) and STP (right panel, n = 5 – 29) during brief (Brief) and prolonged SE (pSE) in **A.** young rats and **C.** adult rats. Fold change in ED_{50} for DZP and STP during prolonged SE relative to brief SE in **B.** young rats and **D.** adult rats.

different (ED_{50} = 377.6 (256.4 – 555.9) mg/kg after brief SE; ED_{50} = 397.2 (290.4 – 544.5) mg/kg after prolonged SE; Figure 3.2C,D).

3.4.3 BRAIN-PLASMA STP

We next wanted to determine if alterations in the bioavailability of STP during maturation or prolonged SE affected its apparent efficacy. To address this possibility, we measured plasma and brain concentrations of STP following administration of 300 mg/kg STP. An initial pilot study in young rats (23 – 27 days old, n = 4 – 5), showed that exposure to brief seizure activity modified neither STP plasma levels nor the brain/plasma ratios compared to animals that did not experience any seizure activity. To further explore this, a larger study of 43 animals was then carried out. This study revealed that STP plasma concentration was not different in juvenile (96 ± 4.1 μg/mL) or adult (86.8 ± 3.6 μg/mL, p > 0.05) animals (Table 3.1). The brain/plasma ratio of STP was also not different between juvenile (0.98 ± 0.04) and adult (0.91 ± 0.04) animals (p = 0.41) and was similar to that previously reported in adult animals (Shen et al., 1990; Shen et al., 1992; Arends et al., 1994). Following prolonged SE, neither the plasma concentration (100.7 ± 11.4 μg/mL, p = 0.67) nor the brain/plasma ratio (1.01 ± 0.09, p = 0.72 compared to naïve animals) changed in juvenile animals (Table 3.1). In contrast, in adult animals the brain/plasma ratio for STP decreased by approximately 20% following prolonged SE (naïve: 0.91 ± 0.04, post-SE: 0.70 ± 0.11; p = 0.043).

Table 3.1. Plasma and brain concentrations of STP

Treatment Groups	[Brain] μg/mL	[Plasma] μg/mL	Brain/Plasma Ratio
Juvenile, Naïve	94.6 ± 6.2 (n=14)	96.0 ± 4.1 (n=14)	0.98 ± 0.04 (n=14)
Juvenile, prolonged SE	115.3 ± 16.8 (n=9)	100.7 ± 11.4 (n=10)	1.01 ± 0.09 1.02 (n=9)
Adult, Naive	80.1 ± 5.6 (n=12)	86.8 ± 3.6 (n=12)	0.91 ± 0.04 (n=12)
Adult, prolonged SE	68.0 ± 17.6 (n=7)	85.1 ± 12.9 (n=7)	0.70 ± 0.11* (n=7)

*$p < 0.05$

3.4.4 STP POTENTIATES EVOKED GABA$_A$ RECEPTOR MEDIATED IPSCS

Given the ability of STP to potentiate GABA$_A$ receptors (Quilichini et al., 2006; Fisher, 2009), we hypothesized that STP acted as an anticonvulsant in SE by enhancing GABAergic inhibition. To test this hypothesis we characterized the effects of STP on GABA$_A$ receptor mediated currents in dentate granule cells.

The effect of STP on evoked IPSCs in dentate granule cells in young (20 – 26 day old) naïve animals was examined in the presence of glutamate antagonists (CNQX, 50 μM and D-APV, 50 μM) to block AMPA/kainate and NMDA receptors, respectively. Bicuculline methochloride (20 μM) completely blocked this IPSC, indicating that it was mediated by GABA$_A$ receptors. DZP (1 μM) increased its decay time (145.6 ± 10.4% of control, n = 5; $p < 0.01$; Figure 3.3), further confirming that it was mediated by GABA$_A$ receptors. STP (100 μM) had no effect on the IPSC rise time (10 – 90% rise time: 118 ± 11% of control, n = 6; p = 0.13) or amplitude (92 ± 7% of control, n = 6; p = 0.22). However, STP
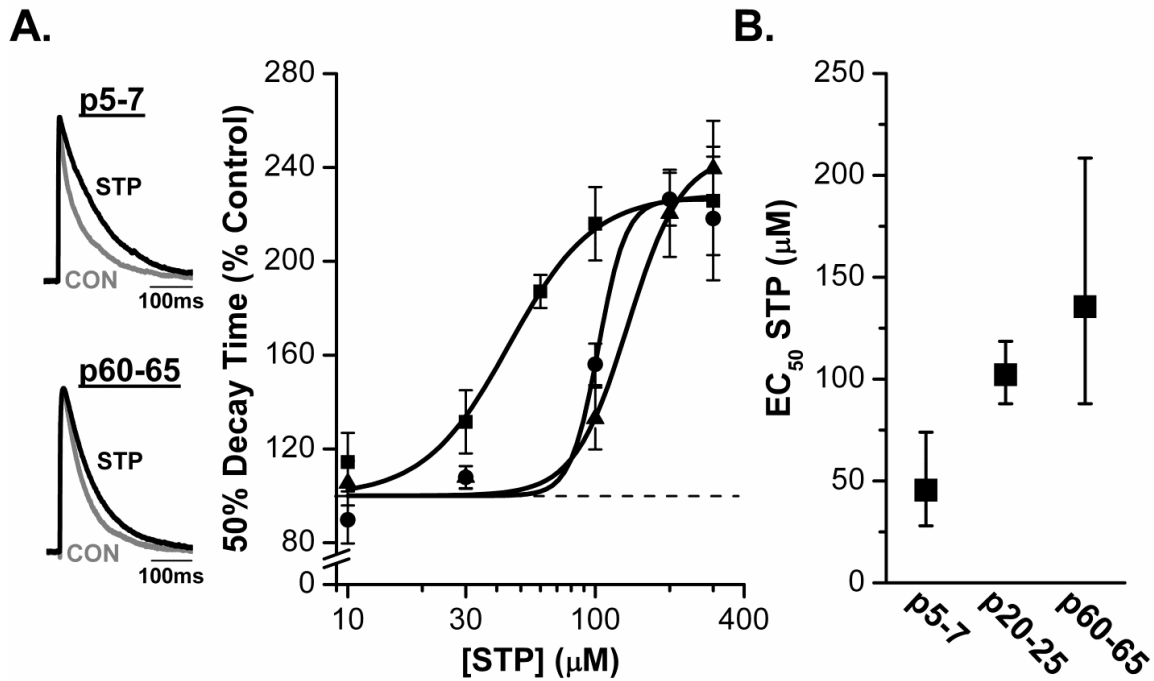
Figure 3.3. STP prolongs IPSC decay in an age-dependent manner. **A.** *Left*, Sample IPSC waveforms from 5 – 7 and 60 – 65 day old animals in the absence (CON) and presence of 100 µM STP (STP). *Right*, Concentration response curves showing the effect of STP on the 50% decay time of the evoked IPSC in the three age groups tested; postnatal day 5 – 7 (*closed squares*, *n* = 4 – 7), postnatal day 20 – 25 (*closed circles*, *n* = 5 – 9) and postnatal day 60 – 65 (*closed triangles*, *n* = 4 – 8). **B.** Plot of the EC_{50} and 95% confidence intervals for each of the three age groups.

significantly prolonged the IPSC decay (156 ± 8.9% of control, n = 9; p < 0.01; Figure 3.3), in agreement with a previous report (Quilichini et al., 2006). This increase in decay time was concentration dependent (EC_{50} = 103 μM (85 − 126 μM), n = 5 − 9). While STP had no effect on IPSC amplitude at lower concentrations, at the highest concentration tested (700 μM) STP significantly reduced IPSC amplitude (62.5 ± 4.6%, n = 6; p < 0.001).

Since the anticonvulsant effect of STP was age-dependent, we compared the ability of STP to prolong IPSC decay time in animals of different ages (5 − 8 days old, 20 − 26 days old, and 59 − 65 days old; Figure 3.3A). STP potentiated IPSCs in granule cells from animals that were postnatal 5 − 8 days to a greater extent than it potentiated IPSCs in animals from either of the other two age groups (Figure 3.3B). The ability of STP to produce greater potentiation of IPSCs in younger animals was similar to the age-dependence of its anticonvulsant effect.

3.4.5 ACTION OF STP IS INDEPENDENT OF THE BZD SITE ON THE GABA$_A$ RECEPTOR

Stiripentol is approved in Europe for use as add-on therapy in Dravet Syndrome, and is commonly administered with the 1,5-benzodiazepine, clobazam (CLB) (Chiron et al., 2000; Ng and Collins, 2007). We investigated whether CLB and STP act through a common site on the GABA$_A$ receptor by measuring whether a maximally effective concentration of CLB occluded the effect of STP. STP alone prolonged IPSC decay time (153.3 ± 15.2% of control,

p < 0.001). CLB maximally potentiates GABA$_A$ currents at a concentration of about 10 µM (Nakamura et al., 1996). At this concentration CLB alone also significantly prolonged IPSC decay time (187.4 ± 17.8% of control, p < 0.001; Figure 3.4A). Application of STP (100 µM) in the presence of CLB (10 µM) significantly increased the IPSC decay time beyond that of CLB alone (350.8 ± 38.2% of control, p < 0.001; Figure 3.4A), suggesting that these drugs act at separate loci.

In further support of this conclusion, we found that the benzodiazepine-site antagonist flumazenil (FMZ) had no effect on IPSC decay time (94.3 ± 3% of control, p > 0.05), but blocked IPSC prolongation produced by 1 µM DZP (85.7 ± 3.2%, p > 0.05) and 10 µM CLB (96.5 ± 4.6%, p > 0.05; Figure 3.4B). However, FMZ did not prevent IPSC prolongation by STP (200.5 ± 18.3%, p < 0.001; Figure 3.4B). In agreement with Quilichini et al. (2006), these findings support the conclusion that STP does not interact with the BZD site of the GABA$_A$ receptor.

3.4.6 GABAERGIC POTENTIATION BY STP REMAINS EFFECTIVE FOLLOWING PROLONGED SE

The ability of STP to retain anticonvulsant potency during prolonged SE could be explained by the continued ability of the drug to potentiate the IPSC during SE. To investigate this possibility we compared the effect of STP on IPSCs in dentate granule cells from naïve animals and from animals that had experienced 45 min of continuous seizure activity (Figure 3.5A). STP (100 µM) significantly potentiated the IPSC in granule cells from both groups of animals
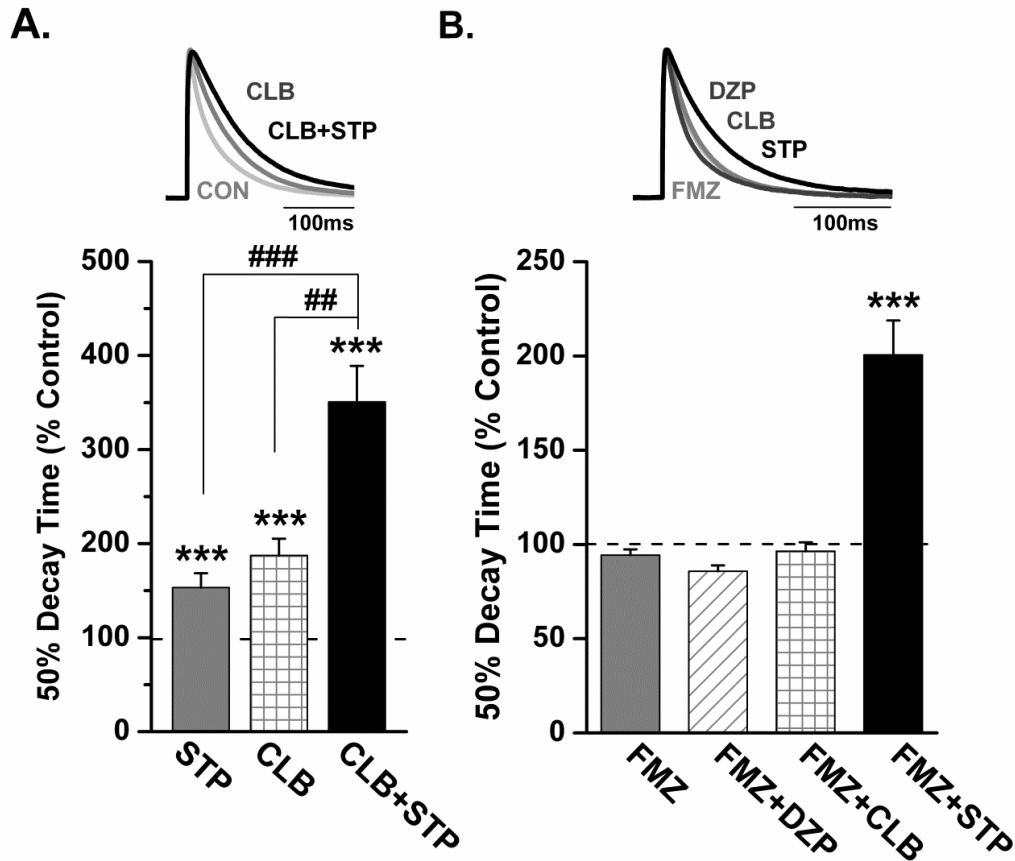
Figure 3.4. STP potentiation of GABAergic transmission is independent of the BZD-binding site. **A.** STP (100 µM, n = 7; ***p < 0.001) and CLB (10 µM, n = 7; ***p < 0.001) increased the 50% decay time of the evoked IPSC. Co-application of STP (100 µM) and CLB (10 µM) significantly enhanced decay time compared to control (n = 8; ***p < 0.001) as well as compared to either STP (###p < 0.001) or CLB alone (##p < 0.01). **B.** FMZ (10 µM) alone did not alter IPSC decay time (n = 10; p > 0.05), but blocked potentiation by DZP (1 µM, n = 4) or CLB (10 µM, n = 4). In contrast, STP (100 µM, n = 4) continued to prolong IPSC decay in the presence of 10 µM FMZ (***p < 0.001).
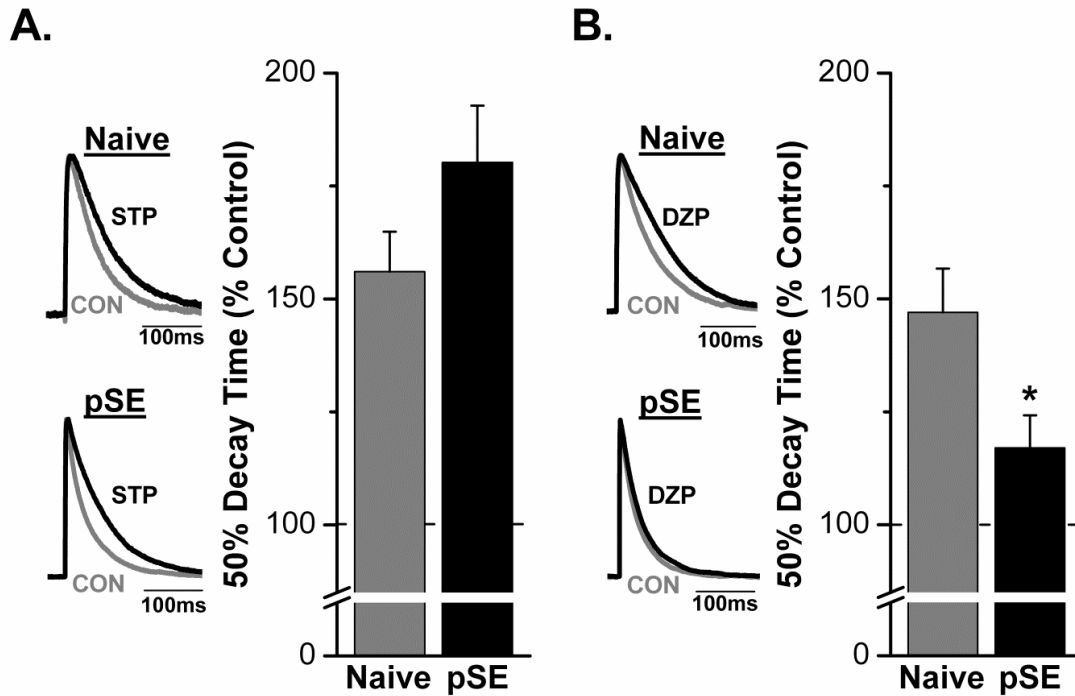
Figure 3.5. STP continues to potentiate phasic GABAergic transmission following prolonged SE. **A.** *Left*, Sample traces from naïve and prolonged SE-experienced animals demonstrate similar potentiation by 100 µM STP. *Right*, STP (100 µM) similarly potentiates IPSC decay in both naïve (*gray bar, n* = 9) and prolonged SE-experienced animals (*black bar, n* = 4). **B.** *Left*, Sample traces from naïve and prolonged SE-experienced animals demonstrate a lack of potentiation by 300 nM DZP in prolonged SE-experienced animals. *Right*, Potentiation of IPSC decay by DZP (300 nM) following prolonged SE (*black bar, n* = 10) is significantly (*$p < 0.05$) reduced compared to naïve animals (*gray bar, n* = 5).

(naïve: 156 ± 8.9%, p < 0.01; prolonged SE: 180.2 ± 12.5%, p < 0.001). The extent of potentiation in both conditions was not different (p = 0.15). In contrast, IPSC potentiation by DZP (300 nM) was significantly reduced in granule cells from animals that had experienced prolonged SE (naïve: 147.0 ± 9.7%; prolonged SE: 117.0 ± 7.2%, p < 0.05; Figure 3.5B). This reduction in DZP potency during SE agrees with previous reports (Walton and Treiman, 1988; Kapur and Macdonald, 1997). These findings demonstrate that STP, but not DZP retains potency following prolonged SE and suggests that the continued ability of STP to potentiate GABAergic IPSCs underlies its ability to remain effective during prolonged SE.

3.4.7 STP, BUT NOT DZP, REMAINS EFFECTIVE IN POTENTIATING TONIC CURRENT FOLLOWING PROLONGED SE

Tonic inhibition in the dentate gyrus is mediated by GABA$_A$ receptors containing α4 and δ subunits as well as those containing α5 and γ2 subunits (Zhang et al., 2007). The ability of STP to act at receptors containing these subunits (Fisher, 2009) suggests that potentiation of tonic GABA currents may contribute to the anticonvulsant effect of STP. We examined the effect of STP on tonic GABA current in dentate granule cells in young (21 – 23 days old) naïve animals. Tonic currents were studied in the presence of 50 µM CNQX, 50 µM D-APV/10 µM MK-801, and 1 µM TTX to block AMPA/kainate receptors, NMDA receptors and Na$^+$ channels, respectively. Tonic currents were blocked by bicuculline methochloride (20 µM), indicating that they were mediated by GABA$_A$

receptors. As predicted, STP (100 µM) significantly potentiated the tonic current (185.5 ± 22.6%, p < 0.01). We then compared the effect of STP on tonic current in naïve animals to that in animals that had experienced prolonged SE (naïve: 20.4 ± 3 pA, n = 10; prolonged SE: 36.6 pA, n = 9; p < 0.05; Figure 3.6A), a finding in agreement with a previous study (Naylor et al., 2005). Despite the increase in tonic current, STP still potentiated this current following prolonged SE (175.4 ± 23.8%, p < 0.05) by a similar amount to that in naïve animals (p = 0.76; Figure 3.6A). By comparison, DZP (1 µM) potentiated tonic GABA current (155.3 ± 18%, p < 0.05) in granule cells in naïve animals, likely by acting on α5/γ2-containing $GABA_A$ receptors (Zhang et al., 2007). However, following prolonged SE, DZP no longer increased the tonic current (95 ± 9.1%, p = 0.60; Figure 3.6B).

3.4.8 STP RETAINS ITS ABILITY TO POTENTIATE mIPSCs FOLLOWING PROLONGED SE

STP can increase GABA release by acting presynaptically on inhibitory terminals (Quilichini et al., 2006) and this action could contribute to both the ability of STP to prolong the IPSC decay and the anticonvulsant effect of the compound. We examined the presynaptic action of STP by measuring its effect on the frequency of miniature IPSCs (mIPSCs). Spontaneous mIPSCs were recorded in the presence of glutamate antagonist (CNQX, 50 µM and D-APV, 50 µM) and TTX (1 µM), to block Na^+ channels. In young animals (19 – 26 days old) STP (100 µM) did not alter the amplitude of mIPSCs (106.8 ± 13.8%, p > 0.05)

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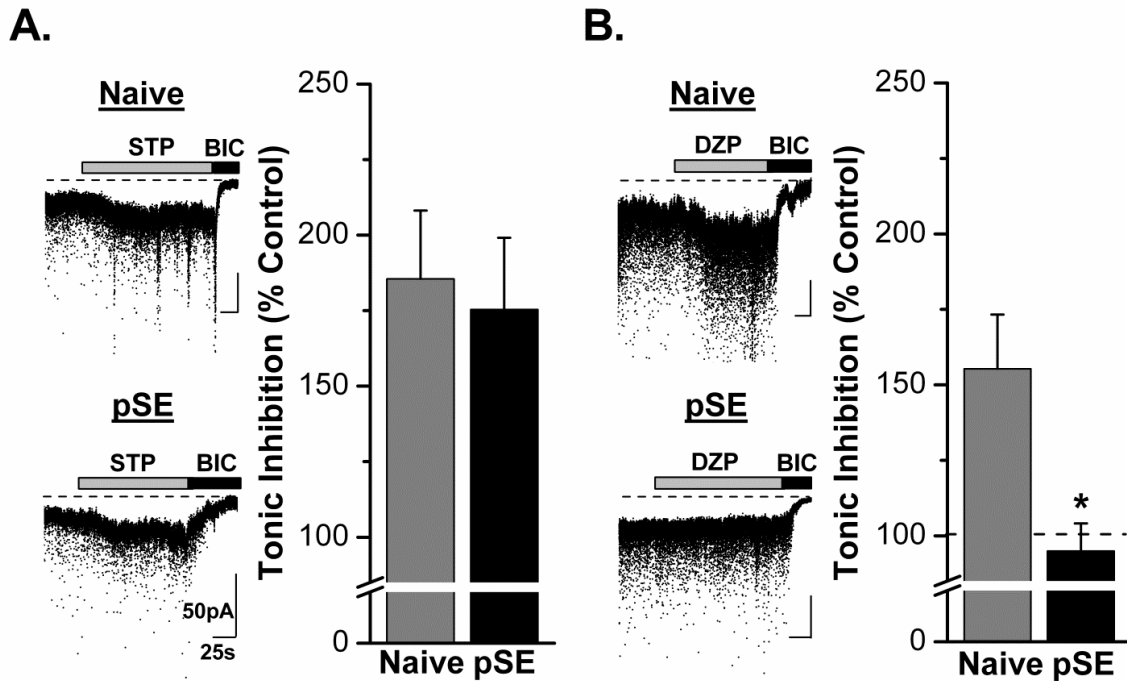
Figure 3.6. STP, not DZP, continues to enhance tonic GABAergic inhibition following prolonged SE. A. *Left*, Sample traces from naïve and prolonged SE-experienced animals demonstrate similar potentiation of tonic $GABA_A$ currents by 100 µM STP. Tonic $GABA_A$ current is blocked by 30 µM bicuculline (BIC). *Right*, STP (100 µM) similarly potentiates tonic $GABA_A$ currents in naïve (*gray bar, n* = 6) and prolonged SE-experienced animals (*black bar, n* = 5). **B.** *Left*, Sample traces demonstrate that DZP (1 µM) potentiates tonic $GABA_A$ current in dentate granule cells in naïve animals and that this potentiation is lost following prolonged SE. *Right*, DZP (1 µM) enhances tonic $GABA_A$ current in naïve animals (*gray bar, n* = 4), but does not potentiate this current following prolonged SE (*black bar, n* = 4), a value significantly different from naïve (*$p < 0.05$).

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but increased both the frequency (131.5 ± 5.8%, $p < 0.001$) and the decay time of events (150.1 ± 12.1%, $p < 0.01$; Figure 3.7A). Similarly, in dentate granule cells from animals that had experienced prolonged SE, 100 µM STP continued to potentiate both the frequency (140.3 ± 13.6%, $p < 0.05$) and decay time (140.7 ± 7.5%, $p < 0.001$) of the mIPSCs, without affecting mIPSCs amplitude (101.5 ± 10.5%, $p > 0.05$; Figure 3.7A). These findings indicate that STP acts presynaptically to enhance GABA release and this effect does not change during prolonged SE. In contrast, in naïve animals DZP (1 µM) significantly potentiated the decay time (156.7 ± 8.2%, $p < 0.001$) of mIPSCs, without affecting mIPSC frequency (95.5 ± 9.6%, $p > 0.05$; Figure 3.7B), supporting the conclusion that its action was entirely postsynaptic on $GABA_A$ receptors. During prolonged SE, the effect of DZP on mIPSC decay (118.1 ± 3.8%, $p < 0.001$) was significantly reduced (Figure 3.7B).

The mechanism by which STP enhances GABA release is unknown. However, given its ability to act presynaptically, we also assessed whether it could enhance glutamate release. The effect of STP on glutamate release was measured by its ability to alter the frequency of spontaneous miniature excitatory post-synaptic currents (mEPSCs). mEPSCs were studied in the presence of the $GABA_A$ receptor antagonist bicuculline methochloride (30 µM) and TTX (1 µM). In young animals (19 – 26 days old) STP (100 µM) significantly increased mEPSC frequency (128 ± 4.5%, $p < 0.001$) without altering the amplitude of decay time of events (Figure 3.8A). Following prolonged SE, the effect of STP on
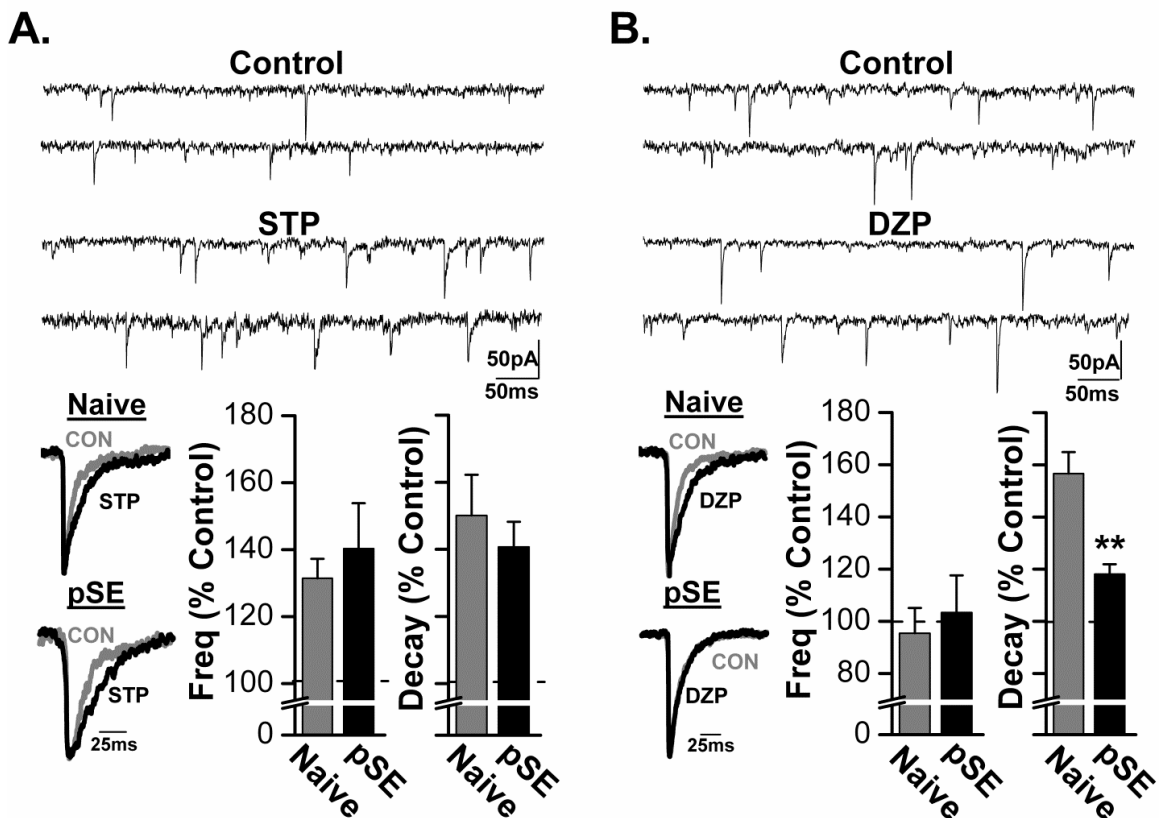
Figure 3.7. STP, not DZP, remains effective in potentiating mIPSCs following prolonged SE. A. *Top*, Sample waveforms from naïve and prolonged SE-experienced animals before and after application of 100 µM STP showing increased mIPSC frequency. *Lower Left*, Superimposed traces of averaged mIPSCs in the absence and presence of STP (100 µM) in naïve and prolonged SE (pSE) animals. *Lower Right*, STP (100 µM) similarly increased mIPSC frequency and decay time in both naïve (*gray bars, n* = 5) and prolonged SE-experienced animals (*black bars, n* = 6). **B.** *Top*, Sample waveforms from naïve and prolonged-SE experienced animals before and after application of 1µM DZP, showing no change in mIPSC frequency. *Lower Left*, Superimposed traces of averaged mIPSCs in the absence and presence of DZP (1 µM) in naïve and prolonged SE (pSE) animals. *Lower Right*, DZP (1 µM) had no effect on mIPSC frequency in naïve (*gray bar, n* = 5) or prolonged SE-experienced animals (*black bar, n* = 6). DZP (1 µM) significantly prolonged SE-experienced mIPSC decay time in dentate granule cells from naïve animals (*gray bar, n* = 5). Following prolonged SE, this potentiation was significantly reduced (*black bar, n* = 6; ***p* < 0.01).
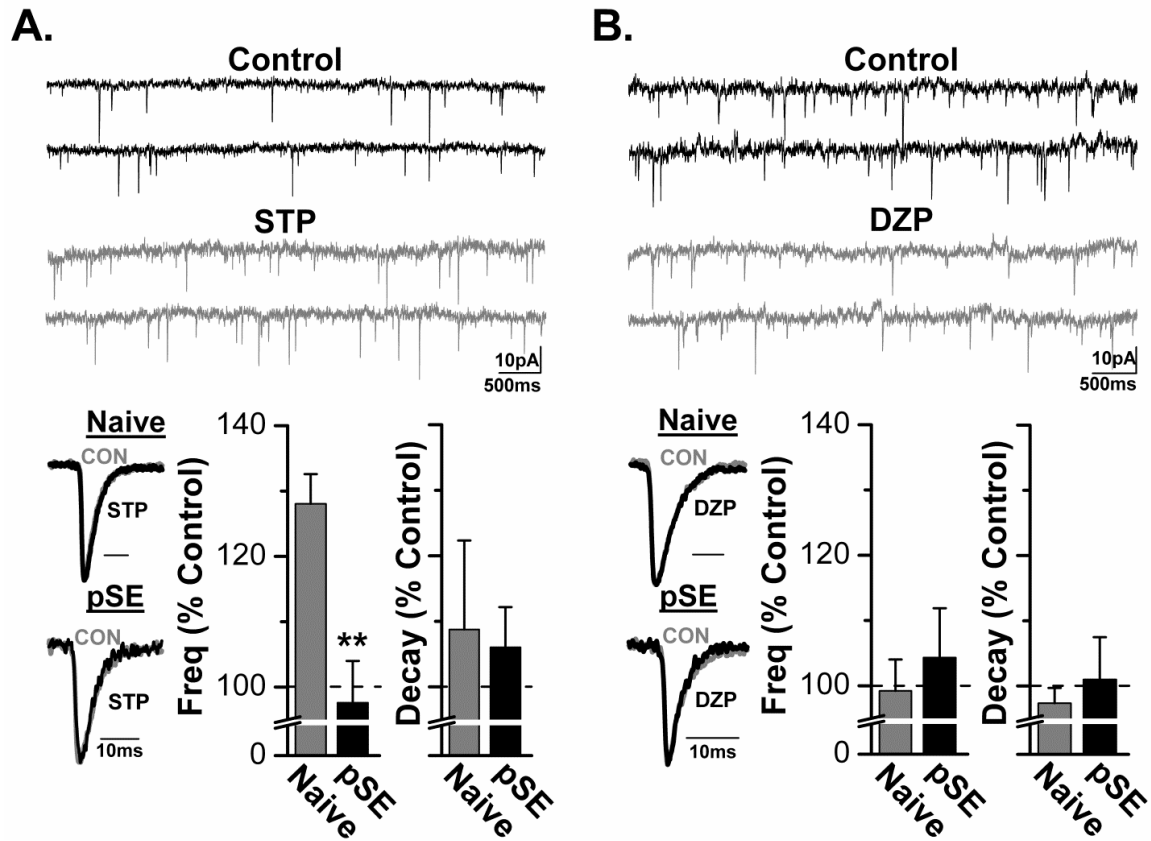
Figure 3.8. Neither STP nor DZP alter mEPSCs following prolonged SE A. *Top*, Sample waveforms from naïve and prolonged SE-experienced animals before and after application of 100 μM STP, showing increased mEPSC frequency. *Lower Left*, Superimposed traces of averaged mEPSCs in the absence and presence of STP (100 μM) in naïve and prolonged SE (pSE) animals. *Lower Right*, STP (100 μM) significantly potentiated mEPSC frequency in naïve animals (*gray bars, n* = 5), but did not alter frequency following prolonged SE (*black bars, n* = 6), a significant difference from naïve animals (***p < 0.01). STP had no effect on mEPSC decay time in naïve or prolonged SE-experienced animals. **B.** DZP (1 μM) did not alter mEPSC frequency or decay time in naïve (*gray bars, n* = 5) or prolonged SE-experienced animals (*black bars, n* = 6).

mEPSC frequency was significantly reduced ($p < 0.01$). In contrast, DZP (1 µM) did not affect the frequency or decay time of mEPSCs in either naïve animals or prolonged SE-experienced animals (Figure 3.8B). These findings indicate that STP affects both inhibitory and excitatory transmission, but that its effects on inhibitory and excitatory terminals are differentially regulated during SE.

3.5. DISCUSSION

The results of this study suggest that STP may be effective in terminating prolonged SE, a time when seizures have become resistant to BZDs. Specifically, this study demonstrates that at therapeutically relevant concentrations STP terminates both brief and prolonged behavioral SE. These actions of STP are due to its enhancement of GABA$_A$ receptor mediated transmission, as STP acts both postsynaptically to prolong IPSC decay and presynaptically to enhance GABA release. Despite a significant reduction in IPSC potentiation by DZP, the actions of STP on GABAergic transmission are preserved during prolonged SE. Postsynaptically STP potentiates both phasic and tonic GABAergic currents by acting on the GABA$_A$ receptor at a locus separate from the BZD site. This study also demonstrated that the anticonvulsant effects of STP, as well as its effects on IPSC decay, are age-dependent with greater effects in younger animals. The results of this study suggest the potential for the use of STP in treatment of BZD-resistant SE. Figure 3.9 summarizes the changes in GABA$_A$R pharmacology during prolonged-SE.
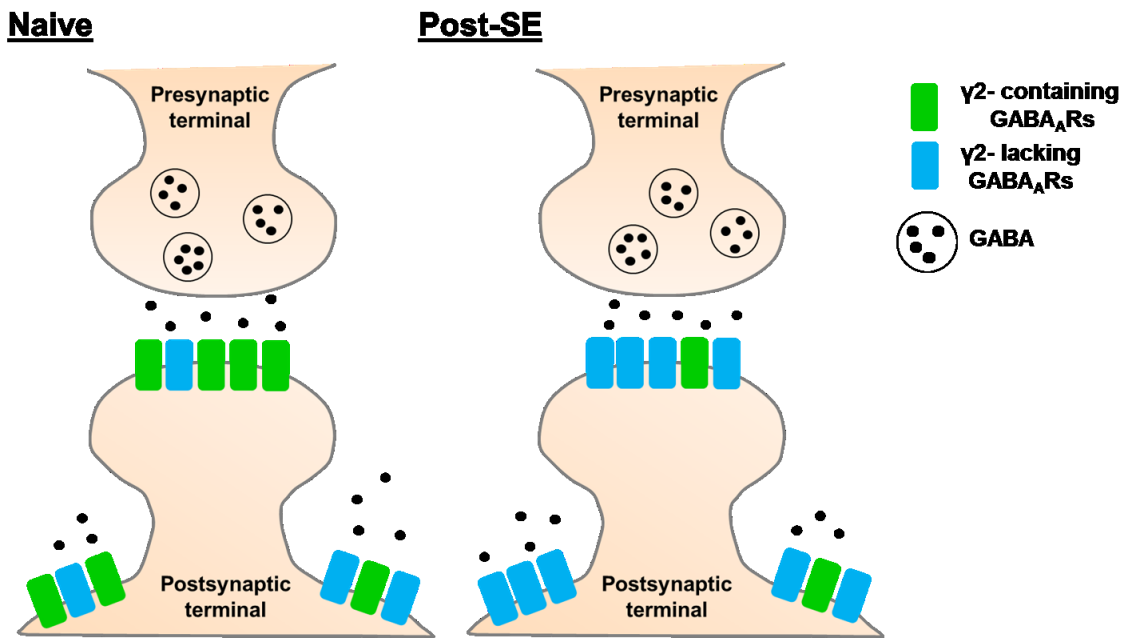
Figure 3.9. Summary of changes in GABA$_A$ receptor pharmacology following prolonged-SE. In naïve animals BZD-sensitive γ2-containing GABA$_A$R dominate the postsynaptic membrane. Extrasynaptic receptors are composed of a combination of γ2-containing and γ2-lacking GABARs. In prolonged-SE experienced animals (Post-SE), the γ2 subunit is internalized leaving mostly BZD-insensitive γ2-lacking GABA$_A$Rs at synaptic and extrasynaptic sites. The pharmacological profile of STP preserves its effectiveness in both conditions while significant pharmacoresistance develops to BZDs.

3.5.1 STATUS EPILEPTICUS INDUCED RESISTANCE TO BZDS

The initiation and maintenance of SE is regulated by GABAergic inhibition within the dentate gyrus and CA1 region of the hippocampus (Kapur and Lothman, 1989; Kapur et al., 1989). Seizures become self-sustaining because with time seizures progressively reduce $GABA_A$ receptor-mediated inhibition in the hippocampus. This reduction in GABAergic transmission leads to the development of further seizures. BZDs, such as DZP, are anticonvulsant by potentiating γ2-containing $GABA_A$ receptor currents in this region. However, the pharmacoresistance to BZDs during prolonged SE results from the rapid activity-dependent decrease in surface expression of the BZD-sensitive γ2-containing $GABA_A$ receptors (Naylor et al., 2005; Goodkin et al., 2008). In contrast, BZD-insensitive $GABA_A$ receptors containing α4 and δ subunits are preserved (Goodkin et al., 2008).

3.5.2 STP POTENTIATION OF BOTH PHASIC AND TONIC GABAERGIC INHIBITION

The results of this study suggest that the ability of STP to remain effective during prolonged SE is due to its unique subunit selectivity profile. STP is a positive allosteric modulator of $GABA_A$ receptors containing any of the α, β or γ subunits (Fisher, 2009). $GABA_A$ receptors containing α4 subunits can mediate phasic inhibition and remain at the membrane surface following prolonged SE. STP may retain its ability to potentiate phasic inhibition during prolonged SE by acting at receptors containing these subunits.

In addition to potentiating phasic inhibition, the results of this study demonstrate that STP strongly potentiates tonic inhibition. This finding is in support of its activity at δ-containing GABA$_A$ receptors (Fisher, 2009). The ability of STP to enhance tonic GABA$_A$ currents may have contributed to its anticonvulsant effect. The continued effectiveness of STP on tonic inhibition during prolonged SE was likely due to the persistence of δ-containing GABA$_A$ receptors on the membrane surface (Goodkin et al., 2008). In contrast, DZP does not act at δ-containing receptors. The observed potentiation of tonic GABA$_A$ current by DZP has been reported to occur through the activity of the drug at α5/γ2-containing extrasynaptic GABA$_A$ receptors (Zhang et al., 2007). However, this effect of DZP was lost during prolonged SE due to internalization of γ2-containing receptors (Naylor et al., 2005). Thus, STP, not DZP, retained efficacy during prolonged SE. While it seems likely that STP potentiated tonic inhibition by acting at δ-containing GABA$_A$ receptors, the possibility that the presynaptic action of STP contributed cannot be excluded. The present data suggest that the ability of STP to remain effective during prolonged SE is likely due to its activity at BZD-insensitive GABA$_A$ receptors containing α4 and δ subunits as well as its presynaptic effect.

3.5.3 EFFECTS OF STP ARE DEVELOPMENTALLY REGULATED

STP has been shown to act at all α subunits, however it has the highest activity at α3-containing GABA$_A$ receptors (Fisher, 2009). In rat, expression of the α3 subunit is strongly developmentally regulated. In embryonic and newborn

brain $\alpha3$ subunits are expressed in most regions (Laurie et al., 1992). However, with maturation $\alpha3$ mRNA expression decreases becoming restricted to neurons primarily in the cortex and thalamus. The developmental loss of $\alpha3$ subunit expression likely explains the observed age dependence in both the ability of STP to potentiate the IPSC and its anticonvulsant activity. However, it is important to note that the developmental profile of $\alpha3$ subunit expression in humans has not been reported. Furthermore, the mechanism by which a developmental loss of $\alpha3$ subunits could cause a shift in STP potency has not been explored. Further studies will be required to resolve these issues.

In the present study the ED_{50} for DZP to terminate SE and the extent of the rightward shift in the DZP ED_{50} during prolonged SE are in agreement with previous studies in the Li-Pilo model (Walton and Treiman, 1988; Kapur and Macdonald, 1997; Jones et al., 2002; Goodkin et al., 2003). The observed shift in DZP ED_{50} was similar in both juvenile and adult animals. Under the same conditions, STP displayed markedly less pharmacoresistance than DZP at both developmental time points. However, in juvenile animals there was a slight trend for the STP ED_{50} to increase (~ 2 fold) after prolonged SE; a trend which disappeared in adult animals. This observation in juvenile animals may be explained by the developmental decrease in $\alpha3$ subunit expression. Thus, in juvenile animals the action of STP on $\alpha3$-containing $GABA_A$ receptors may have accounted for a greater proportion of the drugs effect than in adult animals. Consequently, internalization of $\alpha3$-containing $GABA_A$ receptors during SE would have caused have caused a greater impact on the anticonvulsant activity of STP

in juvenile than in adult animals.

3.5.4 STP IS EFFECTIVE AT THERAPEUTICALLY RELEVANT CONCENTRATIONS

STP is used clinically to treat Dravet Syndrome in children at doses that result in plasma concentration of 4 – 22 µg/mL (Farwell et al., 1993; Chiron et al., 2000), a value that corresponds to approximately 16 – 90 µM. The present study determined that in granule cells from 5 to 7 day old rats STP potentiated the IPSC with an EC_{50} of 45 µM, a value well within the established therapeutic range.   Previous studies examining the effects of STP have focused on its anticonvulsant effects in adult animals. The concentrations of STP used in these reports are similar to those used in adult animals in the present study. For example, Shen et al. (1990) found that STP applied alone produced a maximal anticonvulsant effect in adult animals at concentrations above 120 µg/mL, equivalent to ~500 µM. In agreement, the present study reported that STP terminated SE in adult animals with an ED_{50} of 378 mg/kg, corresponding to a plasma concentration of approximately 110 µg/mL. In younger rats (20 – 25 days old), STP became more potent, terminating SE with an ED_{50} of 100 mg/kg. This corresponds to a plasma concentration of 30 µg/mL, equivalent to ~120 µM. The concentration corresponding to this ED_{50} for the anticonvulsant effect of STP measured *in vivo* agreed well with the EC_{50} of 102 µM determined for STP prolongation of the IPSC *in vitro* from animals of the same age. The similarity between these values is consistent with the idea that potentiation of GABAergic transmission underlies the anticonvulsant effect of STP.

3.5.5 STP ACTS AT A DISTINCT SITE ON THE GABA$_A$ RECEPTOR

The site of action of STP on the GABA$_A$ receptor is not known. The actions of STP on the IPSC were similar to those of BZDs in that they both prolonged the decay of the IPSC without increasing its peak amplitude. However, results of the present study, as well as previous work (Quilichini et al., 2006; Fisher, 2009), indicate that STP acts at a distinct site on the GABA$_A$ receptor; a site separate from BZD binding sites as well as binding sites for neurosteroids and some anesthetics. In support of this, the present study determined that the actions of STP were not blocked by a BZD site antagonist, and were additive with co-applied BZDs. This finding suggests a potential role for STP as add-on therapy with BZDs for the treatment of SE.

The current study also reported that at concentrations above 300 µM, STP depressed the evoked IPSC. This finding is consistent with the previous suggestion that STP acts as a partial agonist (Fisher, 2009). The ability of STP to directly activate the receptor may have contributed to the strong sedation produced by the drug at high doses. Based on similarities to the actions of barbiturates, Quilichini et al. (2006) suggested that STP may act at the barbiturate site. However, unlike barbiturates, STP did not increase the peak amplitude of the IPSC. Fisher (2009) also reported that the subunit dependence of STP is distinct from that of the barbiturates (Thompson et al., 1996), suggesting that STP acts at a unique site on the GABA$_A$ receptor. Barbiturates and STP also differ in their activity at other sites. For example, barbiturates decrease glutamate release and block AMPA receptors (Ho and Harris, 1981;

Miljkovic and MacDonald, 1986), whereas the present study determined that STP enhanced glutamate release. STP also enhanced GABA release and this effect persisted during prolonged SE, suggesting that it could have contributed to the anticonvulsant effect of the drug. This presynaptic effect differentiates STP from both barbiturates and BZDs, further demonstrating the unique pharmacological profile of STP.

3.6 SIGNIFICANCE

The present study determined that STP increased GABA release, potentiated both phasic and tonic GABAergic currents and was anticonvulsant in the Li-Pilo model of SE. Most notably, STP remained effective during prolonged SE at a time when seizures had become pharmacoresistant to BZDs. The actions of STP were age-dependent with the greatest effect in younger animals. These findings suggest that STP may be effective in treatment of prolonged SE. The need for novel therapies for prolonged SE is highlighted by clinical studies showing that the majority of patients undergoing SE do not receive treatment within 30 min (Jordan, 1994; Pellock et al., 2004; Shinnar et al., 2008). Results of the present study suggest that STP, either alone or as add-on therapy, would be effective in treating prolonged and BZD-resistant seizures.

CHAPTER 4

TEMPORAL ALTERATIONS IN KAINATE RECEPTOR SUBUNIT EXPRESSION FOLLOWING STATUS EPILEPTICUS

4.1 INTRODUCTION

Alterations in both glutamatergic and GABAergic receptor populations have been identified in patients suffering from seizure disorders such as status epilepticus (SE) (Grigorenko et al., 1997; Mathern et al., 1998; Mathern et al., 1999; Das et al., 2012; Houser et al., 2012). While some of these modifications can occur during a single SE event others emerge during the progression of the disease, representative of enduring alterations in receptor expression. The ability to develop effective therapeutics for the treatment of SE and epilepsy rests in our understanding of the diseased brain and the available receptor targets.

Kainate receptors (KARs) contribute to excitatory neurotransmission in the cerebrum, specifically throughout the hippocampus, a region central to the development of epilepsy (Chang and Lowenstein, 2003; Sloviter, 2005). In particular, KARs densely populate the mossy fiber – CA3 pathway, a region of the hippocampus prone to excitotoxicity and seizure development (Nadler et al., 1978). KARs in this pathway are believed to be composed of GluK2/3, GluK4 and GluK5 receptor combinations (Darstein et al., 2003). KARs are also responsible for mediating a portion of the excitatory circuit that appears in the epileptic brain following the aberrant sprouting of mossy fibers into the inner molecular layer of

the dentate gyrus (DG) (Epsztein et al., 2005).

In addition to the role of KARs in regulating neurotransmission, direct activation of the receptor via kainate has long been used as a model of SE and leads to the development of epilepsy (Nadler, 1981; Ben-Ari, 1985). In agreement, knockout of the GluK2 subunit increases seizure threshold, demonstrating that low doses of kainate initiate seizures through GluK2-containing KARs (Mulle et al., 1998; Fritsch et al., 2014). KARs have also been implicated in several comorbidities and possible disorders associated with epilepsy. Alterations in KAR subunit expression and localization have been observed in disorders with increased prevalence in epilepsy, including anxiety and depression (Catches et al., 2012; Lerma and Marques, 2013). Together, these studies create a framework in which the KAR could serve as a potential therapeutic target for the treatment of epilepsy and related comorbidities.

Despite this potential relationship few studies have examined possible alterations in KAR subunit expression post-SE. Studies from patients with epilepsy have consistently demonstrated that KAR subunit expression is altered in the epileptic brain (Grigorenko et al., 1997; Mathern et al., 1998; Li et al., 2010; Das et al., 2012). However the observed changes in individual subunit expression remain inconsistent, likely due to differences in seizure etiology and treatment methods, as well as differences in the duration of the disorder. In agreement with human studies, animal models of SE have also demonstrated that KAR subunit expression and localization is altered (Kamphuis et al., 1995; Porter et al., 1996; Lason et al., 1997; Tandon et al., 2002; Zhang et al., 2004;

Ullal et al., 2005; Porter et al., 2006). However, the literature on animal studies is also severely inconsistent, likely due to different animal models utilized as well as the lack of exploring region-specific changes.

The goal of the current study was to explore how the expression of GluK2/3, GluK4 and GluK5 KAR subunits are altered at three distinct time-points following the induction of SE. Expression of the GluK1 subunit was not explored as its expression is essentially absent from postsynaptic neurons and is predominately expressed in hippocampal interneurons (Wisden and Seeburg, 1993). In addition, this study explored region specific alterations by examining tissue from microdissected DG, CA3 and CA1 hippocampal subfields.

Together, the data collected from this study provide the first long-term, region-specific exploration of alterations in KAR subunit expression following the induction of SE and the development of epilepsy.

4.2 HYPOTHESIS

Collectively, the scientific literature demonstrates that in both human and animal studies, KAR subunit expression and localization is altered following SE. Based on the limited human and animal studies, we hypothesize that distinct changes in KAR subunit expression will be observed within each of the three different hippocampal subfields explored. In addition, we postulate that these changes will vary as the disease progresses following the induction of SE.

4.3 MATERIALS AND METHODS

4.3.1 ANIMAL MODEL OF SE

All animal care and use procedures were carried out in accordance with protocols written under the guidance of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of South Carolina. Following induction of SE, rats were housed 1 per cage with *ad libitum* access to food and water. Rats were housed in a climate controlled facility with a light/dark cycle of 12/12 hours.

SE was induced in adult male Sprague-Dawley rats (Harlan, Indianapolis, IN). Animals received intraperitoneal (i.p.) injections of scopolamine methyl bromide and terbutaline hemisulfate (2 mg/kg) followed 30 – 45 min later by pilocarpine hydrochloride (390 mg/kg). After 2 hours of continuous SE, seizures were terminated by diazepam (DZP, 25 mg/kg). This group of animals served as the 'pilo' group. A separate group of animals received identical treatment with the exception of receiving saline (390 mg/kg) instead of pilocarpine hydrochloride. This group of animals served as the 'sham' group. More detailed methods concerning induction of SE are described in Chapter 2.3.

4.3.2 WESTERN BLOT ANALYSIS

Animals 5, 60, and 200 days post-SE were anesthetized, the brain removed, and immediately placed on dry ice. Brains were sectioned at 100 μM on a freezing microtome (Microm, Waldorf, Germany). Tissue from hippocampal

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regions- DG, CA3 and CA1, in addition to prefrontal cortex, amygdala and striatum were collected. Microdissected regions collected from the hippocampus are outlined in Figure 2.3. Individual regions were homogenized with the resulting pellet resuspended in PBS and stored at -80°C prior to use.

Aliquots of 2 – 10 µg of crude plasma membrane protein were loaded onto a 4 – 15% pre-cast polyacrylamide gel. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins prior to being transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with TBS + 5% non-fat dry milk for 60 min at room temperature followed by primary antibody incubation overnight at 4°C. Primary antibodies are listed in Table 2.2. Enhanced chemiluminescence reagents (ECL, Pierce) were used for antibody detection. Images were captured on Kodak BioMax XAR film and densitometric analysis performed using ImageJ 1.47v (National Institutes of Health, USA). More detailed methods concerning western blot analysis are described in Chapter 2.8.

4.3.3 IMMUNOHISTOCHEMISTRY

Animals 60 and 200 days post-SE were anesthetized and perfused for 4 – 5 minutes with ice cold, oxygenated (95% O_2 / 5% CO_2) PBS followed by ice cold 4% paraformaldehyde for approximately 20 minutes. Whole brains were removed and post-fixed at 4°C in 4% paraformaldehyde or 3 hours and then transferred to 0.1M PB until sectioned at 50 µM thick coronal sections on a vibratome (Leica,

VT1000S, Nussloch, Germany). Slices were stored at 4°C in 0.1M PB and 0.1% sodium azide until used for immunohistochemical processing.

Free floating sections were washed for approximately 40 minutes in TBS. Tissue was then exposed to an antigen retrieval step in which sections were placed for 5 min in Na-Citrate buffer heated to 85°C and then cooled to room temperature for 5 – 10 min. Primary antibodies utilized for immunohistochemical analysis are listed in Table 2.3. Tissue was processed using a standard immunohistochemical protocol described in detail in Chapter 2.9, and then cover-slipped using cover glass and DPX mounting solution (Sigma, St. Louis, MO). Slides were left to dry completely prior to analysis with MCID Elite 7.0 (Imaging Research, Inc., St. Catharines, Canada) or ImageJ 1.47v (National Institutes of Health, USA).

4.4 RESULTS

4.4.1 ALTERATIONS IN KAR SUBUNIT EXPRESSION IN THE DENTATE GYRUS

As the first stop in the trisynaptic pathway, the DG serves to gate information entering the hippocampus from the entorhinal cortex (EC; Figure 1.2) (Henze et al., 2002; Acsady and Kali, 2007). This feature is critical in that it allows dentate granule cells (DGCs) to resist the propagation of seizures that may originate from the EC (Collins et al., 1983; Stringer and Lothman, 1989; Lothman et al., 1992). However in human temporal lobe epilepsy, as well as the pilocarpine model of SE, dramatic alterations in the anatomy and circuitry of the DG have been observed. One such change involves the aberrant sprouting of

mossy fibers into the inner molecular layer of the DG (Figure 1.6) (Tauck and Nadler, 1985; Represa et al., 1987; Sutula and Dudek, 2007). This pathological sprouting creates a recurrent excitatory network between granule cells and likely serves to facilitate the synchronous firing of DGCs and promote seizure propagation (Tauck and Nadler, 1985; Gabriel et al., 2004; Timofeeva and Nadler, 2006). In addition, KARs have been shown to mediate a portion of this new excitatory network (Epsztein et al., 2005). We explored the impact of pilocarpine-induced SE on the KAR subunit expression within the DG, in hopes of shedding light on the subunit composition mediating this newly formed excitatory circuit in the epileptic brain.

We determined that expression of the GluK2/3 subunit is significantly reduced in the DG at 5 days post-SE (70.0 ± 5.2% of control, $n = 7 - 8$; $p < 0.01$) and remains significantly reduced at 60 and 200d post-SE (60d: 56.8 ± 7.1% of control, $n = 8$; $p < 0.01$; 200d: 71.1 ± 4.8% of control, $n = 6 - 10$; $p < 0.001$; Figure 4.1A). Our observation that the GluK2/3 subunit is reduced shortly after SE is supported by a previous study in which GluK2/3 subunit expression was also decreased in the DG at 20 days following induction of SE (Porter et al., 2006). We failed to observe any alterations in GluK4 subunit expression at 5d (91.7 ± 9.1% of control, $n = 7 - 8$; $p > 0.05$) and 60d post-SE (93.8 ± 14.2% of control, $n = 8$; $p > 0.05$). However, by 200d post-SE a significant reduction in GluK4 expression was observed in the DG (63.1 ± 4.8% of control, $n = 6 - 9$; $p < 0.01$); Figure 4.1B).The lack of change in GluK4 expression at 5 and 60d time-points is supported by previous findings in which expression of the GluK4 subunit

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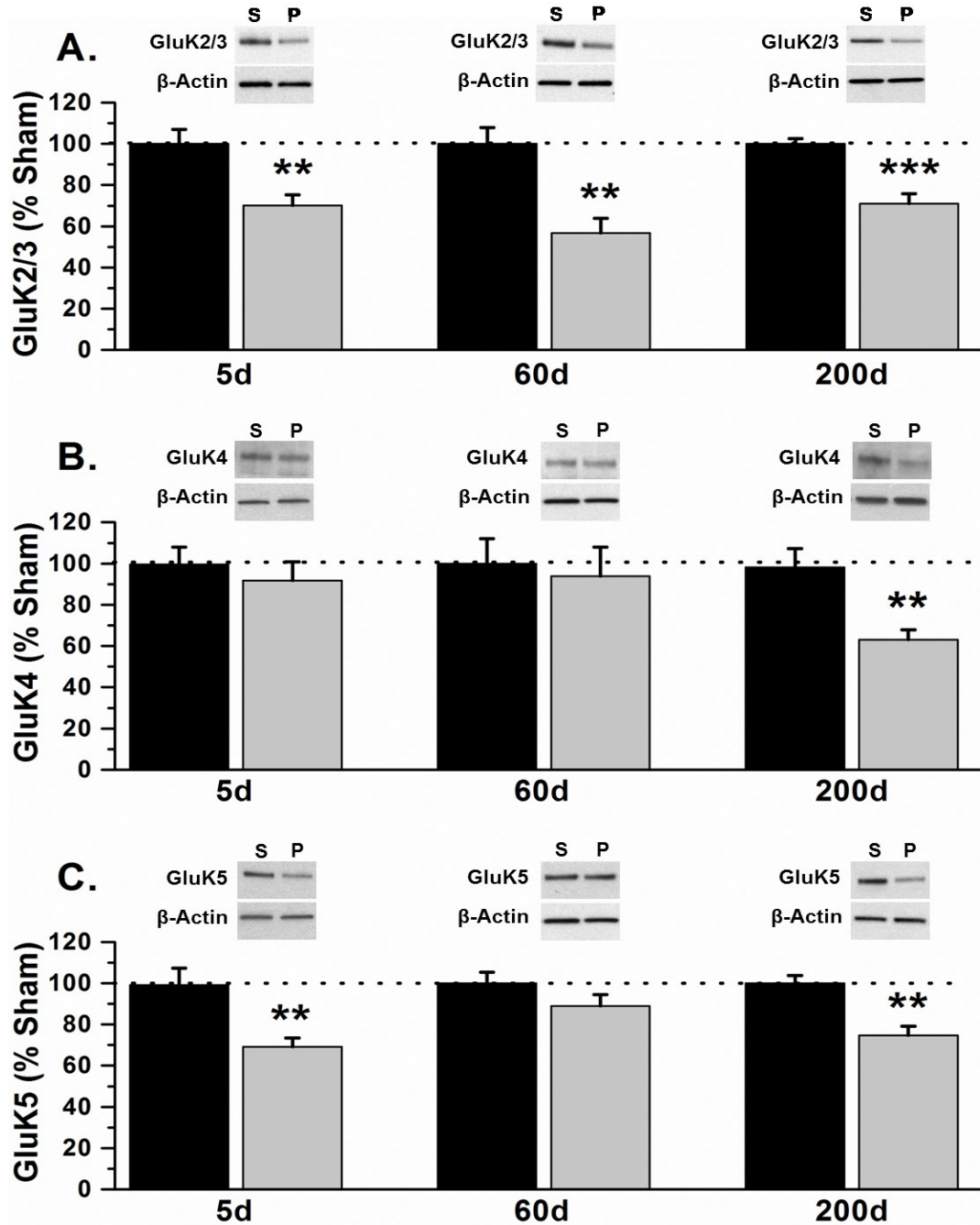
Figure 4.1. Alterations in KAR subunit expression in the dentate gyrus in animals post-SE. Western blot analysis of microdissected DG from animals 5d, 60d and 200d post pilocarpine-induced SE. **A.** Expression of the GluK2/3 subunit was significantly reduced at all three time-points examined. **B.** GluK4 subunit expression was unchanged at 5d and 60d post-SE and significantly decreased by 200d post-SE. **C.** GluK5 subunit expression was initially decreased at 5d post-SE, recovered to baseline by 60d and was decreased at 200d post-SE. S, sham-treated; P, pilo-treated. $n = 6 - 10$; **$p < 0.01$; ***$p < 0.001$, t-test.

was unaltered days to weeks following induction of SE (Kamphuis et al., 1995; Porter et al., 1996).

We observed that the GluK5 subunit is significantly decreased at 5d post-SE (69.2 ± 4.2% of control, n = 7; p < 0.01), and recovers to baseline by 60d (88.9 ± 5.5% of control, n = 7; p > 0.05). However, by 200d post-SE we observed another significant decrease in expression (74.7 ± 4.5% of control, n = 6 – 10; p < 0.01; Figure 4.1C). The observed decrease in GluK5 subunit expression within the DG following induction of SE disagrees with Porter et al. (2006), in which GluK5 expression was increased in DGCs at 20 days post-SE. This disparity may be attributed to differences in tissue preparation as Porter et al. (2006) utilized isolated DGCs and the current study employed microdissected DG which also contained portions of the supragranular layer and hilar interneurons.

To substantiate the above findings immunohistochemical analysis was performed on tissue collected from animals 60d post-SE (Figure 4.2). In agreement with previous studies, we observed that GluK2/3, GluK4 and GluK5 subunit proteins were widely expressed throughout the DG, including robust labeling throughout the polymorphic layer and hilar interneurons (Wisden and Seeburg, 1993; Darstein et al., 2003). Of particular interest was the observation that GluK2/3 labeling within the molecular layer was not uniform, as was observed for GluK4 and GluK5 subunits. In contrast, GluK2/3 expression appeared higher in the outer molecular layer, creating a distinct separation between the inner and outer molecular layers of the DG. In animals 60d post-SE, there is a striking decrease in labeling of hilar interneurons for each of the
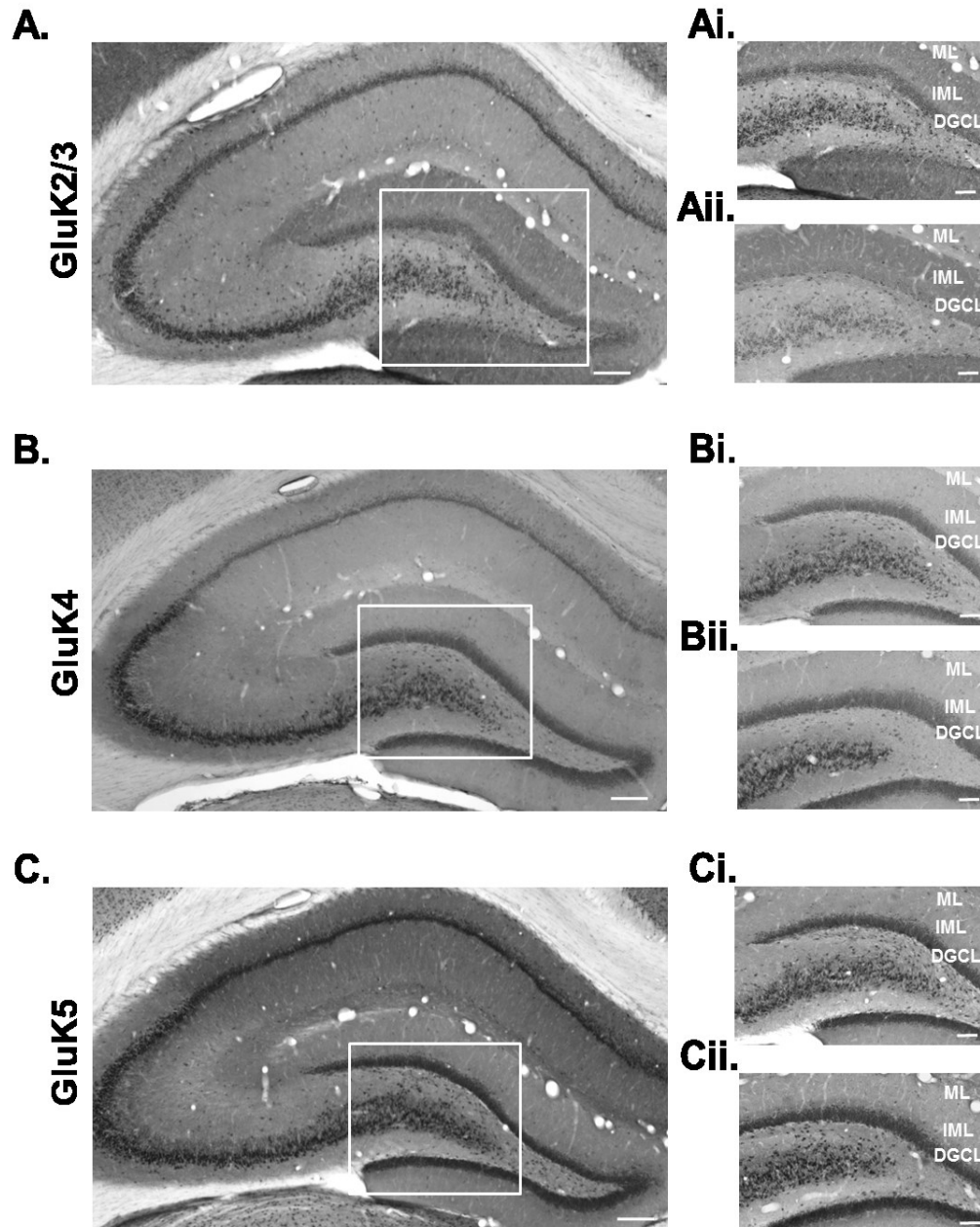
Figure 4.2. KAR subunit immunoreactivity in the hippocampus and DG in animals 60d post-SE. GluK2/3, GluK4 and GluK5 subunits exhibit distinct hippocampal labeling patterns. **A.** GluK2/3 expression was significantly reduced between **Ai.** sham-treated and **Aii.** pilocarpine-treated animals 60d post-SE. **B.** GluK4 immunoreactivity between **Bi.** sham-treated and **Bii.** pilocarpine-treated animals was unaltered within the DG. **C.** There was no change in GluK5 immunoreactivity in the DG among **Ci.** sham-treated and **Cii.** pilocarpine-treated animals. ML, molecular layer; IML, inner molecular layer, DGCL, dentate granule cell layer. Scale bars: A – C, 200 µM; Ai – Cii, 100 µM.

three subunits, particularly GluK4 labeling which appears nearly absent. In support of western blot analysis demonstrating a decrease in GluK2/3 and GluK5 expression in the DG, we observed that GluK2/3 and GluK5 labeling was dramatically reduced throughout the dentate granule cell layer. In addition, we noted that GluK2/3 labeling throughout the molecular layer appeared more uniform post-SE. This observation is suggestive of the idea that dendritic localization of the GluK2/3 subunit is altered post-SE, and may play a role in mossy fiber sprouting and KAR-mediated neurotransmission.

4.4.2 ALTERATIONS IN KAR SUBUNIT EXPRESSION IN AREA CA3

Hippocampal region CA3 is composed primarily of CA3 pyramidal cells and *stratum lucidum* (Figure 1.4). The well characterized mossy fiber – CA3 pathway, the second synapse within the trisynaptic pathway, serves as the primary means of communication between the DG and area CA3 (Figure 1.2). This synapse has been referred to as the 'detonator synapse' in that input from a single mossy fiber is sufficient to fire a CA3 pyramidal cell (Urban et al., 2001). The strength of this mossy fiber input contributes to the highly susceptible nature of CA3 to epileptogenic activity and the associated excitotoxicity (Nadler et al., 1978; Lothman et al., 1991; Ben-Ari and Cossart, 2000). The mossy fiber – CA3 pathway was also the first synapse demonstrated to utilize KARs as a means of regulating excitatory neurotransmission (Castillo et al., 1997; Vignes and Collingridge, 1997). Immunohistochemical and electrophysiological analysis further revealed that KARs, likely composed of GluK2, GluK4 and GluK5

subunits, densely populate area CA3 contributing to transmission on mossy fiber axons, presynaptic mossy fiber terminals and postsynaptic CA3 pyramidal cells (for review see, Lerma and Marques, 2013). Given the various avenues in which area CA3 may be implicated in seizures and the extensive role of KARs within this region, we explored how pilocarpine-induced SE and the development of epilepsy altered KAR subunit expression in area CA3.

Similar to findings in the DG, we determined that at 5 days post-SE in area CA3 there is a significant decrease in GluK2/3 subunit immunoreactivity (57.0 ± 5.6% of control, $n = 7 – 8$; $p < 0.001$). This decrease persists at both the 60 day (56.7 ± 6.3% of control, $n = 7 – 8$; $p < 0.001$) and 200 day time points (67.6 ± 7.0% of control, $n = 6 – 8$; $p < 0.01$; Figure 4.3A). In contrast to the dramatic decrease in GluK2/3 expression, the GluK4 subunit remains unaltered at 5 days post-SE (91.3 ± 10.4% of control, $n = 7 – 8$; $p > 0.05$), but is significantly reduced by 60d (59.4 ± 3.4% of control, $n = 7 – 8$; $p < 0.001$) and remains reduced at 200d post-SE (54.0 ± 7.1% of control, $n = 6 – 10$; $p < 0.001$; Figure 4.3B). In agreement, a decrease in GluK4 subunit expression in area CA3 was observed in a separate seizure model (Porter et al., 1996). Alterations in GluK5 subunit expression appear almost opposite to those of the GluK4 subunit in that expression is significantly decreased at 5d post-SE (63.1 ± 5.1% of control, $n = 7 – 8$; $p < 0.001$), but returns to baseline by the 60 and 200d time-points (60d: 85.5 ± 8.1% of control, $n = 8$; $p > 0.05$; 200d: 82.9 ± 7.0% of control, $n = 6 – 10$; $p > 0.05$; Figure 4.3C). The overall decrease in KAR subunit expression, particularly in GluK2/3 and GluK4 subunits, following pilocarpine-induced SE is in agreement
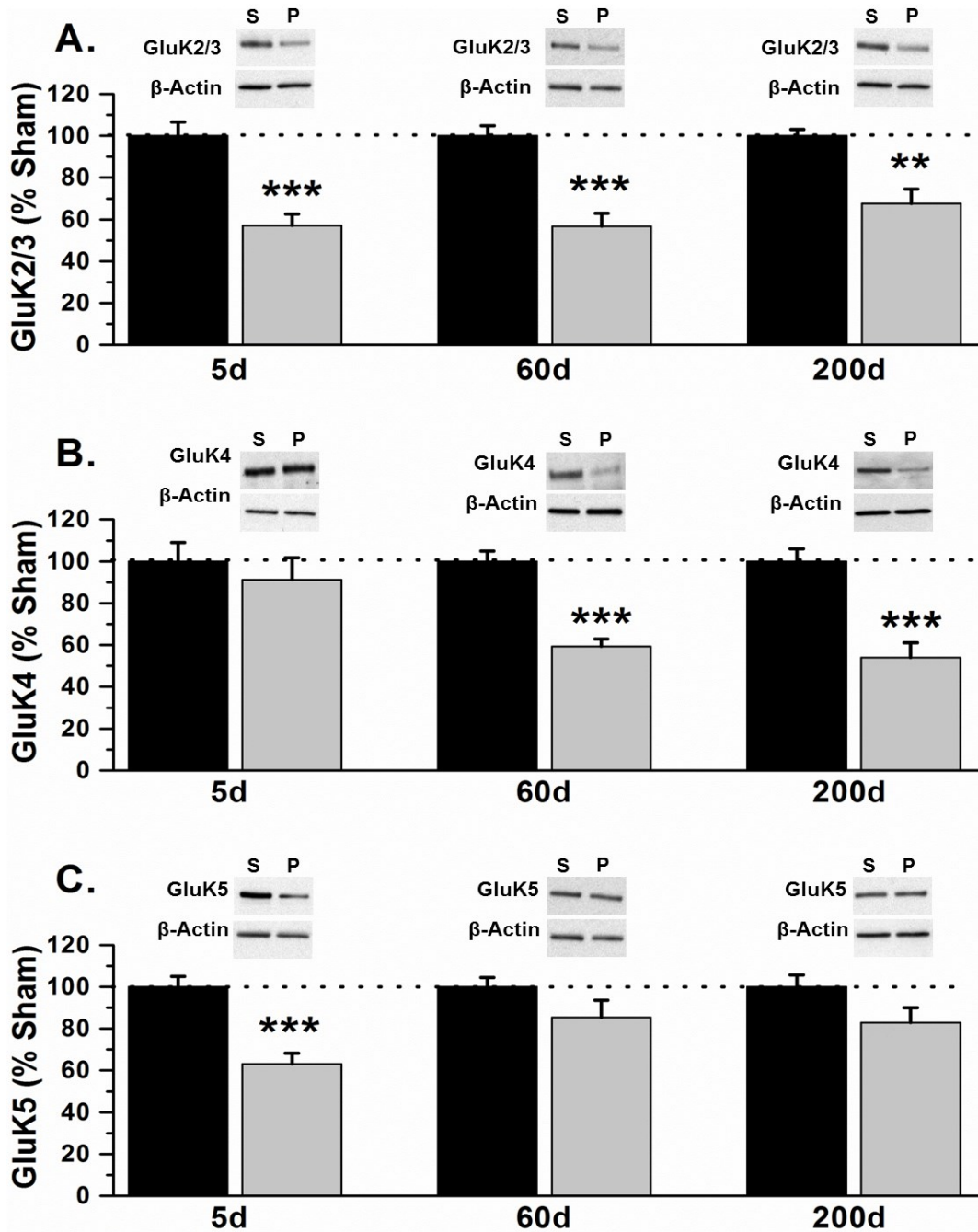
Figure 4.3. Alterations in KAR subunit expression in area CA3 in animals post-SE. Western blot analysis of microdissected CA3 from animals 5d, 60d and 200d post pilocarpine-induced SE. **A.** Expression of the GluK2/3 subunit was significantly reduced at all three time-points examined. **B.** GluK4 subunit expression was unchanged at 5d post-SE and was significantly reduced by 60d and 200d post-SE. **C.** GluK5 subunit expression was initially decreased at 5d post-SE and recovered to baseline by 60d and 200d post-SE. S, sham-treated; P, pilo-treated. $n = 6 - 10$; **$p < 0.01$; ***$p < 0.001$, t-test.

with a previous finding in which a significant decrease in ^{3}H-kainate binding was observed in area CA3 following repeated injection of kainate (Tandon et al., 2002).

The observed decrease in GluK2/3 and GluK4 subunit expression in area CA3 is supported by immunohistochemical analysis of animals 60d post-SE, (Figure 4.4). In agreement with previous studies we observed intense labeling of GluK2/3, GluK4 and GluK5 subunits within CA3 pyramidal cells and *stratum lucidum* (Wisden and Seeburg, 1993; Darstein et al., 2003). Labeling of GluK2/3 and GluK5 was also observed in interneurons located in *stratum radiatum* and to a lesser degree *stratum oriens*, with no apparent interneuron labeling by GluK4. In support of western blot analysis at 60d post-SE, we observed a dramatic decrease in GluK2/3 and GluK4 labeling within individual CA3 pyramidal cells, as well as a reduction in labeling throughout *stratum lucidum*. Expression of the GluK5 subunit did not appear to change following SE, a finding also in support of western blot analysis.

4.4.3 ALTERATIONS IN KAR SUBUNIT EXPRESSION IN AREA CA1

Neurotransmission between area CA3 and CA1 via the Schaffer collaterals serves as the final synapse within the trisynaptic pathway (Figure 1.2). Epileptogenic activity that initiates in area CA3, or propagates to this area from the DG, likely spreads to hippocampal area CA1. While KAR subunits are localized in area CA1, particularly mRNA for the GluK2 and GluK5 subunits, and to a lesser extent the GluK4 subunit, they are dramatically less abundant than in
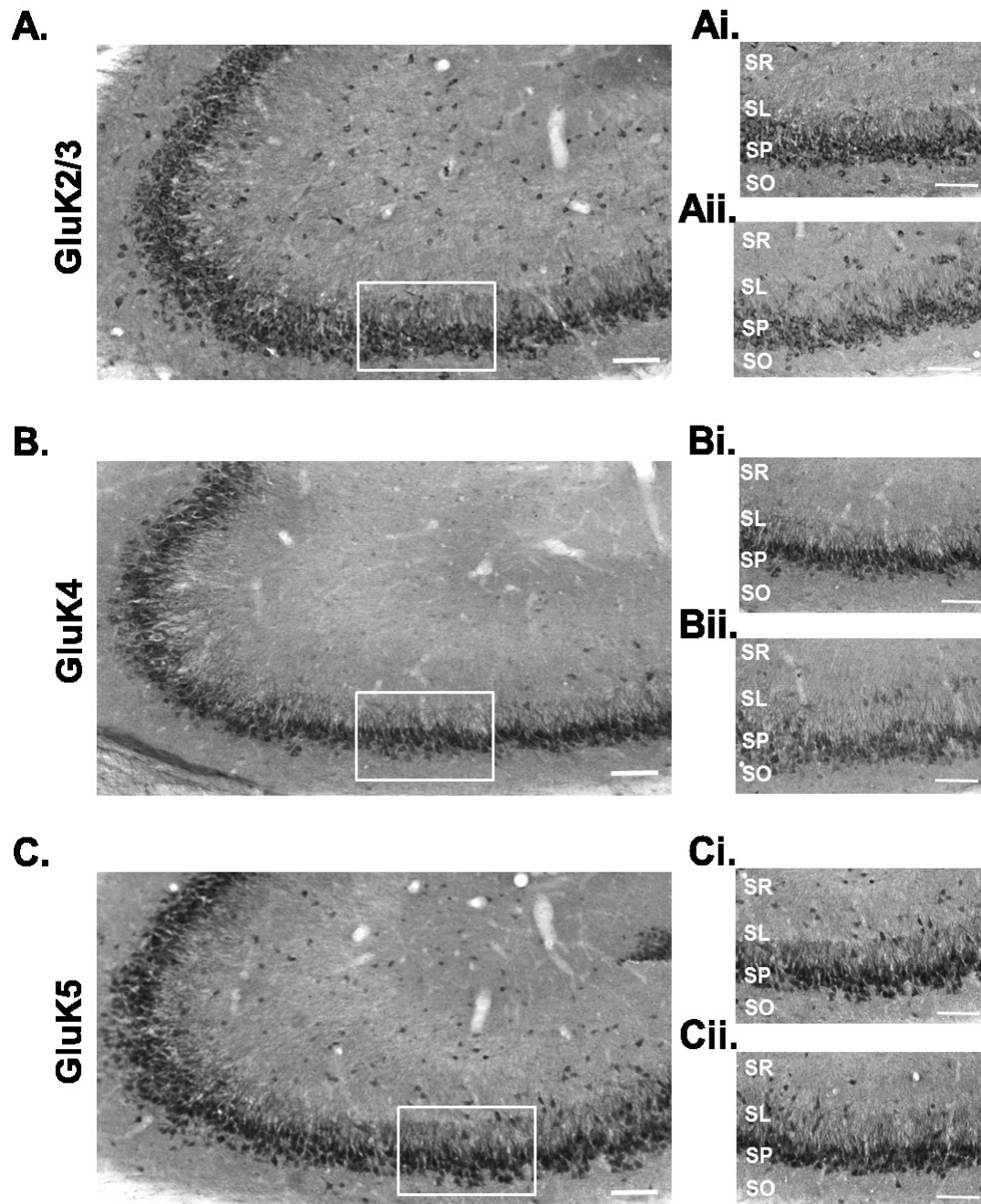
Figure 4.4. KAR subunit immunoreactivity in area CA3 in animals 60d post-SE. Distinct KAR subunit labeling in area CA3. **A.** GluK2/3 subunit expression was reduced in SP and SL between **Ai.** sham-treated and **Aii.** pilocarpine-treated animals 60d post-SE. **B.** GluK4 immunoreactivity among **Bi.** sham-treated and **Bii.** pilocarpine-treated animals was noticeably reduced within SP and SL. **C.** No alteration in GluK5 immunoreactivity within area CA3 was observed between **Ci.** sham-treated and **Cii.** pilocarpine-treated animals. SR, *stratum radiatum*; SL, *stratum lucidum*; SP, *stratum pyramidale*; SO, *stratum oriens*. Scale bars, 100 µM.

neighboring CA3 and DG (Bahn et al., 1994; Bureau et al., 1999; Paternain et al., 2000; Darstein et al., 2003; Isaac et al., 2004). KARs have been shown to regulate excitatory current in CA1 interneurons (Cossart et al., 1998; Frerking et al., 1998) and as demonstrated by knockout studies, GluK2-containing KARs are capable of producing inward current in CA1 pyramidal cells (Bureau et al., 1999). Despite the availability of seemingly functional KARs in CA1, they have not been shown to contribute to synaptic transmission. However, applied kainate and synaptically released glutamate have been shown to act on second-messenger metabotropic KARs to inhibit the slow afterhyperpolarization (AHP) (Melyan et al., 2002; Melyan et al., 2004). Reduction of the slow AHP increases the ability of neurons to fire more rapidly and thus can contribute to the propagation of seizures through area CA1 (Robinson and Deadwyler, 1981; Ashwood et al., 1986). While distinct from their role in CA3 and DG, KARs localized in CA1 may contribute to the spread of seizures. We investigated alterations in KAR subunit expression in area CA1 following pilocarpine-induced SE.

In agreement with the immediate and persistent decrease observed in area CA3 and the DG, we determined that GluK2/3 subunit expression is significantly reduced in area CA1 at all three time points explored (5d: 59.8 ± 9.2% of control, $n = 8$; $p < 0.01$; 60d: 59.2 ± 4.0% of control, $n = 7 – 8$; $p < 0.01$; 200d: 63.4 ± 8.5% of control, $n = 7 – 8$; $p < 0.01$; Figure 4.5A). Alterations in GluK5 subunit expression are similar to those observed in area CA3 in that expression is significantly decreased at 5d post-SE (52.3 ± 8.8% of control, $n = 7$
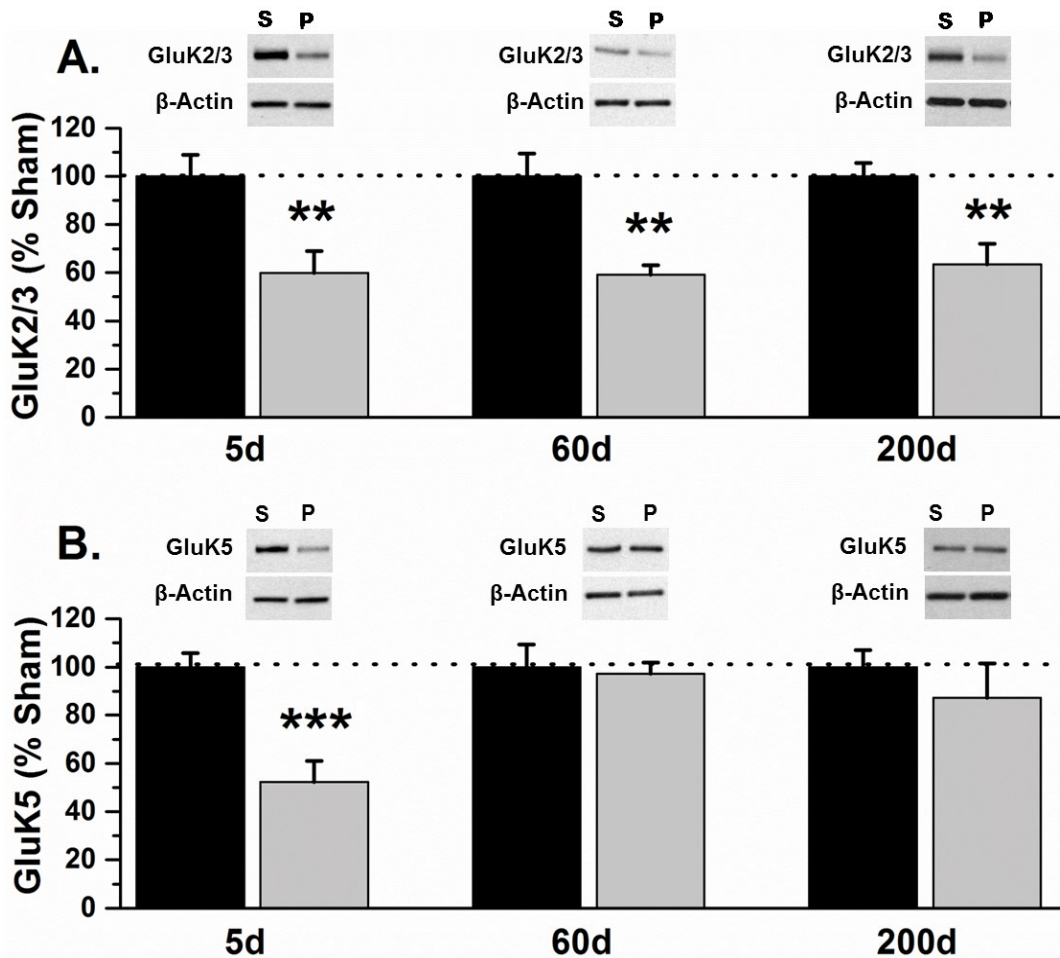
Figure 4.5. Alterations in KAR subunit expression in area CA1 in animals post-SE. Western blot analysis of microdissected CA1 from animals 5d, 60d and 200d post pilocarpine-induced SE. **A.** Expression of the GluK2/3 subunit was significantly reduced at all three time-points examined. **B.** GluK5 subunit expression was initially decreased at 5d post-SE and recovered to baseline by 60d and 200d post-SE. S, sham-treated; P, pilo-treated. *n* = 6 − 8; **p < 0.01; ***p < 0.0001, t-test.

– 8; p < 0.001) and then recovers to baseline by 60d (97.3 ± 4.5% of control, n = 6 – 8; p > 0.05) and 200d post-SE (87.2 ± 14.2% of control, n = 7 – 8; p > 0.05; Figure 4.5B). Western blot analysis utilizing a GluK4 antibody failed to produce a detectable signal on tissue collected from area CA1.

Immunohistochemical analysis of tissue collected 60d post-SE demonstrated robust labeling of GluK2/3 and GluK5 subunits Figure 4.6. We observed immunoreactivity of the GluK2/3 subunit throughout area CA1, including labeling in the somata and dendrites of pyramidal cells, as well as robust labeling of interneurons throughout *stratum radiatum* and *stratum oriens* (Figure 4.6A). In agreement with mRNA expression, we observed the strongest immunoreactivity with GluK5 labeling throughout area CA1 (Wisden and Seeburg, 1993). In addition we observed GluK5 immunoreactivity throughout the dendrites of CA1 pyramidal cells, extending into *stratum radium*. GluK5 expression was also noted in interneurons throughout *stratum radiatum* and *stratum oriens* (Figure 4.6C). Our observed expression of GluK2/3 and GluK5 immunoreactivity throughout area CA1, including interneuron labeling, is in agreement with previous studies (Wisden and Seeburg, 1993; Paternain et al., 2000; Christensen et al., 2004). In animals 60d post-SE we observed a decrease in GluK2/3 immunoreactivity in both CA1 pyramidal cells as well as the remaining interneurons, and no apparent difference in GluK5 immunoreactivity, findings in support of western blot analysis of CA1 tissue.

It is worthwhile noting that while our GluK4 antibody failed to detect protein expression in western blots from area CA1, we did observe distinct
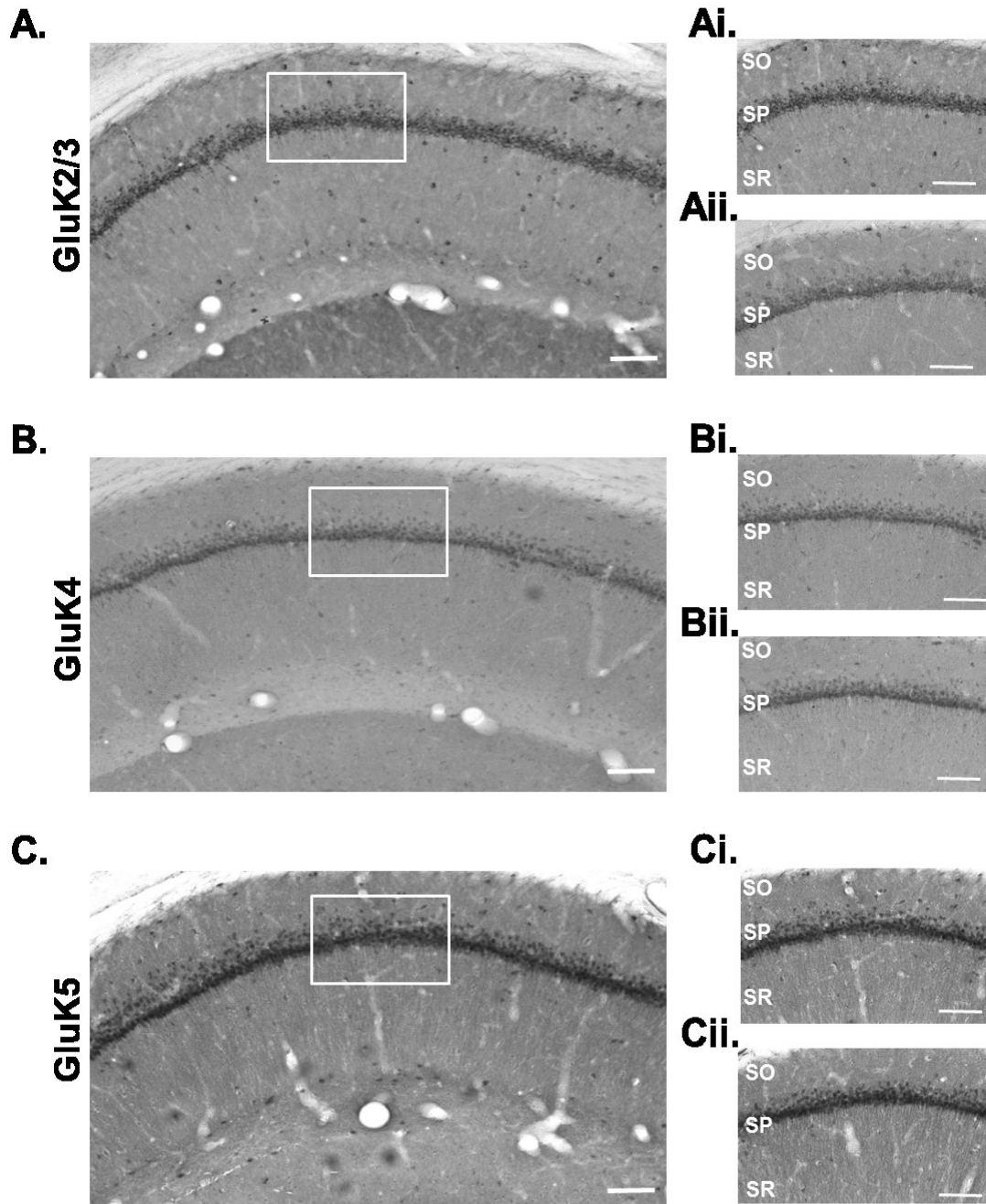
Figure 4.6. KAR subunit immunoreactivity in area CA1 in animals 60d post-SE. Distinct KAR subunit labeling in area CA1. **A.** GluK2/3 subunit expression was dramatically reduced in SP in **Ai.** sham-treated and **Aii.** pilocarpine-treated animals. **B.** GluK4 immunoreactivity among **Bi.** sham-treated and **Bii.** pilocarpine-treated animals was weak and did not appear to change post-SE. **C.** GluK5 immunoreactivity remained unaltered in **Ci.** sham-treated and **Cii.** pilocarpine-treated animals. SO, *stratum oriens;* SP, *stratum pyramidale*; SR, *stratum radiatum.* Scale bars, 100 µM.

labeling of CA1 pyramidal cells in immunohistochemical analysis (Figure 4.6B). In support of our observation, previous studies have also reported the presence of GluK4 immunoreactivity in area CA1 (Fogarty et al., 2000; Darstein et al., 2003; Vargas et al., 2013), but also see Wisden and Seeburg (1993). Based on immunohistochemical observations we did not observe any noticeable alterations in GluK4 immunoreactivity in area CA1 from animals 60d post-SE. Our ability to detect GluK4 in immunohistochemical analysis, but not through western blots, may be due to differences in tissue preparation and handling or antibody selectivity.

4.4.4 REDUCTION IN GLUK2/3 SUBUNIT EXPRESSION IS RESTRICTED TO LIMBIC REGIONS

We observed a significant decrease in GluK2/3 subunit expression in each of the three hippocampal regions explored- DG, CA3 and CA1. This decrease was apparent as early as 5 days post-SE and appeared to be permanent, as data collected from 60d and 200d post-SE also displayed a reduction in GluK2/3 expression. We collected tissue from different brain regions from animals 60 days post-SE to determine if the decrease in GluK2/3 subunit expression was part of a global reduction. We found that similar to hippocampal tissue, there was a significant reduction in GluK2/3 expression within the amygdala (68.2 ± 4.1% of control, $n = 6$; $p < 0.01$) and prefrontal cortex (PFC; 48.4 ± 7.4% of control, n = 5 – 7; $p < 0.05$; Figure 4.7). In contrast, GluK2/3 subunit expression remained unaltered in tissue collected from striatum (90.0 ± 14.1% of control, $n = 8$; p >
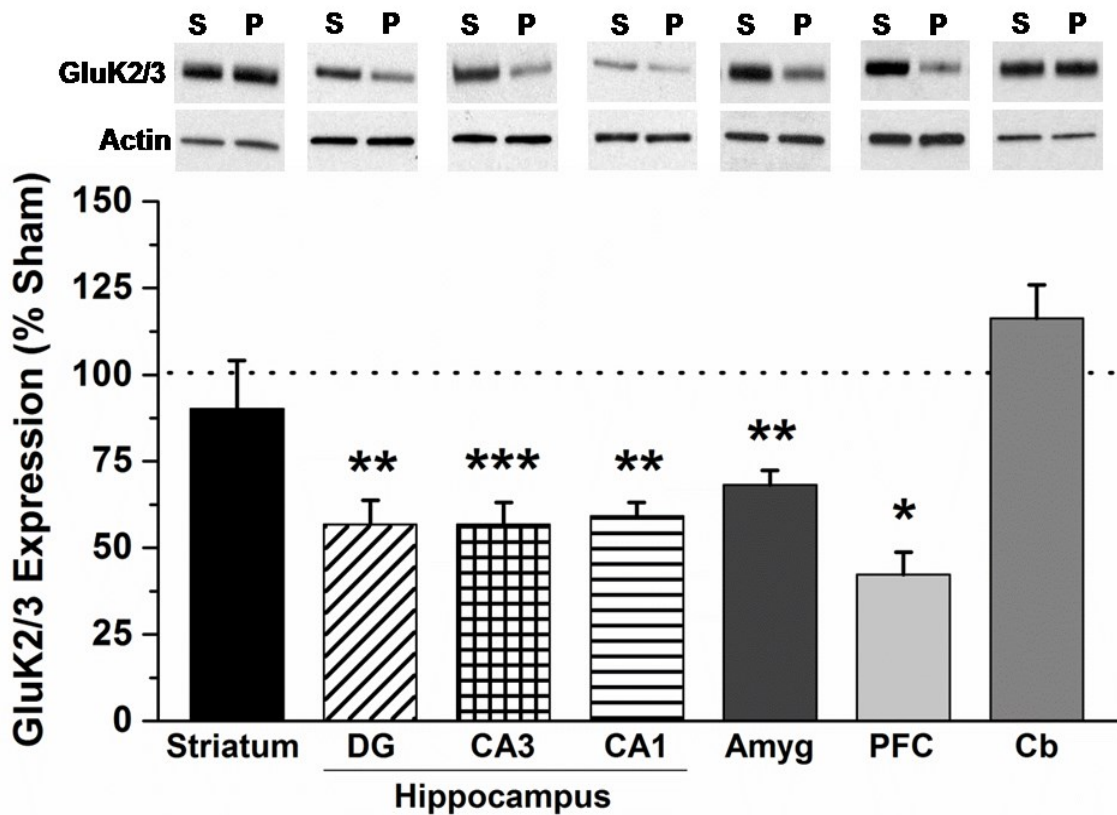
Figure 4.7. Reduction in GluK2/3 subunit expression is restricted to limbic regions. Western blot analysis of microdissected or punched tissue from sham or pilocarpine-treated animals 60d post-SE. GluK2/3 subunit expression is significantly reduced within limbic regions including the hippocampus (DG, CA3 and CA1 regions), amygdala and prefrontal cortex. However GluK2/3 expression was not altered in other brain regions (striatum and cerebellum), demonstrating the impact of SE on GluK2/3 subunit expression throughout the limbic system. S,sham-treated; P, pilo-treated Amyg, amygdala; PFC, prefrontal cortex; Cb, cerebellum. $n = 5 – 8$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$, t-test.

0.05) and cerebellum (Cb; 116.3 ± 9.6% of control, n = 8; p > 0.05; Figure 4.7). These results demonstrate that GluK2/3 expression does not appear to be global, in that the observed reduction was restricted to structures involved in the limbic system- hippocampus, cortex and amygdala (Noebles et al., 2012). These limbic regions are tightly interconnected to memory formation as well as the emotional response of anxiety (Shin and Liberzon, 2010). The dramatic reduction in GluK2/3 subunit expression within these regions may be associated to comorbid conditions of cognitive impairment and increased levels of anxiety reported by patients (Jacoby and Austin, 2007).

4.4.5 GFAP LABELING IS SIGNIFICANTLY INCREASED IN ANIMALS POST-SE

Many studies of epileptic tissue have explored alterations in neuronal cell loss and mossy fiber sprouting, while the role of reactive gliosis is fairly limited. Given that astrocytes are ubiquitously expressed throughout the CNS and play critical roles in regulating synaptic transmission, alterations in their expression in the epileptic brain may have dramatic consequences. Studies from both human and animal models of epilepsy have established that astrogliosis is significantly increased, even as early as one week post-SE (Babb et al., 1996; Schmidt-Kastner and Ingvar, 1996; Binder and Steinhauser, 2006; Vargas et al., 2013). We determined that GFAP labeling of astrocytes was significantly increased in areas CA1, CA3 and DG at all three time-points examined (Figure 4.8). Within area CA1 we found a significant increase in GFAP labeling at 5d (206.4 ± 24.1% of control, n = 8; p < 0.01) 60d (197.9 ± 30.2% of control, n = 7 – 8; p < 0.05) and
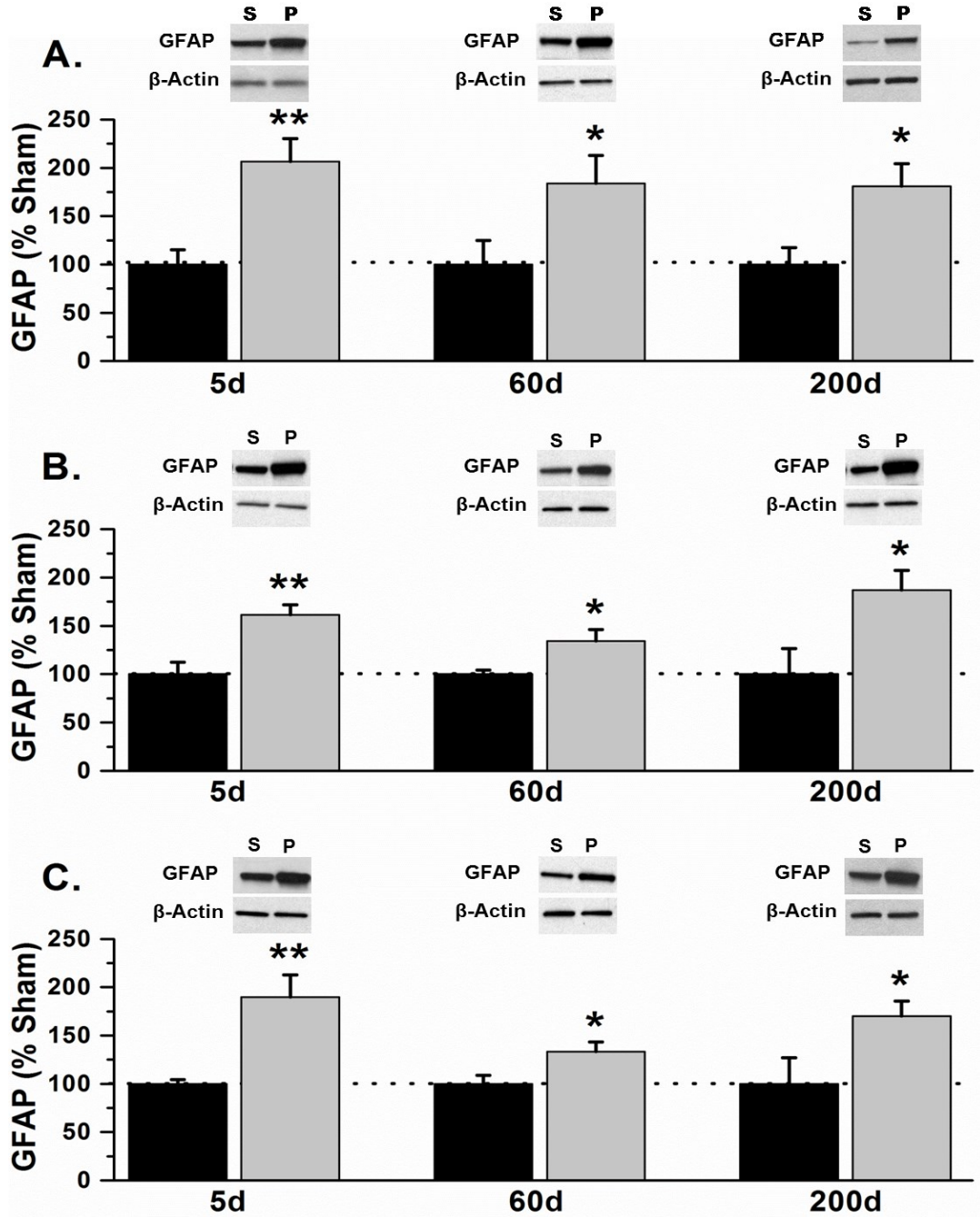
Figure 4.8. GFAP expression is increased in all hippocampal regions post-SE. Western blot analysis of microdissected **A.** CA1 **B.** CA3 and **C.** dentate gyrus from animals 5d, 60d and 200d post-SE demonstrate significant increase in GFAP labeling at all three time-points examined. S, sham-treated; P, pilo-treated. *n* = 6 – 9; *p < 0.05; **p < 0.01, t-test.

200d post-SE (181.0 ± 23.4% of control, *n* = 7; p < 0.05; Figure 4.8A). Similarly, a significant increase was observed in area CA3 at all three points explored (5d: 161.3 ± 10.7% of control, *n* = 8; p < 0.01; 60d: 134.4 ± 11.8% of control, *n* = 8; p < 0.05; 200d: 187.2 ± 20.2% of control, *n* = 6 – 9; p < 0.05; Figure 4.8B). In agreement with the observed increases in CA1 and CA3, we saw significant increases in GFAP labeling throughout the DG at all time-points (5d: 189.7 ± 23.1% of control, n = 6 – 7; p < 0.01; 60d: 133.2 ± 10.0% of control, n = 6 – 7; p < 0.05; 200d: 170.0 ± 15.6% of control, n = 5 – 10; p < 0.05; Figure 4.8C). Immunohistochemical analysis of GFAP expression is depicted in Figure 1.7). While the dramatic increase in GFAP labeling throughout the hippocampus as early as 5d post-SE was shocking, it is in agreement with previous studies and emphasizes the potential impact that astrogliosis can have on neuronal signaling in the epileptic brain.

4.5 DISCUSSION

The results of this study provide the first long-term examination of region-specific changes in hippocampal KARs following pilocarpine induction of SE. These findings are summarized in Figure 4.9. We determined that as early as 5 days post-SE there is a universal downregulation of the GluK2/3 subunit throughout the hippocampus. This decrease appears permanent as it was observed at both 60d and 200d time-points. In contrast to this dramatic reduction in GluK2/3 expression, the apparent decrease in GluK4 and GluK5 subunit expression appear more time and region specific. In addition to our findings
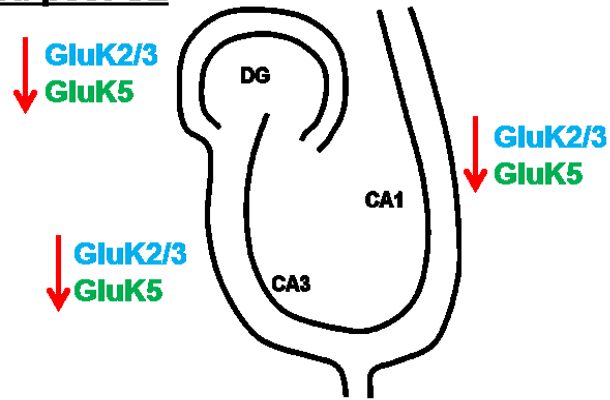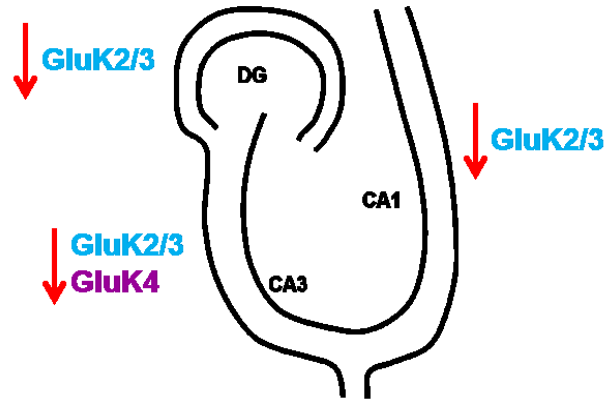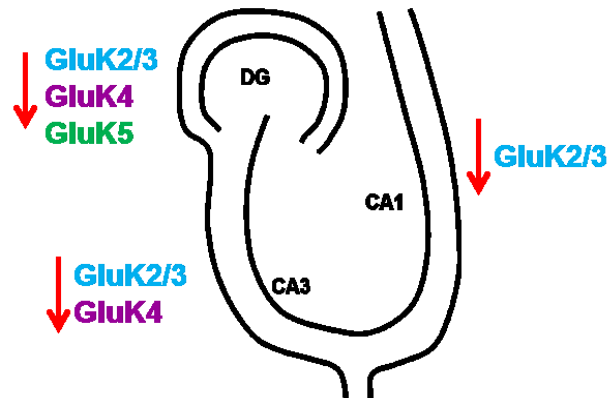
Figure 4.9. Summary of region specific reductions in KAR subunit expression in animals 5d, 60d and 200d post-SE.

regarding alterations in KAR subunit expression, we also observed significant increases in GFAP labeling of astrocytes as early as 5 days post-SE in all three hippocampal regions examined. This increase in GFAP was consistently observed at 5d, 60d and 200d post-SE. Our findings from western blot and immunohistochemical analysis definitively demonstrate that pilocarpine induced SE produces significant alterations in KAR subunit expression throughout the hippocampus and possibly throughout surrounding limbic regions.

4.5.1 EPILEPTOGENESIS AND ASSOCIATED CHANGES IN HIPPOCAMPAL CIRCUITRY

We examined alterations in KAR subunit expression at three distinct time-points following induction of SE. These points were chosen based on known alterations in hippocampal circuitry as well as the development of epileptogenesis following SE. Epileptogenesis refers to the interval between the occurrence of a brain insult, such as SE, and the development of spontaneously occurring seizures. It is widely believed that during this 'latent period' a host of changes occur that transform the healthy, non-epileptic brain into one characterized by recurring seizures (Herman, 2002; Loscher, 2002; Pitkanen, 2002; Stables et al., 2002; Walker et al., 2002; Andre et al., 2007; Pitkanen et al., 2007; Dichter, 2009b, a; Jacobs et al., 2009; Pitkanen and Lukasiuk, 2009; Pitkanen, 2010). The process of epileptogenesis has been well documented in the patient population, with the development of seizures occurring an average of 7.5 years following the precipitating event (French et al., 1993). Several animal

models, including the pilocarpine model of SE utilized in the current study, have also been identified as producing periods of epileptogenesis. Following induction of SE through administration of pilocarpine animals typically experience a seizure free 'latent period' ranging from days to several weeks prior to the development of spontaneous recurring seizures (Walker et al., 2002; Stables et al., 2003; Morimoto et al., 2004; Curia et al., 2008).

Following the latent period and the beginning of spontaneous seizures both human and animal studies demonstrate the appearance of mossy fiber sprouting, which serves as an anatomical hallmark of epilepsy (Sutula et al., 1989; Houser et al., 1990; Babb et al., 1991). As described in detail in Chapter 1.7, aberrant mossy fibers synapse within the inner molecular layer of the DG, creating a new excitatory circuit partially mediated by KARs (Cronin et al., 1992; Wuarin and Dudek, 1996; Sutula et al., 1998; Epsztein et al., 2005). This well characterized occurrence can be seen as early as several days post-SE, but tends to plateau by 2 – 3 months post-SE (Mello et al., 1993; Okazaki et al., 1995). In addition to mossy fiber sprouting neuronal cell loss and the proliferation of astrocytes is also evident within the first few weeks to months following SE (Corsellis and Bruton, 1983; Mathern et al., 1997; Garzillo and Mello, 2002; Binder and Steinhauser, 2006).

In regards to these dramatic alterations in hippocampal circuitry and anatomy following SE definitive time-points were examined in the current study. The 5d post-SE time-point corresponds to a time when animals have recovered from the precipitating SE insult, but are extremely unlikely to be experiencing

spontaneously occurring seizures. In contrast, by 60d post-SE animals have developed spontaneous seizures, and are likely reaching the height of mossy fiber sprouting, neuronal cell loss as well as astrocyte proliferation. In addition to these two distinct time-points we also explored alterations in KAR subunits in animals 200d post-SE. This point provides an extremely chronic view of epilepsy and is likely more similar to the human condition than earlier time-points.

4.5.2 Reduced KAR subunit expression in DG despite mossy fiber sprouting

Mossy fiber sprouting into the inner molecular layer of the DG has become an anatomical hallmark of epilepsy in both humans and animal models (Tauck and Nadler, 1985; Represa et al., 1987; Sutula and Dudek, 2007). Previous studies, as well as those detailed in Chapter 5, have demonstrated that KARs mediate a portion of this newly formed excitatory circuit (Epsztein et al., 2005). Conflicting with these studies is the current observation that KAR subunit expression is significantly decreased within the DG in animals both 60d and 200d post-SE. Several factors could contribute to this apparent discrepancy.

In the present study western blot analysis was performed in microdissected hippocampal tissue. The section of tissue collected for each region is identified in Figure 2.3. In contrast to areas CA1 and CA3, which predominately contain pyramidal cell layers, the region collected for DG analysis also contains a large portion of the supragranular layer as well as area CA4. Therefore the observed alterations in DG samples may not be reflective of pure

dentate granule cells. Another possibility is that the observed reduction in KAR subunits is reflective of extrasynaptic KARs, whereas synaptic KARs, which contribute to the newly formed mossy fiber synapse, remain intact.

In addition, KAR subunit localization and antibody selectivity could also contribute to the incongruity between anatomical and electrophysiological observations. While the GluK2 subunit is expressed throughout the hippocampus, GluK3 expression is essentially restricted to the DG (Bureau et al., 1999; Darstein et al., 2003). The present study utilized an antibody that targeted both GluK2 and GluK3 KAR subunits. Therefore, while conclusions reached about GluK2/3 immunoreactivity and expression in area CA1 and CA3 are reflective of the GluK2 subunit, the presence of GluK3 within the DG complicates our results. It is possible that our findings actually represent a reduction in GluK2 *or* GluK3 subunit expression, while the other subunit may actually increase or remain unaltered. More selective antibodies or analysis of mRNA expression could be used to determine which low affinity KAR subunit is actually impaired in animals post-SE.

4.5.3 TEMPORAL ALTERATIONS IN KAR SUBUNIT EXPRESSION WITHIN THE DG

Through western blot analysis we observed distinct alterations in KAR subunit expression following the induction of SE. These time-sensitive changes were most apparent within the DG. In this region at 60d post-SE we observed significant reduction in the expression of the GluK2/3 subunit only. However, by 200d post-SE all three subunits, GluK2/3, GluK4 and GluK5 were significantly

reduced. By 60d post-SE animals were extremely likely to experience spontaneous seizures, so the decrease at 200d post-SE was likely not due to seizure activity. However, it has been suggested that seizure frequency and severity increases in chronically epileptic animals (Loscher and Brandt, 2010), which may further impact KAR subunit expression in this region.

Another possible, and more likely, explanation for the delay in KAR subunit expression may involve the process of neurogenesis, the generation of new neurons. This process has been well established within the DG as the appearance of newly formed DGCs persists into adulthood (Eriksson et al., 1998; Gould et al., 1999; Cameron and McKay, 2001; Kempermann, 2002). It has been demonstrated that SE significantly increases the number of DGCs by increasing the process of neurogenesis 5 – 10x following the latent period of SE (Parent et al., 1997; Gray and Sundstrom, 1998). However by 3 – 4 weeks post-SE the rate of DGC proliferation returns to baseline (Parent et al., 1997), and by 5 months post-SE there is a significant reduction in adult dentate granule cell proliferation believed to be associated with chronic epilepsy (Hattiangady et al., 2004).

Based on these studies we suggest that at 60d post-SE animals likely experience fairly robust neurogenesis. Porter et al. (2006) determined that GluK2 subunit expression was significantly reduced in newly born DGCs following SE. In agreement with this study we also observed a reduction in GluK2/3 expression at 60d post-SE. However, by 200d post-SE the process of neurogenesis in our animals was likely significantly impaired. Thus the reduction in KAR subunit

expression could be affected. In addition, the observed reduction could also be due to fewer DGCs than what is seen at 5 or 60d post-SE.

4.5.4 ASTROCYTE PROLIFERATION IN ANIMALS POST-SE

Astrocytes are star-shaped glial cells that essentially cover the entire CNS. While not widely discussed, astrocytes are believed to play a prominent role in synaptic transmission through their release and regulation of active molecules including glutamate and GABA (Nedergaard et al., 2003; Halassa et al., 2007; Shigetomi et al., 2008; Perea et al., 2009). The extent of their role has given rise to the term 'tripartite synapse' which proposes that astrocytes play an essential role in regulating synaptic neurotransmission (Araque et al., 1999; Halassa et al., 2007; Perea et al., 2009). Both human and animal models of epilepsy have demonstrated robust increases in astrogliosis (Babb et al., 1996; Binder and Steinhauser, 2006), even as early as one week post-SE (Schmidt-Kastner and Ingvar, 1996; Vargas et al., 2013). In agreement with these previous studies we observed significant increases in GFAP labeling of astrocytes in all three hippocampal brain regions at all time-points explored.

It should be noted that a recent publication also demonstrated an increase in GFAP labeling post kainate-induced SE but also reported colocalization of KAR subunits GluK1, GluK2/3, GluK4 and GluK5 in GFAP-positive astrocytes in area CA1 (Vargas et al., 2013). In direct contrast, we did not observe any immunoreactivity in cells resembling astrocytes in any brain region at any of the three time-points explored. Several factors could contribute to these apparent

differences. Vargas et al. (2013) utilized the kainate model of SE as well as enriched astroglial tissue fractions for western blot analysis. The current study employed the pilocarpine model of SE, thus avoiding direct activation of KARs. In addition we performed western blot analysis on crude plasma membrane fractions not enriched with astroglia and were still able to detect dramatic increases in GFAP labeling in tissue collected from animals post-SE. While our findings regarding the increase in GFAP labeling are in agreement with previous findings, we do not have any evidence supporting the notion that KAR subunitsare colocalized with astrocytes (Vargas et al., 2013).

4.5.5 KAR SUBUNIT EXPRESSION AND COMORBIDITIES

While epilepsy is first and foremost considered a seizure disorder, it is also accompanied by a variety of comorbidities including alterations in psychiatric and cognitive functioning. The diagnosis of psychiatric comorbidities is significantly increased in patients with epilepsy, with nearly 50% of patients reporting depressive-like symptoms (Jackson and Turkington, 2005). In addition, patients commonly report impairments in memory, attention and describe a feeling of general mental slowness (van Rijckevorsel, 2006). Studies examining the role of hippocampal KARs have begun to elucidate a possible correlation between KARs and comorbidities associated with epilepsy.

Of note is the recent identification that the GluK4 subunit is likely involved in regulating anxiety and depressive-like phenotypes (Catches et al., 2012), however findings from this study do not rule out the likely role of the GluK5

subunit. The GluK4 subunit has also been linked to response to antidepressant treatment in patients (Paddock et al., 2007). In addition to the possible correlation between altered KAR subunit expression and disorders associated with epilepsy, KARs have also been linked to other severe disorders such as bipolar disorder and schizophrenia (for review see Lerma and Marques, 2013). Given the role of KARs in a variety of diseases, the findings of this study suggest that the dramatic reduction in KAR subunit expression we identified may be implicated in epilepsy comorbidities. Thus, KARs may serve as potential targets for treatment of these disorders.

4.5.6 ALTERED KAR SUBUNIT EXPRESSION AND CA3 NEUROTRANSMISSION POST-SE

In the current study we observed significant reductions in both GluK2/3 and GluK4 subunit expression in area CA3 from epileptic animals (60d and 200d post-SE). Previous studies have demonstrated that the GluK5 subunit contains several endoplasmic reticulum (ER) retention motifs that block surface expression unless bound to a GluK1 – 3 subunit, thus promoting the formation of heteromeric KARs (Gallyas et al., 2003; Ren et al., 2003). However, recent findings have demonstrated that agonist binding to the GluK5 subunit was sufficient to promote heteromeric receptor trafficking (Fisher and Housley, 2013). In light of these studies we suggest that in epileptic animals there may be fewer functional KARs, and that the KARs that do remain likely contain GluK2/5

subunits, a shift from predominately GluK2/4-containing receptors in control animals. The potential shift from Glu2/4 to GluK2/5-containing KARs would dramatically impact receptor pharmacology as well as the kinetics of the KAR-mediated current.

4.6 SIGNIFICANCE

The results of this study demonstrate that pilocarpine-induced SE produces dramatic alterations in KAR subunit expression in both a region- and time-specific manner. Hippocampal KARs have been implicated in seizure disorders as well as comorbidities such as anxiety and depression (Catches et al., 2012; Lerma and Marques, 2013). The observed alterations in KAR subunit expression following SE may shed light on the role of individual KAR subunits in the generation and propagation of seizures as well as observed comorbidities. We suggest that these alterations likely influence KAR-mediated neurotransmission in both the DG and area CA3 following pilocarpine-induced SE, a topic explored in detail in Chapter 5. Together these results provide further evidence that healthy and diseased brains are strikingly different and that understanding these differences may be the key to the development of therapeutics for treatment of diseases such as epilepsy.

CHAPTER 5

ALTERATIONS IN KAINATE RECEPTOR MEDIATED NEUROTRANSMISSION AT THE MOSSY FIBER – CA3 SYNAPSE OF EPILEPTIC ANIMALS

5.1 INTRODUCTION

Kainate receptors (KARs) are ionotropic glutamate receptors that mediate fast excitatory neurotransmission. Functional KARs are tetramers that can be composed of a homomeric combination of the low glutamate affinity GluK1 – 3 subunits. The GluK4 and GluK5 subunits have higher glutamate sensitivity and only form functional receptor complexes when paired with GluK1 – 3 subunits. KARs are widely distributed throughout the CNS with the strongest expression noted within the dentate gyrus (DG) and area CA3 of the hippocampus (Wisden and Seeburg, 1993; Bahn et al., 1994; Darstein et al., 2003).

The role of hippocampal KARs has been most strongly characterized at the mossy fiber – CA3 synapse. In this region postsynaptic KARs mediate a small excitatory current with slow decay kinetics, which allows for temporal summation and serves to enhance the depolarization envelope (Castillo et al., 1997; Vignes and Collingridge, 1997; Frerking and Ohliger-Frerking, 2002). These characteristics of postsynaptic KARs emphasize their potential contribution to a hyperexcitable network. In contrast to their widespread role in area CA3, KARs have not been found to contribute to synaptic transmission in the naïve DG. The lack of KAR-mediated transmission within the DG is also

noted in area CA1, where despite an abundance of KAR subunits there is no synaptic contribution (Bureau et al., 1999).

The DG has been identified as the 'gatekeeper' of the hippocampus, acting to restrict excitatory transmission entering area CA3, a region prone to hyperexcitability and epileptiform activity (Nadler et al., 1978). An anatomical hallmark of epilepsy is the appearance of sprouted mossy fibers in the inner molecular layer of the DG (Tauck and Nadler, 1985; Represa et al., 1987; Sutula and Dudek, 2007). In addition to this axonal rewiring, KARs have been implicated in mediating a portion of the newly formed excitatory circuit in the epileptic brain (Epsztein et al., 2005). Most notably, KARs located on these aberrant mossy fibers have been shown to increase the hyperexcitability and synchronization of DGCs by altering their intrinsic firing pattern (Artinian et al., 2011). These actions significantly impair the ability of the DG to properly gate excitatory neurotransmission in epilepsy.

These alterations in the 'gatekeeper' function of the DG likely impact transmission and epileptiform activity throughout the other regions of the hippocampus, most notably, area CA3. In addition, given the dramatic shift in the role of KARs within the epileptic DG, it is likely that KARs located in area CA3 are also modified. The goal of this study was to explore these possible alterations in KAR-mediated neurotransmission at the mossy fiber – CA3 synapse in epileptic animals. Findings from this study have provided the first in-depth look at the impact of epilepsy on the function of KARs localized to area CA3 and provide evidence for their divergent role within hippocampal mossy fibers.

5.2 HYPOTHESIS

Anatomical data presented in Chapter 4 demonstrated a robust and significant decrease in both GluK2/3 and GluK4 subunit expression in area CA3 in epileptic animals. Based upon these data we hypothesize that KAR-mediated neurotransmission in the mossy fiber – CA3 pathway will be reduced in epileptic animals. Given the significant increase in excitatory drive within the DG, the observed reduction in KAR-mediated transmission in area CA3 of epileptic animals may serve as a compensatory modification to limit hyperexcitability and the generation of epileptiform activity.

5.3 MATERIALS AND METHODS

5.3.1 ANIMAL MODEL OF SE

All animal care and use procedures were carried out in accordance with protocols written under the guidance of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of South Carolina. Following induction of SE, rats were housed 1 per cage with *ad libitum* access to food and water. Rats were housed in a climate controlled facility with a light/dark cycle of 12/12 hours.

SE was induced in adult male Sprague-Dawley rats (Harlan, Indianapolis, IN). Animals received intraperitoneal (i.p.) injections of scopolamine methyl bromide and terbutaline hemisulfate (2 mg/kg) followed 30 – 45 min later by pilocarpine hydrochloride (390 mg/kg). After 2 hours of continuous SE, seizures

were terminated by diazepam (DZP, 25 mg/kg). This group of animals served as the 'pilo' group. A separate group of animals received identical treatment with the exception of receiving saline (390 mg/kg) instead of pilocarpine hydrochloride. This group of animals served as the 'sham' group. More detailed methods concerning induction of SE are described in Chapter 2.3.

5.3.2 WHOLE CELL ELECTROPHYSIOLOGY

Transverse (300 µM thick) hippocampal slices were prepared from sham- and pilocarpine-treated adult male Sprague-Dawley rats as described in detail in Chapter 2.6. Briefly, animals were deeply anesthetized with isoflurane and transcardially perfused with a sucrose-based aCSF. Slices were prepared in ice cold (4°C) sucrose-based aCSF and incubated in room-temperature oxygenated (95% O_2 / 5% CO_2) NaCl-based solution.

Excitatory postsynaptic currents (EPSCs) recorded from dentate granule cells from animals 169 ± 10 days post-treatment were evoked with a 50 ms puff of 10 µM domoate applied every 60 seconds through a Pneumatic PicoPump (PV800, WPI, Sarasota, FL), located approximately 70 µM from the recorded cell. EPSCs were pharmacologically isolated in an antagonist cocktail containing bicuculline methochloride (Bic, 15 µM), CGP (1 µM), MK-801 (10 µM) and TTX (1 µM). KAR-mediated currents were isolated using GYKI53655 (50 µM). CNQX (100 µM) was applied at the end of the experiments to block any residual glutamatergic transmission. All drugs were purchased from Abcam with the exception of bicuculline methochloride (Ascent Scientific).

5.3.3 FIELD POTENTIAL ELECTROPHYSIOLOGY

Transverse (500 µM thick) hippocampal slices were prepared from sham- and pilocarpine-treated adult male Sprague-Dawley rats as described in detail in Chapter 2.7. Briefly, animals were deeply anesthetized with isoflurane and transcardially perfused with a sucrose-based aCSF. Slices were prepared in ice cold (4°C), and then incubated for 30 minutes in heated (37°C) oxygenated (95% O_2 / 5% CO_2) sucrose-based aCSF prior to a gradual transition to room-temperature oxygenated (95% O_2 / 5% CO_2) NaCl-based solution.

Field EPSPs (fEPSPs) were evoked from animals 157 ± 9 days post-treatment in the DG and area CA3 using two distinct paradigms. 1) Mossy fiber – CA3 fEPSPs were evoked in the dentate gyrus granule cell layer every 30 sec and recorded in CA3 *stratum lucidum*. 2) Recurrent mossy fiber fEPSPs in the dentate gyrus of epileptic animals were evoked in the inner one-third of the molecular layer every 60 sec and recorded in the granule cell layer. Figure 2.2 depicts each of the two experimental paradigms utilized, blue and green electrodes, respectively. fEPSPs were evoked at 50% of the maximal response determined through an I/O curve. fEPSPs evoked from mossy fibers were identified based upon their large paired pulse facilitation (PPF) as well as inhibition by the group II metabotropic glutamate receptor antagonist DCG-IV, which selectively inhibits mossy fiber-evoked, but not fEPSPs evoked from stimulation of associational/commissural fibers (Yeckel et al., 1999). In our hands DCG-IV (1 µM) blocked 91.8 ± 1.2% of the response in sham (*n* = 6), and 88.9 ± 1.5% in pilo-treated animals (*n* = 6; Figure 5.1A). In addition, we utilized
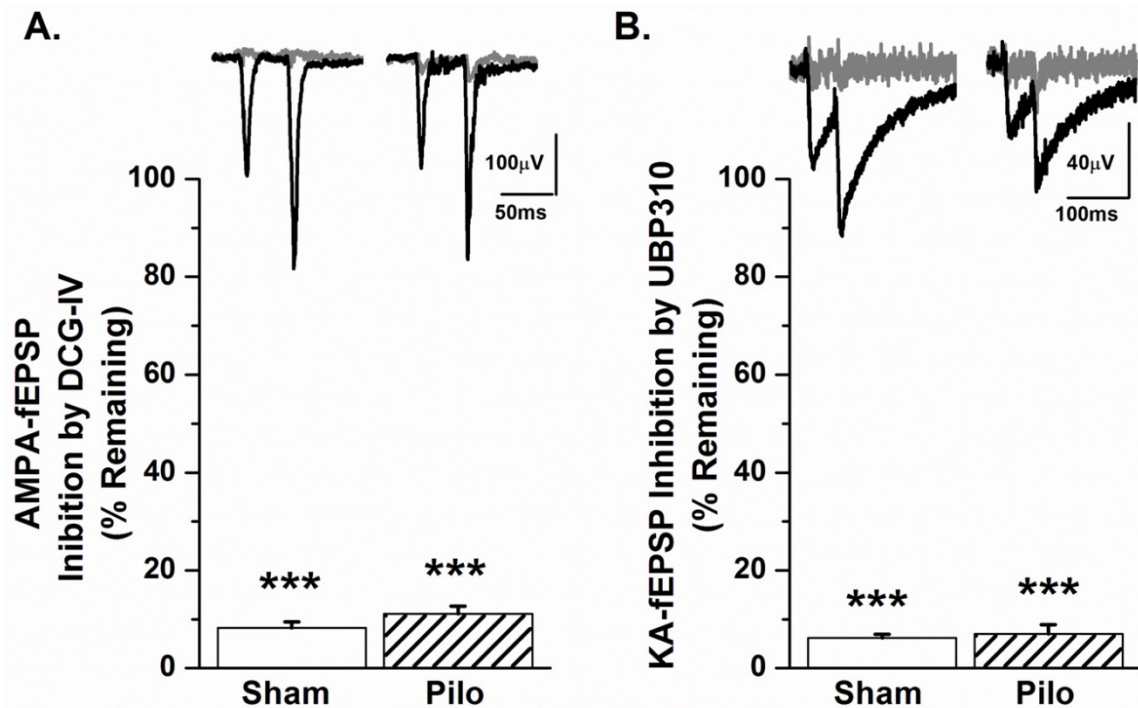
Figure 5.1. Selective inhibition of fEPSPs. **A.** DCG-IV (1 µM) inhibited AMPA-fEPSPs evoked from selective stimulation of the mossy fiber – CA3 pathway in sham (*n* = 6) and pilo-treated animals (*n* = 6). Control waveforms evoked from sham and pilo-treated animals demonstrate large PPF (*black traces*) and inhibition by DCG-IV (*gray* traces). **B.** UBP310 (3 µM) inhibits KA-fEPSPs evoked from stimulation of the mossy fiber – CA3 pathway in both sham (*n* = 7) and pilo-treated animals (*n* = 5). Control waveforms evoked from sham and pilo-treated animals demonstrate PPF (*black traces*) and inhibition by UBP310 (*gray traces*). ***p < 0.001, t-test.

willardine derivative UBP310 to inhibit KAR-mediated transmission (Jane et al., 2009; Perrais et al., 2009; Pinheiro et al., 2013). We demonstrated that UBP310 (3 µM) blocked 93.9 ± 0.8% of the response in sham-treated animals ($n = 7$), and 93.0 ± 2.0% in pilo-treated animals ($n = 5$; Figure 5.1B).

fEPSPs were pharmacologically isolated in an antagonist cocktail containing bicuculline methochloride (Bic, 20 µM) and MK-801 (10 µM). AMPA receptors were blocked by application of GYKI53655 (50 µM), and KARs were blocked by UBP310 (3 µM) or ACET (300nM). Residual transmission was blocked by application of CNQX (100 µM). All drugs were purchased from Abcam with the exception of bicuculline methochloride (Ascent Scientific) and ACET (Tocris bioscience).

5.4 RESULTS

5.4.1 KAR-MEDIATED TRANSMISSION IS INCREASED IN THE DENTATE GYRUS OF EPILEPTIC ANIMALS

Puff application of the glutamate receptor agonist domoate (10 µM) in the inner molecular layer of the DG elicited a large current mediated by the extrasynaptic receptor population. Application of the AMPA receptor (AMPAR) antagonist GYKI53655 (50 µM) revealed a large KAR-mediated component (Figure 5.2A). This suggests that functional extrasynaptic KARs are present and fail to contribute to synaptic neurotransmission, a finding similar to what has been observed in area CA1 (Bureau et al., 1999). KARs have been shown to contribute to the excitatory circuit formed by the aberrant sprouting of mossy
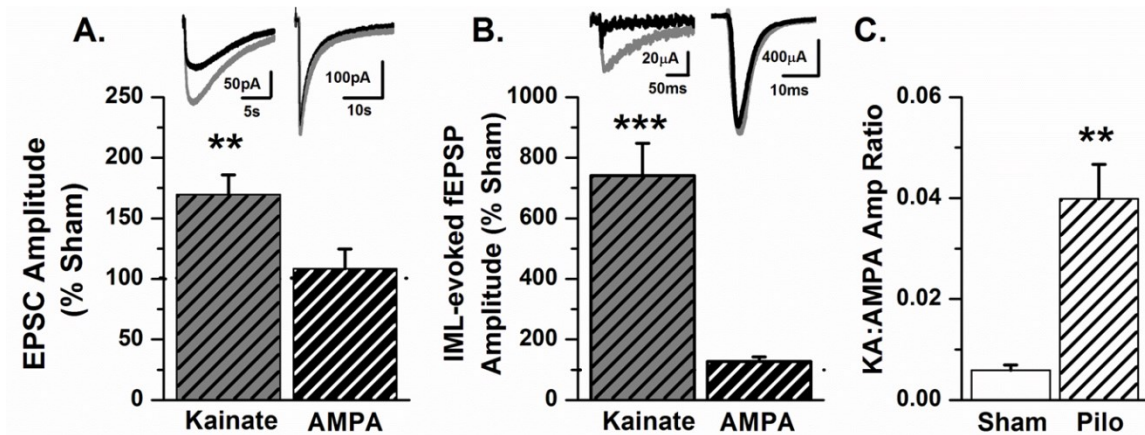
Figure 5.2. KARs mediate transmission in dentate granule cells from epileptic animals. **A.** $EPSC_{KA}$ amplitude is significantly increased in pilo-treated animals following puff application of domoate (10 µM) on dentate granule cells (*n* = 6). No change in the $EPSC_{AMPA}$ amplitude between sham and pilo-treated animals was observed (*n* = 6). Waveforms evoked by domoate in sham (*black traces*) and pilo-treated animals (*gray traces*) demonstrate an increase in $ESPC_{KA}$ but not $EPSC_{AMPA}$. **B.** Stimulation of recurrent mossy fibers elicits a KAR-mediated fEPSP in pilo-treated animals (*n* = 5). No change in the $fEPSP_{AMPA}$ amplitude was observed between sham and pilo-treated animals (*n* = 5 – 6). Waveforms evoked by stimulation of the inner molecular layer demonstrate the appearance of a $fEPSP_{KA}$ in pilo-treated animals (*gray traces*), and no change in the amplitude of the $fEPSP_{AMPA}$. **C.** The KA:AMPA amplitude ratio is significantly increased in pilo-treated animals due to the appearance of a KAR-mediated response (*n* = 5 – 6). **$p < 0.01$; ***$p < 0.001$, t-test.

fibers into the inner molecular layer of the DG in epileptic animals (Epsztein et al., 2005). Therefore we explored the effects of 10 µM domoate on DGCs from pilo-treated animals. We determined that the amplitude of the current was significantly increased following application of 10µM domoate (169.7 ± 16.3% of control, $n = 6$; $p < 0.01$; Figure 5.2A), while the $EPSC_{AMPA}$ was unaltered (108.3 ± 16.4% of control, $n = 6$; $p > 0.05$; Figure 5.2A). These findings were further explored using field potential electrophysiology.

fEPSPs were elicited with a stimulating electrode placed in the inner molecular layer of the DG with the recording electrode placed within the granule cell layer (Figure 2.2, green electrodes). In sham-treated animals we evoked a large fEPSP that was completely inhibited by GYKI53655, a finding in agreement with previous observations demonstrating a lack of synaptic KAR-mediated transmission in the DG. However, in epileptic animals we observed a GYKI-resistant component that was sensitive to the KAR antagonist UBP310 (3 µM), demonstrating the appearance of KAR-regulated synaptic transmission in the DG of pilo-treated animals (740.8 ± 107.3% of control, $n = 5$; $p < 0.001$; Figure 5.2B). Furthermore, we determined that transmission mediated by AMPARs was unaltered (127.3 ± 15.2% of control, $n = 5 - 6$; $p > 0.05$; Figure 5.2B), demonstrating a selective increase in KAR-mediated transmission. In agreement the KA:AMPA amplitude ratio was significantly increased in pilo-treated animals (sham: 0.006 ± .0001, $n = 5$; pilo: 0.04 ± 0.007, $n = 6$; $p < 0.01$; Figure 5.2C).

Input-output (I/O) curves were conducted to examine the impact of stimulation intensity on the observed increase in KAR-mediated transmission.

The fEPSP$_{KA}$ amplitude increased with stimulation strength in pilo-, but not sham-treated animals (n = 3 – 6, Figure 5.3A). At maximal stimulation strength the fEPSP$_{KA}$ evoked from pilo-treated animals was significantly greater than that from sham-treated animals (sham: 31.1 ± 11.5 µV; pilo: 136.4 ± 28.8 µV, n = 3 – 5; p < 0.05; Figure 5.3A). In agreement with our observation that the increase in KAR-mediated transmission is a selective modification, no difference in the amplitude of the fEPSP$_{AMPA}$ was observed over many stimulus intensities, even at the highest intensity (sham: 187.6 ± 84.3 µV; pilo: 237.2 ± 56.2 µV, n = 3 – 5, p > 0.05; Figure 5.3B). These findings are in agreement with observations by Epsztein et al. (2005) demonstrating the appearance of a KAR-mediated response in the inner molecular layer of the DG in epileptic animals.

5.4.2 SYNAPTIC INTEGRATION IS IMPAIRED IN THE DENTATE GYRUS OF EPILEPTIC ANIMALS

As fast synaptic transmission enters the DG it must be synaptically integrated by the intrinsically sparse firing DGCs, a process that serves to limit transmission (Jung and McNaughton, 1993; Schmidt-Hieber et al., 2007). In epilepsy, the KARs located on aberrant mossy fibers create a shift in the firing properties of DGCs from sparse firing to sustained, rhythmic firing thus increasing the window for synaptic integration and increasing hyperexcitability (Artinian et al., 2011). We examined the contribution of AMPA and KA-receptor mediated transmission at varying frequency intensities in pilo-treated animals. We determined that in pilo-treated animals there was a significant increase in the
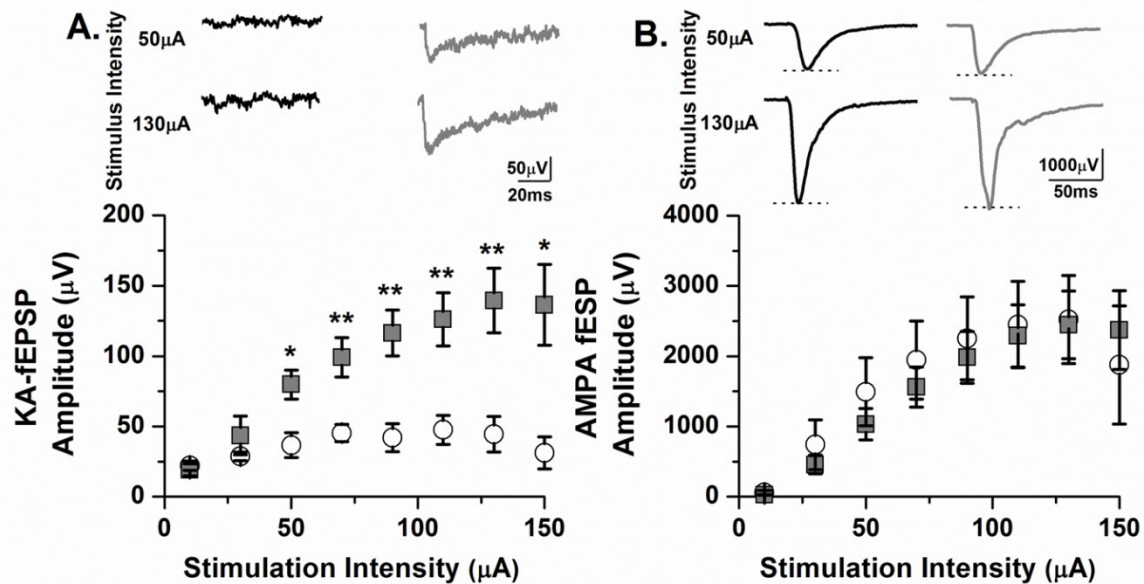
Figure 5.3. I/O curves of fEPSPs in the dentate gyrus demonstrate the appearance of KAR-mediated transmission in epileptic animals. **A.** At stimulus intensities of 50μA and above a KAR-mediated fEPSP is apparent in pilo-treated animals (*closed gray squares*, *n* = 5 – 6) but not sham-treated animals (*open circles*, *n* = 3 – 5). Representative waveforms from sham-treated (*black traces*) illustrate the lack of a fEPSP$_{KA}$ in sham-treated animals, and the development of a fEPSP$_{KA}$ in pilo-treated animals (*gray traces*). **B.** The amplitude of the fEPSP$_{AMPA}$ evoked from pilo-treated animals (*closed gray squares*, *n* = 5 – 6) is not significantly different that the fEPSP$_{AMPA}$ evoked from sham-treated animals (*open circles*, *n* = 3 – 5) at any of the stimulus intensities examined. Representative waveforms from sham-treated (*black traces*) and pilo-treated (*gray traces*) are not different at two different stimulus intensities. *p < 0.05; **p < 0.01, t-test.

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area of the fEPSP$_{AMPA/KA}$ at all frequencies explored (Figure 5.4A) representative of an increase in synaptic integration. In addition, the area of the fEPSP$_{AMPA/KA}$ induced by higher frequencies (10 – 100 Hz) was significantly greater than the area of very low (0.017 Hz) stimuli. We specifically examined a 5 pulse, 50 Hz train, which increased the total MF-evoked area in pilo-treated animals (sham: 26.5 ± 3.9 mV/ms, n = 5; pilo: 60.4 ± 7.1 mV/ms, n = 5; p < 0.01; Figure 5.4B). We further explored the isolated fEPSP$_{KA}$ and fEPSP$_{AMPA}$ in response to a 50 Hz train and determined that the increase in area was due solely to the appearance of a KAR-mediated component (pilo: 20.4 ± 4.6 mV/ms, n = 5; Figure 5.4C) as the area of the isolated AMPAR-mediated train was unaltered (sham: 26.2 ± 2.8 mV/ms; pilo: 28.6 ± 3.1 mV/ms, n = 5; p > 0.05; Figure 5.4C).

To further exemplify the appearance of a KAR-mediated response in pilo-treated animals we used the same 5 pulse, 50 Hz train to elicit a substantial KAR-mediated component and examined inhibition produced by the KAR antagonist UBP310 (Pinheiro et al., 2013). We determined that 3 μM UBP310 significantly inhibited the response in pilo-treated (32.2 ± 8.9% of control, n = 4; p < 0.01; Figure 5.5), but not sham-treated animals (91.9 ± 6.8% of control, n = 4; p > 0.05; Figure 5.5). These findings were in agreement with Pinheiro et al. (2013) in which UBP310 significantly blocked EPSC$_{KA}$ elicited in dentate granule cells at recurrent mossy fiber synapses in epileptic animals. We also explored the ability of a different KAR antagonist, ACET, to block the KAR-mediated response in pilo-treated animals. Our initial data suggested that ACET (300 nM) blocks the response to a similar degree to that of UBP310 (26.8 ± 0.8% of
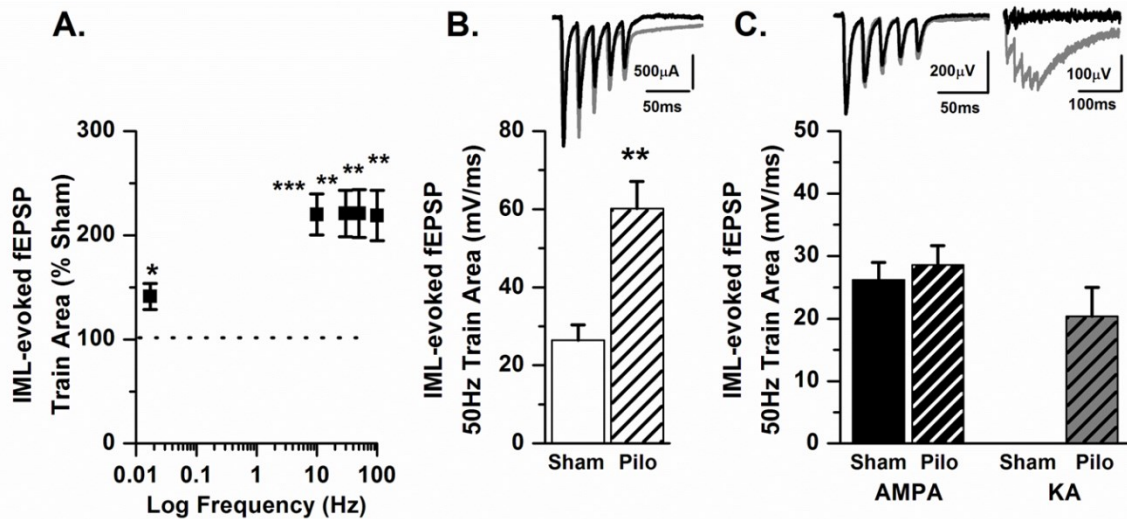
Figure 5.4. Synaptic integration is impaired in the dentate gyrus of epileptic animals. Trains were evoked by stimulation of the inner molecular layer of the dentate gyrus. **A.** The area of the fEPSP$_{AMPA/KA}$ was significantly increased at all frequencies examined in pilo-treated animals (*n* = 5 – 7). The area evoked by higher frequencies (10 – 100 Hz) was significantly increased relative to the lowest frequency examined. **B.** The fEPSP$_{AMPA/KA}$ area of a 5 pulse, 50 Hz train was significantly increased in pilo-treated animals (*n* = 5). Representative waveform demonstrates the increased area and decay of the fESPS$_{AMPA/KA}$ in sham (*black trace*) and pilo-treated animals (*gray trace*). **C.** Area of the fEPSP$_{AMPA}$ is not different between sham and pilo-treated animals (*n* = 5). There is the appearance of a KAR-mediated fEPSP in pilo-treated animals (*n* = 5). Waveforms illustrate no change in the fEPSP$_{AMPA}$ in sham (*black trace*) and pilo-treated animals (*gray trace*) and the appearance of a fEPSP$_{KA}$ in pilo-treated animals (*gray trace*). *p < 0.05; **p < 0.01; ***p < 0.001, t-test.
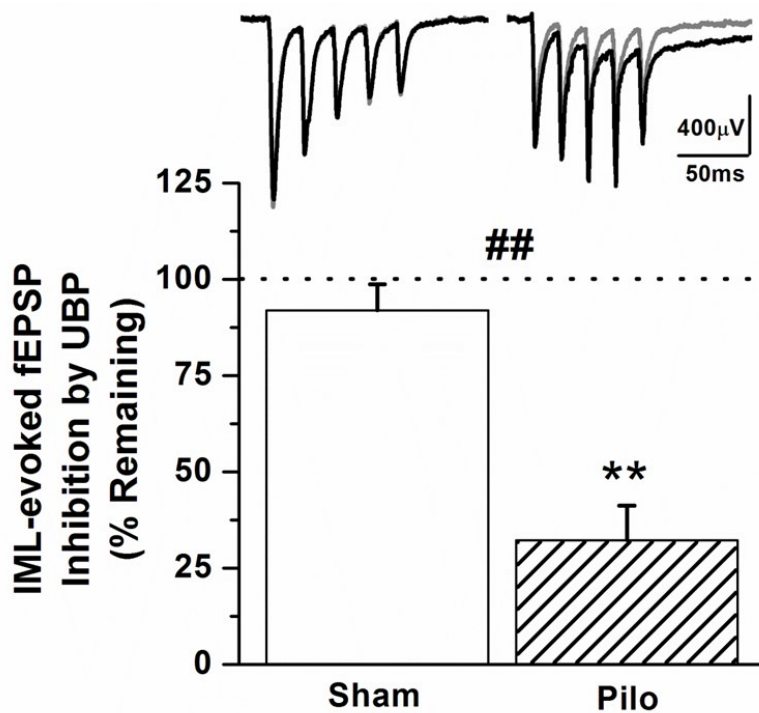
Figure 5.5. UBP310 inhibits KAR-mediated transmission at recurrent mossy fiber synapses. The selective KAR antagonist UBP310 is ineffective in sham-treated animals (n = 4), but significantly inhibits fEPSPs evoked from the recurrent mossy fibers of pilo-treated animals (n = 4). This demonstrates the appearance of a KAR-mediated response in the dentate gyrus of pilo-treated animals. Representative waveforms illustrate the lack of inhibition by UBP (*gray traces*) on control responses (*black traces*) in sham-treated animals, and the significant reduction in the fEPSP in pilo-treated animals. **p < 0.01; ##p < 0.01 t-test.

control, n = 2, p = 0.08, data not shown). These data further demonstrate that a UBP310-sensitive KAR-mediated response appears within the inner molecular layer of pilo-treated animals and contributes to the excitatory circuit. In addition, we demonstrated that this new KAR component significantly contributes to both high and low frequency transmission, which likely contributes to hyperexcitability and the generation of epileptiform activity within the DG.

5.4.3 KAR-MEDIATED TRANSMISSION AT THE MOSSY FIBER – CA3 SYNAPSE IS SIGNIFICANTLY REDUCED IN EPILEPTIC ANIMALS

Mossy fiber – CA3 fEPSPs were elicited with a stimulating electrode placed along the hilar border of the dentate granule cell layer with the recording electrode placed within CA3 *stratum lucidum* (Figure 2.2, blue electrodes). In direct contrast to the increase in KAR-mediated transmission observed in the DG, we observed that the $fEPSP_{KA}$ was significantly reduced in pilo-treated animals (48.9 ± 5.0% of control, n = 7 – 8; p < 0.001; Figure 5.6A). This dramatic decrease in KAR-mediated transmission is selective, in that the $fEPSP_{AMPA}$ was not different in pilo-treated animals (98.3 ± 20.2% of control, n = 5 – 9; p > 0.05; Figure 5.6A). In support of these findings, we observed that the KA:AMPA amplitude ratio was significantly reduced (sham: 0.274 ± 0.026, n = 4; pilo: 0.146 ± 0.02, n = 5; p < 0.01; Figure 5.6B), as well as the KA:AMPA area ratio (sham: 6.63 ± 0.86, n = 3; pilo: 1.71 ± 0.61, n = 5; p < 0.01; Figure 5.6C). I/O curves at stimulus intensities from 10 – 400 μA were performed on isolated KA- and AMPA-receptor mediated responses. We observed a significant difference in the
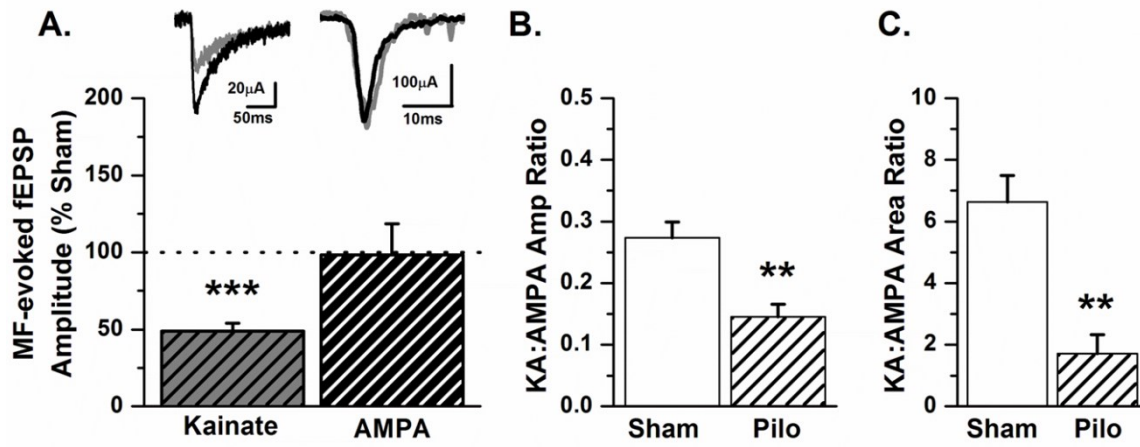
Figure 5.6. KAR-mediated transmission is significantly reduced in the mossy fiber – CA3 pathway of epileptic animals. **A.** The amplitude of the mossy fiber evoked KAR-mediated fEPSP is significantly reduced in pilo-treated animals (n = 7 – 8). No change in the AMPAR-mediated fEPSP was observed between sham and pilo-treated animals (n = 5 – 9). Waveforms evoked by stimulation of the mossy fiber pathway demonstrate the reduction in fEPSP$_{KA}$ amplitude in sham (*black traces*) and pilo-treated animals (*gray traces*), with no apparent change in fEPSP$_{AMPA}$ amplitude. **B.** The KA:AMPA amplitude ratio is significantly reduced in pilo-treated animals due to the decrease of a KAR-mediated response (n = 4 – 5). **C.** The KA:AMPA area ratio is also significantly reduced in pilo-treated animals (n = 3 – 5). **p < 0.01; ***p < 0.001, t-test.

amplitude of the fEPSP$_{KA}$ between sham and pilo-treated animals at stimulus intensities of 70 μA and above (Figure 5.7A). At maximal stimulation the fEPSP$_{KA}$ evoked from pilo-treated animals was significantly smaller than that of sham-treated animals (sham: 177.8 ± 33.2% of control, n = 3; pilo: 59.6 ± 10.2% of control, n = 4; p < 0.05; Figure 5.7A). In support of the selective alteration in KAR-mediated transmission we did not observe any difference in the amplitude of the fEPSP$_{AMPA}$ at any stimulus intensity (Figure 5.7B). These findings demonstrate that there is a downregulation in excitatory neurotransmission mediated by KARs, but not AMPARs. These findings are supported by the anatomical data collected in Chapter 4 demonstrating a significant reduction in GluK2/3 and GluK4 subunit expression in area CA3 of animals 60 and 200d post-SE, Figure 4.3 – 4.4).

5.4.4 SYNAPTIC INTEGRATION AT THE MOSSY FIBER – CA3 SYNAPSE OF EPILEPTIC ANIMALS IS IMPAIRED

The ability of CA3 pyramidal cells to properly integrate information stemming from the DG and synapsing via the mossy fiber pathway is believed to be an integral step in the process of encoding and retrieving episodic memory (Herrmann et al., 2004; Bartos et al., 2007). In addition higher frequency, gamma oscillations have been demonstrated to be necessary for induction of long-term potentiation (LTP), a synaptic correlate of learning (Rosenzweig et al., 1997; Traub et al., 1998). Given the significant decrease in KAR-mediated neurotransmission in area CA3 in pilo-treated animals we explored synaptic
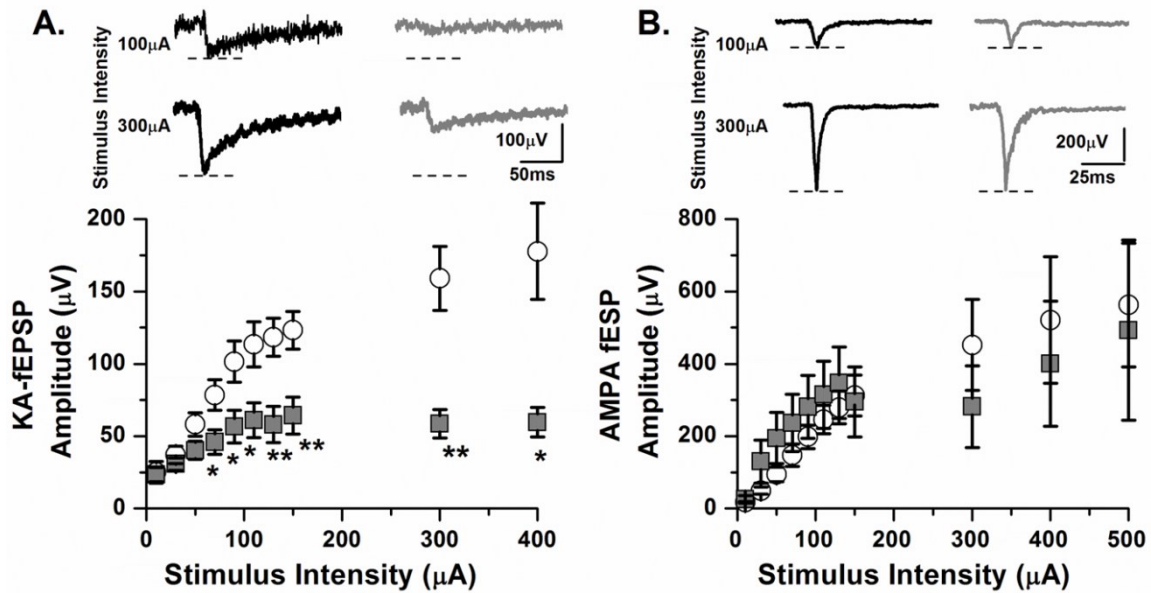
Figure 5.7. I/O curves of mossy fiber evoked fEPSPs demonstrate a selective decrease in KAR-mediated transmission. **A.** The amplitude of the fEPSP$_{KA}$ evoked from pilo-treated animals (*closed gray squares*, n = 4 – 6) is significantly reduced compared to the fEPSP$_{KA}$ evoked from sham-treated animals (*open circles*, n = 3 – 6) at stimulus intensities of 70 – 400 µA. Representative waveforms from sham-treated (*black traces*) and pilo-treated (*gray traces*) demonstrate the reduced potentiation of fEPSP$_{KA}$ at two different stimulus intensities. **B.** The amplitude of the fEPSP$_{AMPA}$ evoked from pilo-treated animals (*closed gray squares*, n = 3 – 5) is not significantly different that the fEPSP$_{AMPA}$ evoked from sham-treated animals (*open circles*, n = 4 – 10) at any of the stimulus intensities examined. Representative waveforms from sham-treated (*black traces*) and pilo-treated (*gray traces*) are not different at two different stimulus intensities. *p < 0.05; **p < 0.01, t-test.

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integration at several different frequencies within the mossy fiber – CA3 pathway (Figure 5.8A). We determined that at lower frequencies (0.03 – 10 Hz) there was no difference in area of $fEPSP_{AMPA/KA}$. However at higher frequencies, closer to that of gamma frequency (30 – 100 Hz), there was a significant decrease in the $fEPSP_{AMPA/KA}$, indicative of a reduction in signal integration within the mossy fiber – CA3 pathway in pilo-treated animals. Similar to data collected from the DG, we examined a 5 pulse, 50 Hz train and concluded that the area of the train was significantly reduced in pilo-treated animals (sham: 56.8 ± 6.2% of control, n = 8; pilo: 27.6 ± 5.0% of control, n = 5; p < 0.01; Figure 5.8B). As anticipated we discovered that this decrease was attributed to the selective reduction in the $fEPSP_{KA}$ (sham: 35.7 ± 4.3 mV/ms, n = 7; pilo: 18.6 ± 3.1 mV/ms, n = 6; p < 0.01; Figure 5.8C) as the $fEPSP_{AMPA}$ remained unaltered (sham: 10.9 ± 2.0 mV/ms, n = 5; pilo: 11.1 ± 2.1 mV/ms, n = 5; p > 0.05; Figure 5.8C).

We examined the ability of the KAR antagonist UBP310 to inhibit the $fEPSP_{AMPA/KA}$ induced by a 5 pulse, 50 Hz train stimuli. Given the large KAR-mediated component at the mossy fiber – CA3 synapse in sham-treated animals, we observed a significant inhibition following application of 3 μM UBP (33.5 ± 6.6% of control, n = 5; p < 0.01; Figure 5.9), a finding in agreement with a previous study demonstrating inhibition of postsynaptic KARs at mossy fiber – CA3 synapses (Pinheiro et al., 2013).Given the significant reduction in KAR-mediated transmission in pilo-treated animals we anticipated that UBP310 (3μM) would produce less inhibition of the $fEPSP_{AMPA/KA}$. We found that UBP310 still blocked a significant portion of $fEPSP_{AMPA/KA}$ in pilo-treated animals (60.3 ± 3.8%
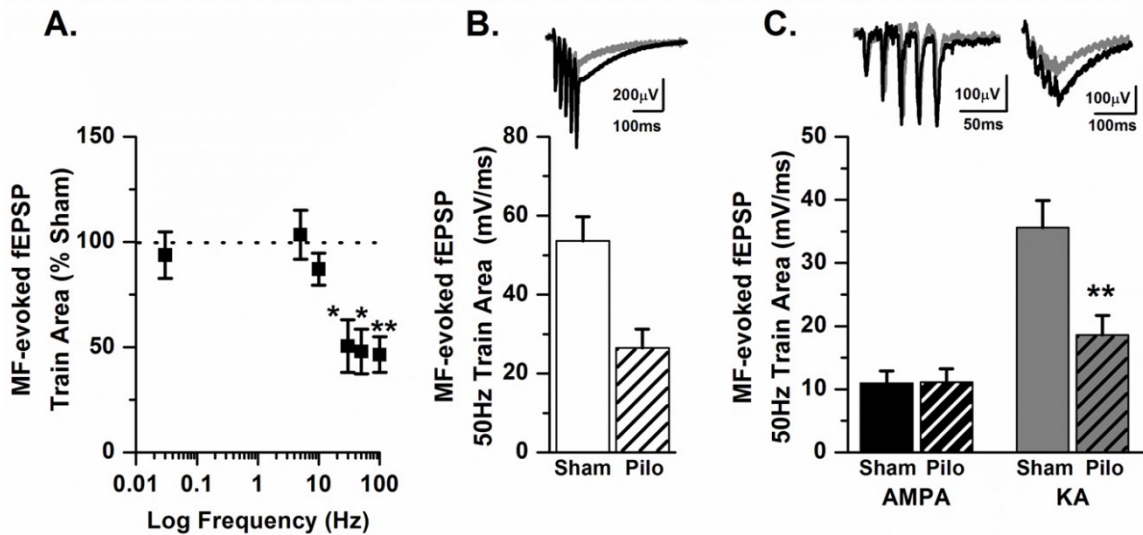
Figure 5.8. Frequency dependent alterations in synaptic integration at the mossy fiber – CA3 synapse of epileptic animals. Trains were evoked by stimulation of the mossy fiber – CA3 pathway. **A.** Frequency-dependent alterations in KAR-mediated transmission were observed in the mossy fiber – CA3 pathway (n = 4 – 8). Significant reduction in the area fEPSP$_{AMPA/KA}$ was observed in pilo-treated animals at higher frequencies (30 – 100 Hz). **B.** The fEPSP$_{AMPA/KA}$ area of a 5 pulse, 50 Hz train was significantly reduced in pilo-treated animals. Representative waveform demonstrates the decrease in area and decay of the fEPSP$_{AMPA/KA}$ in sham (*black trace*) and pilo-treated animals (*gray trace*). **C.** Area of the fEPSP$_{AMPA}$ is not different between sham and pilo-treated animals (n = 5). There is a significant decrease in the fEPSP$_{KA}$ in pilo-treated animals (n = 6 – 7). Waveforms illustrate the lack of change in the fEPSP$_{AMPA}$ in sham (*black trace*) and pilo-treated animals (*gray trace*) and the significant decrease in the area fEPSP$_{KA}$ in pilo-treated (*gray trace*) relative to sham-treated animals (*back trace*). *$p < 0.05$; **$p < 0.01$, t-test.
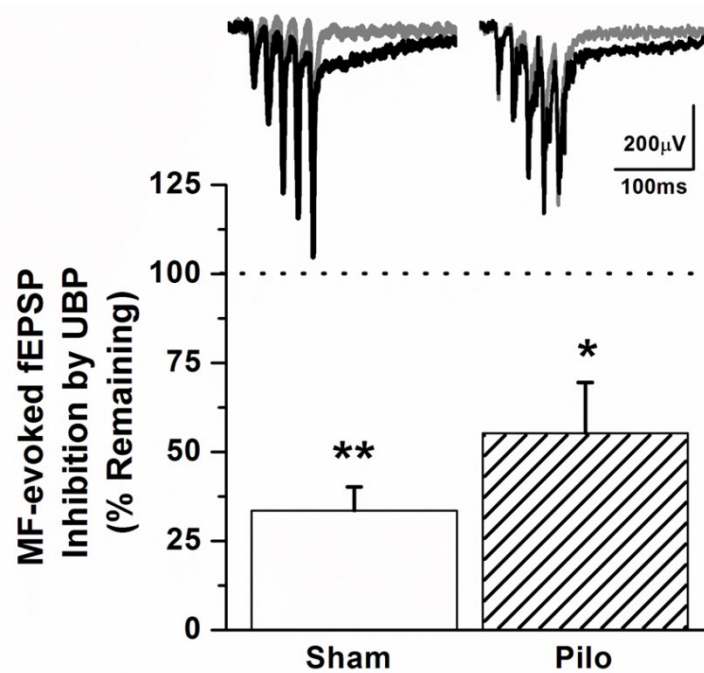
Figure 5.9. UBP310 inhibits KAR-mediated transmission at mossy fiber – CA3 synapses in sham and epileptic animals. The selective KAR antagonist UBP310 significantly inhibits fEPSPs evoked by stimulation of the mossy fiber – CA3 pathway in sham (*n* = 5) and pilo-treated animals (*n* = 4). There was a trend towards a significant reduction in UBP310 inhibition in pilo-compared to sham-treated animals (p = 0.087). Representative waveforms illustrate the inhibition produced by UBP310 (*gray traces*) compared to control traces (*black traces*) in both sham and pilo-treated animals. *p < 0.05; **p < 0.01, t-test.

of control, $n = 4$, $p < 0.05$; Figure 5.9). While not significant, we did observe a trend towards the reduced inhibition by UBP310 in pilo- vs. sham-treated animals ($p = 0.087$). Similar to the actions of UBP310, we observed a significant inhibition by ACET (300nM) on the $fEPSP_{AMPA/KA}$ in both sham (27.2 ± 6.6% of control, $n = 4$; $p < 0.05$) and pilo-treated animals (28.3 ± 2.9% of control, $n = 3$; $p < 0.05$; data not shown). Together these data demonstrate that KAR-mediated transmission within the mossy fiber – CA3 pathway is significantly reduced in pilo-treated animals. This finding is in direct opposition to the observed increase in KAR-mediated transmission within the DG. We suggest that this decrease may serve as a compensatory mechanism to limit hippocampal excitability and the development of seizures within the highly epileptogenic CA3 region.

5.4.5 PAIRED PULSE FACILITATION IS NOT ALTERED IN EPILEPTIC ANIMALS

A prominent feature of the mossy fiber – CA3 synapse is extremely large PPF (Henze et al., 2000), thought to be mediated in part by KARs (Kamiya et al., 2002). We explored whether PPF at the mossy fiber – CA3 synapse is altered in pilo-treated animals. We determined in sham-treated animals that the paired-pulse ratio (PPR) at 50 ms was significantly increased in both isolated AMPA (1.7 ± 0.1, $n = 8$; $p < 0.001$), and KA-receptor mediated fEPSPs (1.2 ± 0.1, $n = 8$; $p < 0.05$; Figure 5.10A). In addition the PPR mediated by the KAR was significantly lower than that of the AMPAR ($p < 0.01$). These findings are supported by previous studies and have been demonstrated to hold true for a variety of stimulus intervals, demonstrating that during paired stimuli KARs exhibit less
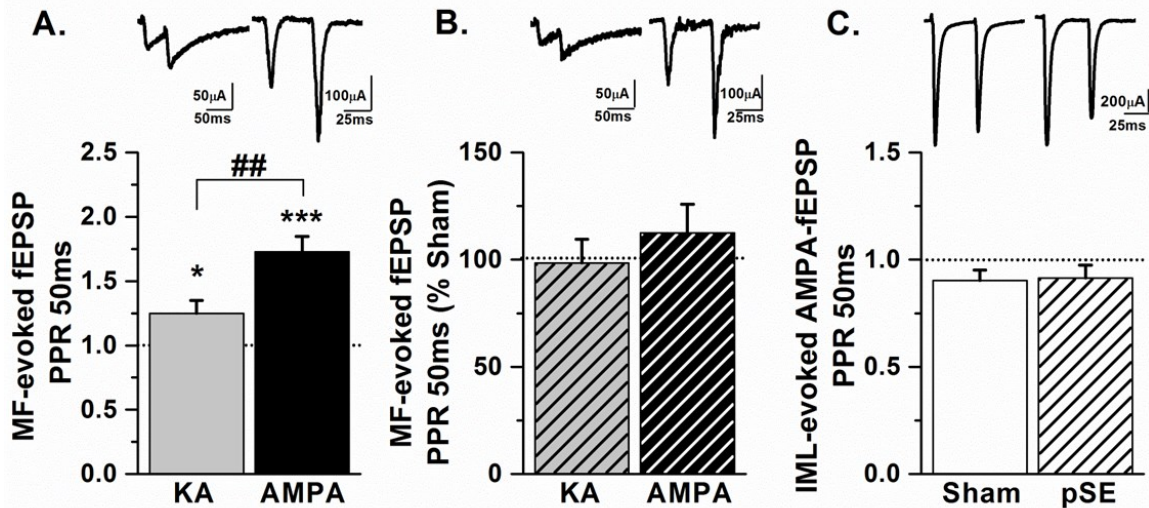
Figure 5.10. Paired pulse facilitation is not altered epileptic animals.
Alterations in the paired pulse ratio at an interval of 50 ms were examined at the mossy fiber – CA3 synapse (**A. and B.)** and in the dentate of sham and pilo-treated animals **(C.)**. **A**. In sham-treated animals the fEPSP$_{KA}$ (*n* = 8) and the fEPSP$_{AMPA}$ (n = 8) PPR50 were significantly increased. The ratio of the fEPSP$_{AMPA}$ was significantly greater than that observed for the fEPSP$_{KA}$. Representative waveforms illustrate PPF mediated by KA and AMPA-receptors. **B.** Relative to sham-treated animals there was no difference in the PPR50 for fEPSP$_{KA}$ (*n* = 5) or fEPSP$_{AMPA}$ (*n* = 5) elicited from pilo-treated animals. Representative waveforms demonstrate similar levels of PPF in pilo-treated animals to what was observed in sham-treated animals. **C.** The fEPSP$_{AMPA}$ PPR50 was not significantly different in sham (*n* = 5) or pilo-treated animals (*n* = 6). Representative waveforms illustrate the lack of PPF or PPD observed within the dentate of sham- and pilo-treated animals. *p < 0.05; ***p < 0.001; $^{##}$p < 0.01, t-test.

facilitation than AMPARs at the mossy fiber synapse (Contractor et al., 2001; Ito et al., 2004). In pilo-treated animals we observed that the PPR for $fEPSP_{KA}$ and $fEPSP_{AMPA}$ were not different than those noted in sham-treated animals (KA: 98.5 ± 11.0% of sham, $n = 5$; $p > 0.05$; AMPA: 112.5 ± 13.4% of sham, $n = 5$; $p > 0.05$; Figure 5.10B). We also explored the ability of UBP310 (3 μM) to alter PPF. In agreement with Perrais et al. (2009), UBP310 had no effect on the PPF in either sham-treated (97.1 ± 3.7, $n = 7$; $p > 0.05$) or pilo-treated animals (89.6 ± 68% of control, $n = 5$; $p > 0.05$), suggesting that presynaptic KARs are composed of the UBP310-insensitive heteromeric GluK2/3, or homomeric GluK2- subunit combination. Together these data demonstrate that the relative contribution of AMPARs and KARs to the presynaptic regulation of PPF is not altered in epilepsy. It should be noted that this observation is in contrast to the report that the PPR of the $fEPSP_{AMPA/KA}$ at the mossy fiber synapse is significantly reduced in kainate-treated animals several weeks post-SE (Goussakov et al., 2000).

Unlike the distinct PPF observed at the mossy fiber – CA3 synapse, stimulation of the perforant path input in the DG is not as well-defined. A general consensus reveals that stimulation of the lateral perforant path input results in PPF, while stimulation of the medial perforant path produces paired-pulse depression (PPD) (McNaughton, 1980; Christie and Abraham, 1992b, a; Colino and Malenka, 1993; Froc et al., 2003). However, these findings have been widely scrutinized and in general it is believed that simply relying on observed PPF or PPD is not sufficient to demonstrate proper stimulation of the lateral or medial perforant path (Petersen et al., 2013). We briefly examined whether the PPR of

fEPSP$_{AMPA}$ was altered in the DG of epileptic animals and determined that the PPR at a 50 ms interval was unaltered in sham-treated (0.9 ± 0.05, n = 5, p > 0.05) and pilo-treated animals (0.91 ± 0.06, n = 6, p > 0.05; Figure 5.10C).

5.5 DISCUSSION

The results of this study provide the first examination of specific alterations in KAR-mediated neurotransmission in area CA3 of epileptic animals. Despite the abundance of KARs in this region and the significant role they play in both pre- and postsynaptic regulation of neurotransmission there are no reports exploring their function in epilepsy. In agreement with previous studies in the DG of epileptic animals we observed the appearance of a KAR-mediated response (Epsztein et al., 2005). In direct contrast to this increase within the DG, we observed for the first time, a significant reduction in KAR-mediated transmission in area CA3 in pilo-treated animals. We believe that these effects are limited to KARs located on postsynaptic CA3 pyramidal cells, as we did not observe any difference in presynaptic regulation of PPF. The results of this study suggest that the observed reduction in KAR-mediated transmission in area CA3 may serve as a compensatory modification in response to the significant increase in synchronization and excitatory transmission observed in the DG of epileptic animals. Figure 5.11 summarizes the principal finding of this study, the reduction in KAR-mediated transmission at the mossy fiber – CA3 synapse.
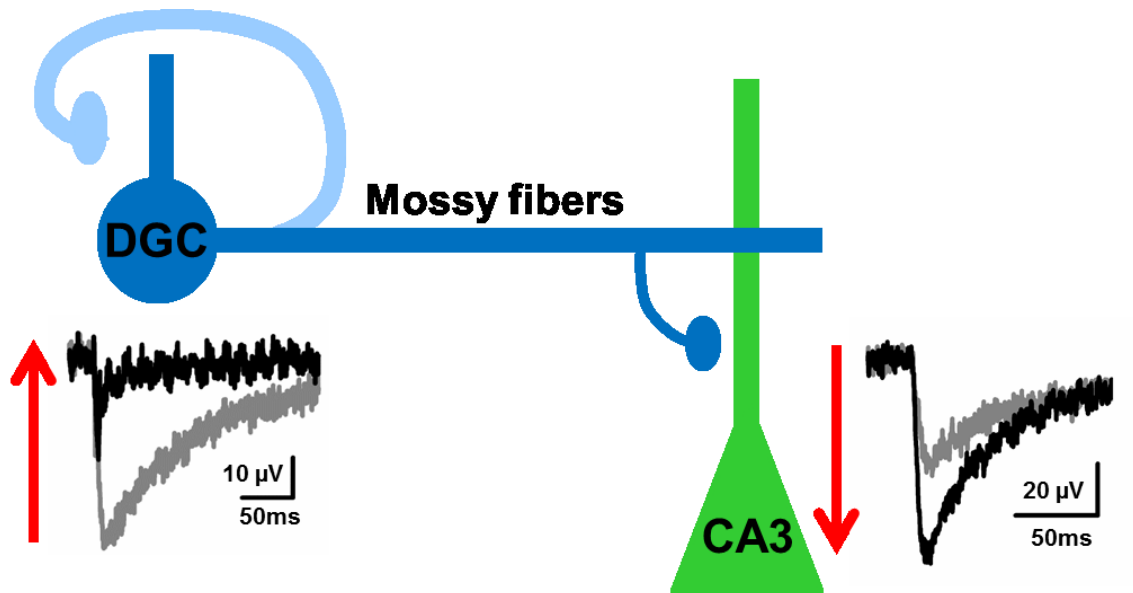
Figure 5.11. Summary of findings detailed in Chapter 5. Epilepsy differentially alters KARs at two synapses of the same axon. In epileptic animals aberrant mossy fiber sprouting *(light blue),* is associated with an appearance in KAR-mediated transmission. In contrast, transmission mediated by KARs at the mossy fiber – CA3 synapse is significantly reduced. Sham fEPSP$_{KA}$: *black traces*; Pilo fEPSP$_{KA}$: *gray traces*.

5.5.1 KAR-MEDIATED TRANSMISSION IS REDUCED AT THE MOSSY FIBER – CA3 SYNAPSE

In pilo-treated animals we observed a selective reduction in the mossy fiber evoked $fEPSP_{KA}$, as the $fEPSP_{AMPA}$ remained unaltered. In agreement, we observed significant reduction in the $fEPSP_{KA}$ of epileptic animals over several different stimulus intensities while the $fEPSP_{AMPA}$ was not changed. This finding clearly demonstrates that KAR-, but not AMPAR-mediated transmission, is significantly reduced in epilepsy. These data are in contrast to the report by Goussakov et al. (2000) in which the mossy fiber evoked fEPSP was increased in the kainate model of SE. The discrepancy between these studies is likely attributed to several factors. The most likely rationale is the age of the animals and their epileptic background. The current study utilized animals considered to be chronically epileptic (189 ± 9 days post-SE), while Goussakov et al. (2000) examined alterations in animals only several weeks post-SE. It is widely noted that while anatomical alterations and neuronal rewiring can occur within days following SE, many of these changes do not plateau until many months after the event (Loscher and Brandt, 2010). Thus it is likely that the current study and that conducted by Goussakov et al. (2000) examined different hippocampal networks.

5.5.2 POTENTIAL ALTERATIONS IN HIPPOCAMPAL KAR SUBUNIT COMBINATION

An underlying goal of this study was to determine if the KAR subunit composition at postsynaptic CA3 cells was altered in the epileptic brain. This type of alteration could have a dramatic impact on receptor agonist affinity,

biophysical properties of the receptor, and the development of potential therapeutics. Given the lack of subunit selective KAR antagonists we attempted to use UBP310 and ACET to determine if KAR subunit composition was altered in area CA3 of epileptic animals.

The willardine-derivative UBP310 has been identified as a potent antagonist at homomeric GluK3 and heteromeric GluK2/5-containing KARs (Perrais et al., 2009; Pinheiro et al., 2013). Furthermore, this compound was shown to be ineffective at blocking homomeric GluK2-containing KARs and heteromeric GluK2/3-containing KARs (Perrais et al., 2009). We observed rapid and robust inhibition of KAR-mediated transmission in area CA3 from both sham and pilo-treated animals following application with UBP310. Furthermore, UBP310 did not affect presynaptically regulated PPF. In agreement with previous studies, these data suggest that presynaptic KARs are likely composed of homomeric GluK2-containing KARs and/or heteromeric GluK2/3-containing KARs, as UBP310 failed to inhibit the response (Perrais et al., 2009; Pinheiro et al., 2013).

In regards to postsynaptic KARs, the observed inhibition by UBP310 suggests they contain GluK2/5 subunits (Pinheiro et al., 2013). However, the ability of UBP310 to inhibit GluK2/4-containing KARs has not been explored, and thus it is quite possible that KARs localized to postsynaptic CA3 cells may contain an abundance of GluK2/4-contaning receptors. In addition, anatomical data from Chapter 4 demonstrated a significant reduction in GluK2/3 and GluK4 subunits, suggesting that residual KARs in area CA3 may be composed of

GluK2/5 subunits. In an attempt to elucidate this possibility we utilized the KAR antagonist ACET. While ACET was initially developed as a GluK1 antagonist (Dolman et al., 2007; Dargan et al., 2009), preliminary data from our lab has suggested that it may selectively inhibit GluK2/4-containing KARs. However, we observed similar inhibition by ACET in both sham and pilo-treated animals. This finding could demonstrate a lack of ACET selectivity, or demonstrate that the KAR subunit composition on the membrane surface is not altered in area CA3 of pilo-treated animals. Given the differences that the GluK4 and GluK5 subunits bestow upon the receptor, having a clear understanding of their expression is essential. However, this lies within our ability to develop subunit-selective KAR antagonists which as of yet has proved quite challenging.

5.5.3 ALTERATIONS IN PRESYNAPTIC KAR-MEDIATED TRANSMISSION AT THE MOSSY FIBER – CA3 SYNAPSE

In addition to their postsynaptic localization KARs are also located on presynaptic mossy fiber terminals where they have been shown to regulate several forms of both short-term and long-term synaptic plasticity (Nicoll and Malenka, 1995; Salin et al., 1996). Synaptically released glutamate can activate these presynaptic KARs to facilitate or depress glutamate release and have also been shown to be integral in the induction of mossy fiber LTP (Bortolotto et al., 1999; Contractor et al., 2001; Lauri et al., 2001; Schmitz et al., 2001a; Schmitz et al., 2003; Pinheiro et al., 2007; Scott et al., 2008). In the current study we examined possible alterations in the presynaptic regulation of PPF at the mossy

fiber – CA3 synapse and did not observe any difference in pilo-treated animals, thus attributing the observed decrease in KAR-mediated transmission to postsynaptic modifications. In contrast to this finding, Goussakov et al. (2000) reported a decrease in PPF at the mossy fiber – CA3 pathway from kainate treated animals. As previously discussed this discrepancy is likely due to the substantial difference in the length in which epilepsy progressed following induction of SE, weeks vs. months.

Previous studies have demonstrated that differential alterations in presynaptically regulated processes such as PPF and frequency facilitation can be observed (Contractor et al., 2001; Fernandes et al., 2009). These observations led to the suggestion that two populations of presynaptic KARs may exist; those that mediate a single release event, and those responsible for mediating low frequency stimulations (Fernandes et al., 2009). While data collected in the current study as well as anatomical data presented in Chapter 4 are supportive of a postsynaptic alteration in KAR function, the possibility that other presynaptically mediated processes, such as frequency facilitation or even mossy fiber LTP, may be altered cannot be completely excluded.

5.5.4 IMPACT OF COMPROMISED DENTATE GYRUS 'GATEKEEPER' FUNCTION

The concept that the DG may serve as a gate to limit or filter transmission into the hippocampus was first established in the 1960s (Andersen et al., 1966). This role was re-examined following the observation that in both patients and animal models of SE there is abnormal sprouting of mossy fibers into the DG

which creates an excitatory network (Tauck and Nadler, 1985; Represa et al., 1987; Sutula and Dudek, 2007). In addition to regulating excitatory neurotransmission mossy fibers, including those sprouted into the DG, release extremely high levels of synaptically sequestered zinc (Perez-Clausell and Danscher, 1985). This release of zinc has been found to accelerate and increase epileptiform bursting within the DG (Timofeeva and Nadler, 2006). More recently it was demonstrated that KARs located on sprouted mossy fibers contribute to regulation of glutamatergic activity and actually alter the intrinsic firing properties of dentate granule cells (Epsztein et al., 2005; Artinian et al., 2011). The switch from the normal sparse firing to more sustained activity significantly increases the synchronization of dentate granule cells and reduces their ability to properly integrate transmission entering from the entorhinal cortex (Artinian et al., 2011).

In agreement with these reports the data in the current study revealed an increase in KAR-mediated transmission with the DG and subsequent impairment of synaptic integration, as demonstrated by a significant increase in the area of the $fEPSP_{KA}$ at varying frequencies. This well-established increase in excitatory transmission within the DG could have huge consequences for the generation of epileptiform activity in area CA3. We suggest that the observed decrease in KAR-mediated transmission in area CA3 may serve as a compensatory mechanism to limit the impact of synchronous activity in the DG. Of particular note in this study is the observation that on two different synapses of the same axon we have observed opposite alterations of the same receptor population. Aberrant mossy fibers synapsing into the inner molecular layer of the DG exhibit

increased KAR-mediated transmission, while mossy fibers synapsing into area CA3 exhibit decreased KAR-mediated transmission. A similar finding has not been observed in other synapses or brain regions, adding to the uniqueness of hippocampal mossy fibers.

5.5.5 COMORBIDITIES AND EPILEPSY

As previously discussed epilepsy is associated with severe comorbidities and recent literature has suggested that KARs and alteration in subunit expression may be implicated. We suggest that the apparent downregulation of KAR subunit expression and KAR-mediated transmission within area CA3, while likely a compensatory modification, may be associated with noted comorbidities. As previously elaborated upon, the reduction in KAR function may be a compensatory mechanism to limit excitability. However the trade-off may be the occurrence of comorbidities, particularly those linked to the mossy fiber – CA3 pathway. While controversy surrounds mossy fiber induced LTP (Henze et al., 2000), it is apparent that the mossy fiber – CA3 synapse demonstrates the ability to undergo long-term or even permanent alterations. The observed decrease in KAR function at this synapse may likely contribute to cognitive impairment, specifically associated with memories. The process of encoding and retrieving memories has been associated with gamma frequencies, ~30 – 80 Hz (Herrmann et al., 2004; Bartos et al., 2007). In the current study we observed a selective reduction in the area of the $fEPSP_{KA}$ at frequencies of 30 – 100Hz, those similar to gamma frequency. This finding suggests that the impairment of signal

integration at frequencies responsible for memory and learning may contribute to the cognitive comorbidities associated with epilepsy.

5.6 SIGNIFICANCE

The results of this study demonstrate that in the epileptic brain KARs localized to mossy fibers are differentially altered. We demonstrated for the first time that KAR-mediated transmission is significantly reduced in area CA3. In stark contrast to the decrease in KARs at the mossy fiber – CA3 synapse, we observed a significant increase in KARs localized to aberrant mossy fibers synapsing in the DG, a finding in agreement with previous reports (Epsztein et al., 2005). We suggest that the reduction in KAR-mediated excitatory transmission in area CA3 is a compensatory modification in response to the dramatic increase in DGC synchronization and inability of the DG to properly integrate transmission. In addition we suggest that this compensatory decrease in KAR-mediated transmission may precede the development of comorbidities associated with epilepsy, including anxiety, depression and cognitive impairment. Given the vast alterations in KAR-mediated transmission within the DG and area CA3, and the potential impact of those alterations on cognitive and psychiatric comorbidities, KARs may prove to be valuable therapeutic targets. However, further investigation into subunit composition and the relationship between KARs and comorbidities associated with epilepsy should be conducted.

CHAPTER 6

ALTERATIONS IN KAINATE RECEPTOR MEDIATED REGULATION OF AXON EXCITABILITY IN EPILEPTIC ANIMALS

6.1 INTRODUCTION

Excitatory neurotransmission mediated by kainate receptors (KARs) has been most thoroughly examined at the hippocampal mossy fiber – CA3 synapse. At this synapse KARs localized to postsynaptic CA3 cells regulate a small, but slowly decaying excitatory current that significantly contributes to temporal summation and a large depolarizing envelope (Castillo et al., 1997; Vignes and Collingridge, 1997; Frerking and Ohliger-Frerking, 2002). KARs localized to presynaptic mossy fiber (MF) terminals have been shown to contribute to forms of both short-term and long-term plasticity, including paired-pulse facilitation, frequency facilitation and long-term potentiation (Bortolotto et al., 1999; Contractor et al., 2001; Lauri et al., 2001; Schmitz et al., 2001a; Kamiya et al., 2002; Pinheiro et al., 2007). Several studies have also examined the role of presynaptic KARs in regulating glutamate release at the MF terminal. These studies report that low concentrations of the KAR agonist kainate (KA) facilitate the release of glutamate, while higher concentrations of glutamate depress transmitter release (Contractor et al., 2000; Kamiya and Ozawa, 2000; Schmitz et al., 2001a). In support of these findings utilizing exogenously applied kainate, it was found that released glutamate following brief and prolonged trains produces

was found that released glutamate following brief and prolonged trains produces similar effects on presynaptic KAR regulation of transmitter release (Bettler et al., 1990; Schmitz et al., 2000; Schmitz et al., 2001a).

In addition to these diverse pre- and postsynaptic roles of KARs at the mossy fiber – CA3 synapse, KARs are thought to be present along the length of mossy fibers as well as along the axons of CA3 pyramidal cells where they have been shown to regulate axon excitability (Kamiya and Ozawa, 2000; Schmitz et al., 2000; Frerking et al., 2001). Similar to the findings of presynaptic KAR-regulated glutamate release, it has been determined that KARs located along MFs regulate excitability in a bidirectional manner, as low concentrations of kainate facilitate and higher concentrations depress axon excitability (Kamiya and Ozawa, 2000; Schmitz et al., 2000). Whereas KARs localized to MFs demonstrate bimodal actions, those receptors along the axons of CA3 pyramidal cells have been shown to purely reduce axon excitability in a concentration dependent manner (Frerking et al., 2001; Mott et al., 2008). It should be noted that KAR regulation of axon excitability, determined through observation of orthodromic fiber volleys or antidromic action potentials is a separate and distinguishable phenomenon than that of presynaptic regulation of glutamate release. However, alterations in axon excitability are typically consistent with observed presynaptic changes in glutamate release.

KARs localized to MF or CA3 axons are likely key regulators of glutamatergic transmission and may ultimately serve to govern excitatory drive through the hippocampus, specifically area CA3 and CA1 via the mossy fiber and

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CA3 axons, respectively. Given observations of dramatic alterations in KAR subunit expression in the hippocampus of status epilepticus-experienced animals (Lason et al., 1997; Tandon et al., 2002; Zhang et al., 2004; Ullal et al., 2005; Porter et al., 2006) and Chapter 4, in conjunction with the hyperexcitable and epileptogenic nature of CA3, it is conceivable that KAR-regulation of axonal excitability may be altered. Therefore the goal of this study was to determine possible alterations in KAR-regulation of axon excitability in epileptic animals.

6.2 HYPOTHESIS

We previously observed significant alterations in KAR subunit expression and localization throughout the hippocampus, specifically a reduction in GluK2/3 and GluK4 within the mossy fibers terminating in CA3 *stratum lucidum* (Chapter 4). In addition, in Chapter 5, we suggested that in epileptic animals the increase in the excitatory circuitry of the dentate gyrus may lead a compensatory downregulation of KAR-mediated excitatory in area CA3. Based on these observations, we hypothesize that the ability of KARs to regulate axon excitability will be reduced in the epileptic brain. This potential outcome could serve to either recover or exacerbate the epileptic condition.

6.3 MATERIALS AND METHODS

6.3.1 ANIMAL MODEL OF SE

All animal care and use procedures were carried out in accordance with protocols written under the guidance of the National Institutes of Health Guide for

the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of South Carolina. Following induction of SE, rats were housed 1 per cage with *ad libitum* access to food and water. Rats were housed in a climate controlled facility with a light/dark cycle of 12/12 hours.

SE was induced in adult male Sprague-Dawley rats (Harlan, Indianapolis, IN). Animals received intraperitoneal (i.p.) injections of scopolamine methyl bromide and terbutaline hemisulfate (2 mg/kg) followed 30 – 45 min later by pilocarpine hydrochloride (390 mg/kg). After 2 hours of continuous SE, seizures were terminated by diazepam (DZP, 25 mg/kg). This group of animals served as the 'pilo' group. A separate group of animals received identical treatment with the exception of receiving saline (390 mg/kg) instead of pilocarpine hydrochloride. This group of animals served as the 'sham' group. More detailed methods concerning induction of SE are described in Chapter 2.3.

6.3.2 FIELD POTENTIAL ELECTROPHYSIOLOGY

Transverse (500 µM thick) hippocampal slices were prepared from sham- and pilocarpine-treated adult male Sprague-Dawley rats 145 ± 10 days post-treatment as described in detail in Chapter 2.7. Briefly, animals were deeply anesthetized with isoflurane and transcardially perfused with a sucrose-based aCSF. Slices were prepared in ice cold (4°C), and then incubated for 30 min in heated (37ºC) oxygenated (95% O_2 / 5% CO_2) sucrose-based aCSF prior to a gradual transition to room-temperate oxygenated Na-Cl based solution.

In contrast to orthodromic stimulation in which action potentials travel away from the soma, antidromic stimulation involves stimulation of the dendrite or axon as the action potential travels backwards towards the soma. This type of stimulation paradigm is commonly used to examine dendritic or axonal projections. The current project utilized antidromic spikes to directly explore KAR-mediated hippocampal axon excitability. Antidromic population spikes were evoked in two distinct pathways. 1) Mossy fibers were stimulated in CA3 *stratum lucidum* and the response was recorded in the dentate granule cell layer. 2) CA3 axons were stimulated in *stratum radiatum* and the response was recorded in the CA3 pyramidal cell layer. Figure 2.2 depicts each of these two experimental paradigms utilized, purple and red electrodes, respectively. Antidromic spikes were pharmacologically isolated in an antagonist cocktail containing bicuculline methochloride (Bic, 20 µM) and MK-801 (10 µM). KAR antagonists, UBP310 (3 µM), ACET (300 nM), and kynurenic acid (KYN, 3 mM) were used to explore kainate-induced axon excitability or depression. All drugs were purchased from Abcam with the exceptions of Bic (Ascent Scientific), ACET (Tocris Bioscience), and KYN (Sigma-Aldrich).

6.4 RESULTS

6.4.1 KAR- MEDIATED FACILITATION, BUT NOT DEPRESSION OF MOSSY FIBER EXCITABILITY IS REDUCED IN EPILEPTIC ANIMALS

KARs located along mossy fibers regulate MF excitability in a bimodal manner, as low concentrations facilitate and high concentrations of kainate

depress excitability (Kamiya and Ozawa, 2000; Schmitz et al., 2000). Alterations in the ability of KARs to properly regulate this action would dramatically impact the excitatory drive into area CA3 resulting in alterations in both short-term and long-term plasticity at this synapse. Antidromic spikes were evoked by stimulation of the mossy fibers in CA3 *stratum lucidum* and recorded in the dentate granule cell layer (Figure 2.2, purple electrodes).

In agreement with previous studies we observed concentration-dependent effects on axon excitability following bath application of KA with low concentrations potentiating the amplitude of the MF antidromic spike, and higher concentrations inhibiting the spike amplitude (Figure 6.1A). However, in pilo-treated animals we found that while depression induced by high KA was intact, the facilitation produced by low KA was completely absent (Figure 6.1A). Based on these studies we further examined alterations in MF excitability produced by 0.3 µM KA due to its selective activation of the KAR at this low concentration (Mulle et al., 1998). We concluded that 0.3 µM KA significantly increases the amplitude (121.5 ± 2.9% of control, n = 7; p < 0.001; Figure 6.1B) and area (117.6 ± 4.4% of control, n = 7, p < 0.01; Figure 6.1C) of the MF antidromic spike. In contrast, the actions of 0.3 µM KA were significantly different in pilo-treated animals, as KA produced no effect on the amplitude (98.9 ± 2.8% of control, n = 5, p > 0.05; Figure 6.1B) or area (95.3 ± 2.0% of control, n = 5; p > 0.05; Figure 6.1C) of the antidromic spike. We also examined possible alterations in the half width of the antidromic spike following application of 0.3 µM and found no difference in sham (97.6 ± 1.3% of control, n = 7; p > 0.05; Figure
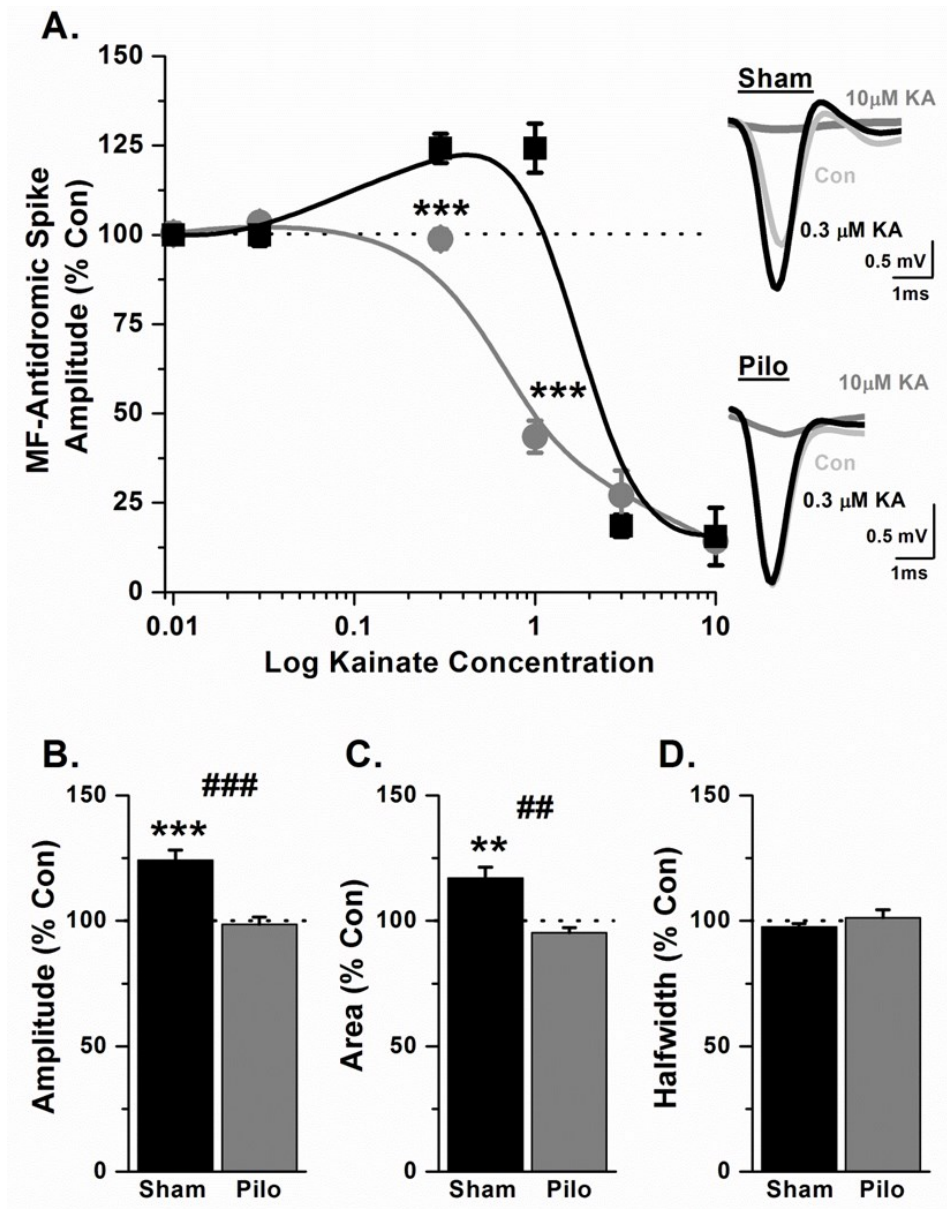
Figure 6.1. KAR-mediated facilitation but not depression of mossy fiber excitability is reduced in epileptic animals. A. Kainate concentration response curves from sham-treated (*black squares, n* = 5 – 7) and pilo-treated animals (*gray circles, n* = 4 – 7). Facilitation produced by low KA was significantly reduced in pilo-treated animals. Depression produced by high KA was not different between groups. *Right,* Waveforms from sham and pilo-treated animals. Baseline antidromic spikes (*Con, light gray traces)*, were potentiated by 0.3 μM KA in sham, but not pilo-treated groups (black *traces)*, and depressed by 10 μM KA (dark *gray traces).* Application of 0.3 μM significantly increased the amplitude **(B.)** and area **(C.)** of sham-treated, but not pilo-treated animals. KA (0.3 μM) had no effect on the half width of the MF antidromic spike **(D.)** **p < 0.01; ***p < 0.001; ##p < 0.01; ###p < 0.001, t-test.

6.1D) and pilo-treated animals (101.2 ± 3.2% of control, *n* = 5; p > 0.05; Figure 6.1D). These findings demonstrate that bimodal regulation of axon excitability is absent in epileptic animals as low concentrations of KA now produce more inhibitory than facilitatory effects. Furthermore we suggest that this loss of KAR-mediated facilitation of MF excitability may be due to a decrease in GluK4 subunit expression in area CA3 (Chapter 4). In support of this notion, it has been reported that facilitation of MF excitability is significantly reduced in GluK4^{-/-} animals (Catches et al., 2012).

6.4.2 UBP310 SELECTIVELY BLOCKS KA-INDUCED FACILITATION, BUT NOT DEPRESSION OF MOSSY FIBER EXCITABILITY

We next explored whether the observed facilitation of MF excitability by low kainate (0.3 µM) or the depression by high KA could be blocked by application of a KAR antagonist. The selective KAR antagonist UBP310, while originally characterized as a GluK1 antagonist, has been shown to potently block current at homomeric GluK3 and heteromeric GluK2/5-containing KARs, while it is ineffective at homomeric GluK2 and heteromeric GluK2/3-containing KARs (Perrais et al., 2009; Pinheiro et al., 2013). It should be noted that while it has not been reported, it is likely that UBP310 also antagonizes GluK4-containing receptors. We first demonstrated that UBP310 (3 µM) was capable of reversing the facilitation produced by 0.3 µM KA in sham-treated animals (Figure 6.2A). We next explored the ability of UBP310 to prevent KA-induced facilitation or depression of the MF antidromic spike (Figure 6.2B). We found that in both sham
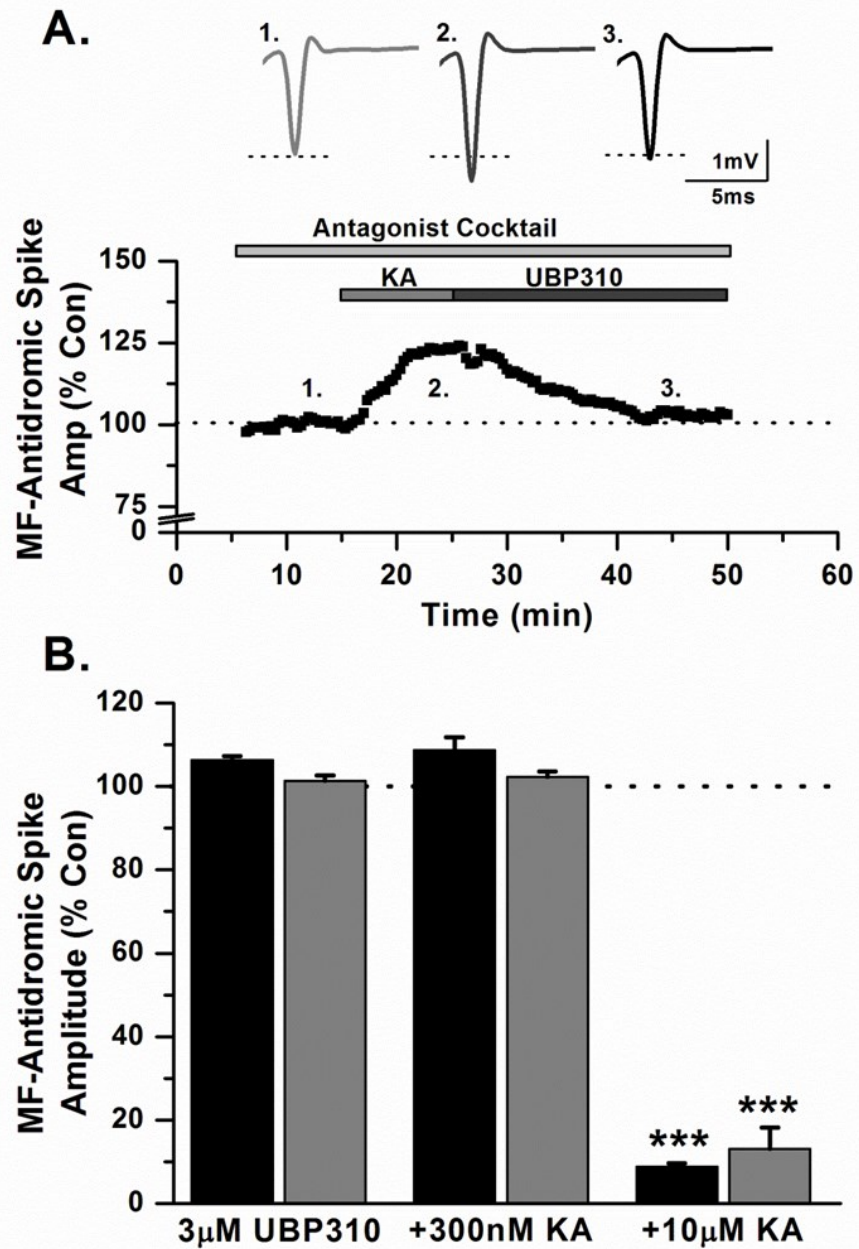
Figure 6.2. UBP310 blocks KA-induced facilitation but not depression of mossy fiber excitability. **A.** Representative time-course experiment demonstrating facilitation induced by low KA (0.3 µM) and reversal by 3 µM UBP310. Sample waveforms illustrate the antidromic spike at each point during the experiment. **B.** Bar graphs demonstrate that that application of 3 µM UBP did not alter the baseline amplitude of the MF-antidromic spike but was able to block the ability of low (0.3 µM KA) to facilitate excitability in sham-treated animals. Depression by high KA (10 µM) was not prevented by UBP310. Sham-treated group: *black bars* (*n* = 5); pilo-treated group: *gray bars* (*n* = 5). ***p < 0.001, t-test.

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(106.3 ± 1.0% of control, n = 5; p > 0.05; Figure 6.2B) and pilo-treated animals (101.3 ± 1.4% of control, n = 5; p > 0.05; Figure 6.2B), bath application of UBP310 (3 µM) had no effect on the baseline antidromic spike amplitude. However, we determined that in sham-treated animals pre-application of UBP310 significantly blocked the anticipated facilitatory effects of 0.3 µM KA (without UBP310: 121.5 ± 2.9% of control; n = 7; with UBP310: 108.7 ± 3.1% of control, n = 5; p < 0.01; Figure 6.2B). In pilo-treated animals the presence of UBP310 did not affect the observed lack of potentiation produced by 0.3 µM KA (p > 0.05; Figure 6.2B).

Given the capacity of UBP310 to block KA-induced facilitation by low KA we also examined its ability to block depression induced by high KA (10 µM). We found that pre-application of UBP310 (3 µM) was unable to block the significant depression induced by 10 µM KA in sham (without UBP: 15.5 ± 8.0% of control, n = 5; with UBP: 8.9 ± 0.8% of control, n = 5; p > 0.05) and pilo-treated animals (without UBP: 14.3 ± 2.2% of control, n = 5; with UBP: 13.1 ± 5.2% of control, n = 5; p > 0.05;Figure 6.2B). Given that UBP310 selectively inhibits KARs over AMPA or NMDA-receptors (Perrais et al., 2009; Pinheiro et al., 2013), these findings demonstrate that facilitation by low KA is in fact KAR-mediated. In addition we suggest that the KARs located on mossy fibers are likely composed of heteromeric GluK2/4 or GluK2/5-containing receptors and possibly homomeric GluK3-cotaining receptors.   However, this is less likely as GluK3 subunit expression is restricted to the dentate gyrus (Wisden and Seeburg, 1993; Bureau et al., 1999; Darstein et al., 2003).

6.4.3 ACET SELECTIVELY BLOCKS KA-INDUCED FACILITATION, BUT NOT DEPRESSION OF MOSSY FIBER EXCITABILITY

Similar to UBP310, the compound ACET (UBP316) was originally characterized as an antagonist of GluK1-containing KARs (Dolman et al., 2007; Dargan et al., 2009). However studies have suggested that ACET may also antagonize KARs composed of homomeric GluK3 subunits (Perrais et al., 2009), and has even shown to be partially effective in blocking transmission at the mossy fiber – CA3 synapse (Pinheiro et al., 2013). In addition, to these studies preliminary data from our lab has suggested that ACET may demonstrate increased selectivity for GluK2/4, over GluK2/5-containing KARs. We examined the ability of ACET to prevent KA-induced facilitation and/or depression of mossy fiber excitability.

A time course experiment demonstrates the ability of ACET (1 µM) to block the facilitation produced by 0.3 µM KA in sham-treated animals (Figure 6.3A). We next explored the ability of ACET to prevent the observed facilitation and depression of MF excitability produced by KA (Figure 6.3B), and found results essentially identical to those produced by application of UBP310. Briefly, bath application of ACET (1 µM) had no effect on the baseline amplitude of the antidromic spike in either sham (99.7 ± 2.4% of control, n = 5; p > 0.05; Figure 6.3B) or pilo-treated animals (109.5 ± 6.8% of control, n = 3; p > 0.05; Figure 6.3B). In agreement with our results with UBP310, we found that ACET significantly prevented the facilitatory action of 0.3 µM KA in sham-treated animals (without ACET: 121.5 ± 2.9% of control; n = 7; with ACET: 101.0 ± 2.6%
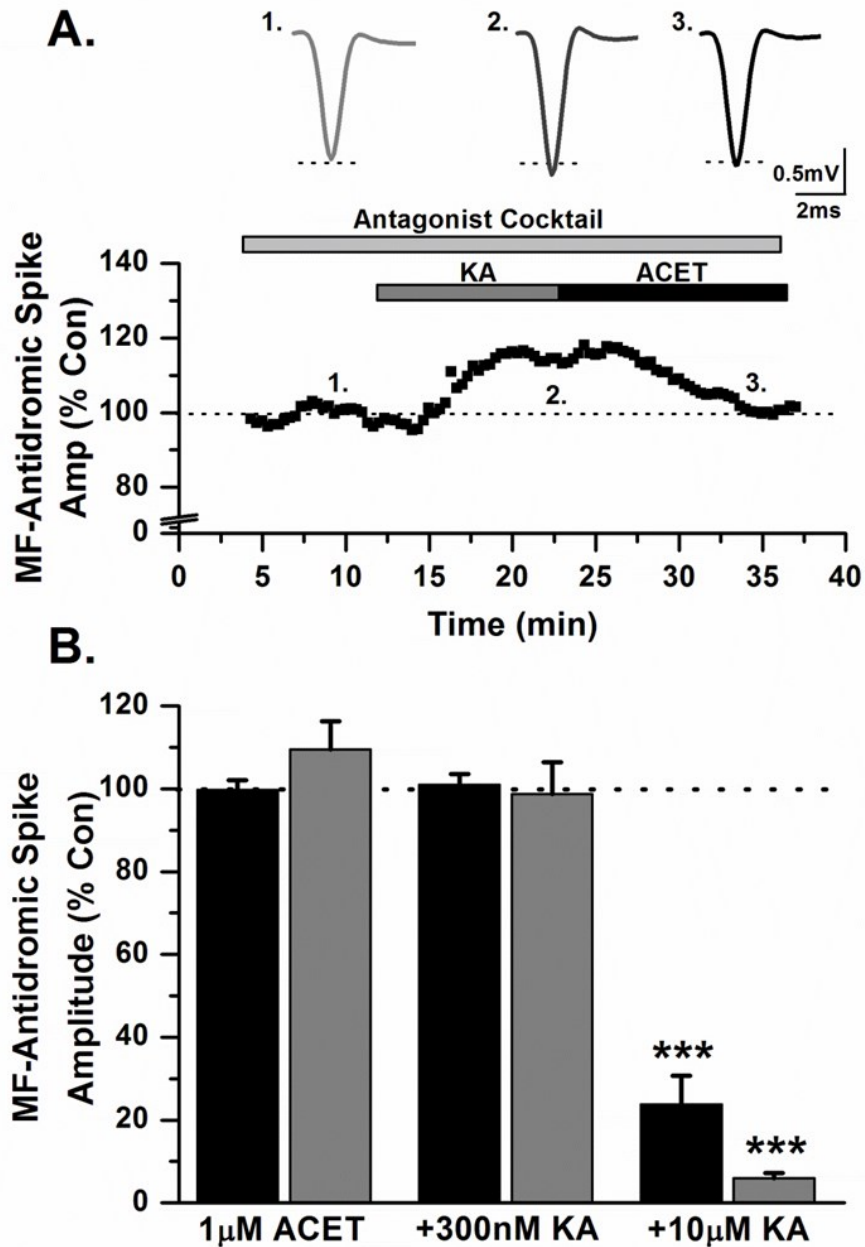
***Figure 6.3. ACET blocks KA-induced facilitation but not depression
of mossy fiber excitability. A.*** Representative time-course experiment
demonstrating facilitation induced by low KA (0.3 µM) and reversal by 1 µM
ACET. Sample waveforms illustrate the antidromic spike at each point during the
experiment. **B.** Bar graphs demonstrate that that application of 1 µM ACET did
not alter the baseline amplitude of the MF antidromic spike but was able to block
the ability of low KA (0.3 µM) to facilitate excitability. Depression by high KA (10
µM) was not prevented by ACET. Sham-treated group: *black bars* (n = 4 – 5);
pilo-treated group: *gray bars* (n = 2 – 3). ***$p < 0.001$, t-test.

of control, $n = 5$; $p < 0.001$; Figure 6.3B). The lack of potentiation by 0.3 µM in pilo-treated animals was not significantly different in the presence of ACET ($p > 0.05$; Figure 6.3B). In addition, we determined that ACET (1 µM) was not able to prevent the depression induced by 10 µM KA in sham (without ACET: $15.5 \pm 8.0\%$ of control, $n = 5$; with ACET: $23.8 \pm 6.9\%$ of control, $n = 5$; $p > 0.05$) or pilo-treated animals without ACET: $14.3 \pm 2.2\%$ of control, $n = 5$; with ACET: $6.0 \pm 1.3\%$ of control, $n = 2$; $p > 0.05$; Figure 6.3B).

The above findings are not surprising given the similarities in the UBP310 and ACET pharmacological profiles. However, the possibility of distinguishing whether the actions of KA on mossy fiber excitability were mediated by GluK2/4- or GluK2/5-containing KARs was encouraging. Regardless, the results from these experiments support the idea that KARs located on mossy fiber axons are likely comprised of the GluK2/4- and/or GluK2/5 subunit combination. However, the inability of either UBP310 or ACET to prevent inhibition by 10 µM KA suggests either that the inhibition is mediated by AMPA receptors and not KARs, or perhaps that the observed inhibition occurs through a metabotropic function.

6.4.4 KYN BLOCKS BOTH KA-INDUCED FACILITATION AND DEPRESSION OF MOSSY FIBER EXCITABILITY

While UBP310 and ACET were originally designed as KAR antagonists, kynurenic acid (KYN) has been characterized as a more general glutamate receptor antagonist (Ganong et al., 1983; Alt et al., 2004). In regards to KARs, it has been demonstrated that KYN has a much higher affinity for GluK2 over

GluK5 KAR subunits (Alt et al., 2004). KYN has also been shown to potentiate current at recombinant GluK2/5 and GluK3/5 KARs, through its removal of GluK2 or GluK3-induced desensitization (Fisher and Mott, 2011). We explored the actions of KYN on KA-induced potentiation and depression of MF excitability.

We first demonstrated that KYN (3 mM) was able to reverse the potentiation observed by 0.3 µM KA in sham-treated animals (Figure 6.4A). Similar to previous experiments we then wanted to determine if pre-application of KYN was able to prevent the observed facilitation or depression of MF excitability by 0.3 µM (Figure 6.4B). In agreement with our previous experiments, we found that KYN (3 mM) did not significantly alter the amplitude of the MF antidromic spike in sham (95.7 ± 2.2% of control, n = 5; p > 0.05) or pilo-treated animals (100.7 ± 2.7% of control, n = 5; p > 0.05; Figure 6.4B). Similar to our studies of UBP and ACET, we found that KYN significantly prevented the expected facilitation induced by 0.3 µM KA in sham-treated animals (without KYN: 121.5 ± 2.9% of control, n = 7; with KYN: 95.7 ± 1.6% of control, n = 5; p < 0.001; Figure 6.4B). In addition the lack of potentiation observed by 0.3 µM KA in pilo-treated animals was not significantly different in the presence of CNQX (p > 0.05; Figure 6.4B).

In stark contrast to the inability of both UBP and ACET to block the depressive effects of high KA, we observed that pre-application of 3 mM KYN significantly blocked the depression induced by 10 µM KA in pilo-treated animals (without KYN: 14.3 ± 2.2% of control, n = 5; with KYN: 87.5 ± 8.0% of control, n = 5; p < 0.001; Figure 6.4B). Perhaps even more intriguing is that in sham-treated
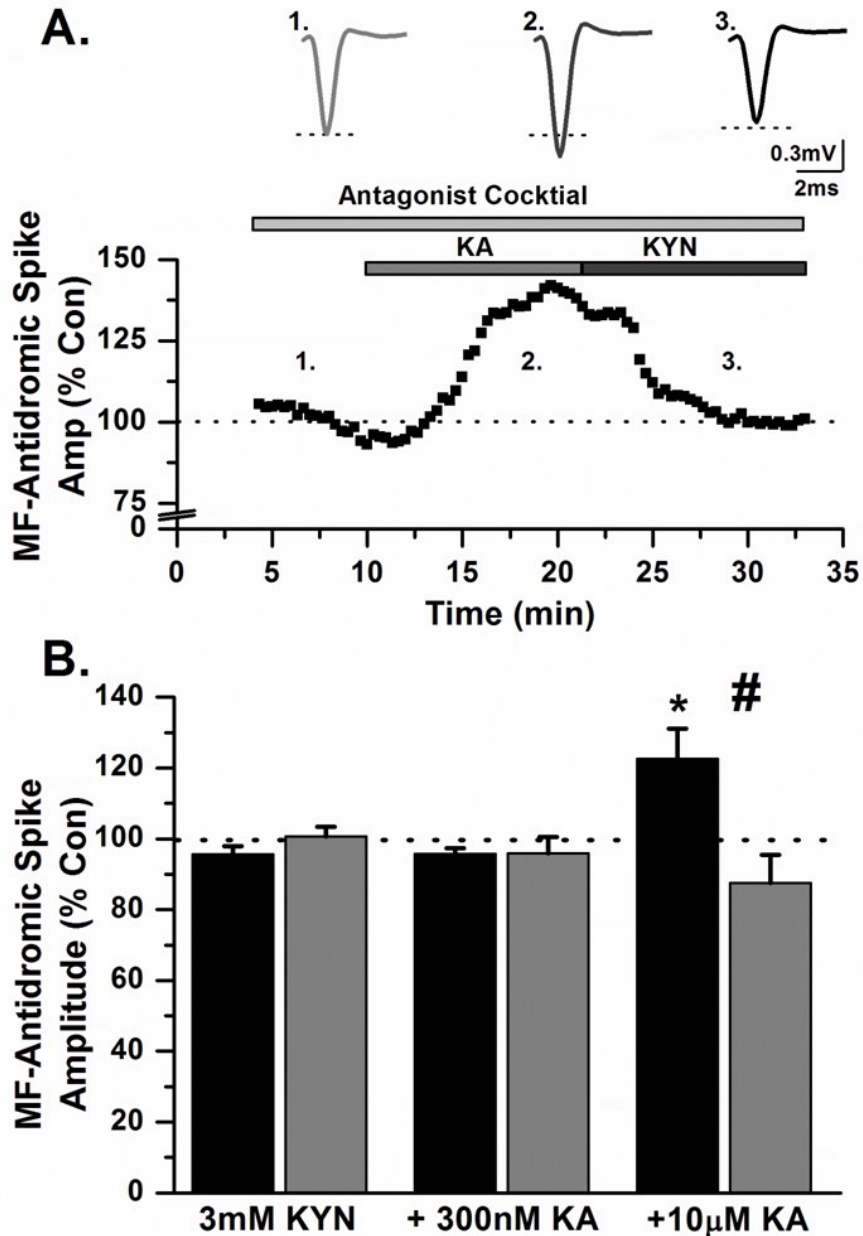
Figure 6.4. Kynurenate prevents KA-induced facilitation and depression of mossy fiber excitability. A. Representative time-course experiment demonstrating facilitation induced by low KA (0.3 µM) and reversal by 3 mM KYN. Sample waveforms illustrate the antidromic at each point during the experiment. **B.** Bar graphs demonstrate that that application of 3 mM KYN did not alter the baseline amplitude of the MF antidromic spike but was able to block the ability of low KA (0.3 µM) to facilitate excitability. In the presence of KYN, 10 µM KA significantly increased the MF-antidromic spike amplitude in sham-treated animals, and prevented inhibition in pilo-treated animals. Sham-treated group: *black bars* (n = 5); pilo-treated group: *gray bars* (n = 5). *$p < 0.05$; [#]$p < 0.05$, t-test.

animals, in the presence of 3 mM KYN, 10 μM KA actually significantly increased the amplitude of the MF antidromic spike (without KYN: 15.5 ± 8.0% of control, n = 5; with KYN: 122.6 ± 8.4% of control, n = 5; p < 0.001; Figure 6.4B).

Based upon the data collected from experiments using UBP310 and ACET, we anticipated the ability of KYN to block the facilitation of MF excitability induced by 0.3 μM KA. Blockade of KA-induced depression by KYN may be reflective of its more general antagonistic action of glutamate receptors, as opposed to selectively targeting KARs. The observed increase in MF excitability in sham-treated animals in the presence of KYN is perplexing, and likely involves a more intricate interaction between KYN and the KAR and/or AMPAR complexes. Nevertheless, this set of experiments clearly demonstrates that depression induced by high KA can be blocked and is perhaps regulated by AMPA receptors.

6.4.5 CNQX SELECTIVELY BLOCKS KA-INDUCED FACILITATION, BUT NOT DEPRESSION OF MOSSY FIBER EXCITABILITY

To explore the possibility that KA-induced depression is possibly mediated by AMPA receptors, we utilized CNQX, an extremely potent antagonist at AMPA receptors with an IC_{50} of ~400 nM (Honore et al., 1988; Sheardown et al., 1990). To ensure absolute blockade of both AMPA and KA-receptors we utilized 100 μM CNQX, an extremely high concentration. Very surprisingly, we found that similar to UBP and ACET, CNQX (100 μM) was able to block the facilitation, but not the depression of MF excitability induced by KA. Bath application of CNQX (100 μM)

did not significantly alter the baseline MF antidromic spike amplitude in sham
($103.5 \pm 9.0\%$ of control, $n = 5$; $p > 0.05$) or pilo-treated animals ($101.5 \pm 7.3\%$ of
control, $n = 3$; $p > 0.05$; Figure 6.5). Furthermore, similar to UBP and ACET, we
determined that CNQX significantly blocked the ability of 0.3 µM KA to potentiate
the antidromic spike (without CNQX: $121.5 \pm 2.9\%$ of control, $n = 7$; with CNQX:
$100.3 \pm 3.0\%$ of control, $n = 5$; $p < 0.001$; Figure 6.5). In addition, the lack of
potentiation by 0.3 µM KA in pilo-treated animals was not significantly different in
the presence of CNQX ($p > 0.05$; Figure 6.5). We next explored the actions of 10
µM KA in the presence of CNQX, fully anticipating CNQX to prevent the dramatic
depression induced by high KA. However, we determined that CNQX (100 µM) in
fact did not prevent the depression in sham (without CNQX: $15.5 \pm 8.0\%$ of
control, $n = 5$; with CNQX: $48.0 \pm 17.0\%$ of control, $n = 5$; $p > 0.05$) or pilo-treated
animals without CNQX: $14.3 \pm 2.2\%$ of control, $n = 5$; with CNQX: $40.7 \pm 14.8\%$
of control, $n = 3$; $p > 0.05$; Figure 6.5).

It is worthwhile noting that in the presence of CNQX the observed
depression in both sham and pilo-treated animals by high KA was quite variable.
In sham-treated animals, in the presence of CNQX, inhibition by 10 µM KA
ranged from a minimal 4.6% to almost complete inhibition at 91.5%. Similarly in
pilo-treated animals, in the presence of CNQX, inhibition by 10 µM KA ranged
from 44.5 – 89%. We suggest that perhaps with additional experiments it would
become clear that while CNQX does not completely block the depressive action
of 10 µM KA, it is likely that it would at least limit the extent of this depression.
However, these results further suggest that the facilitatory actions of low KA
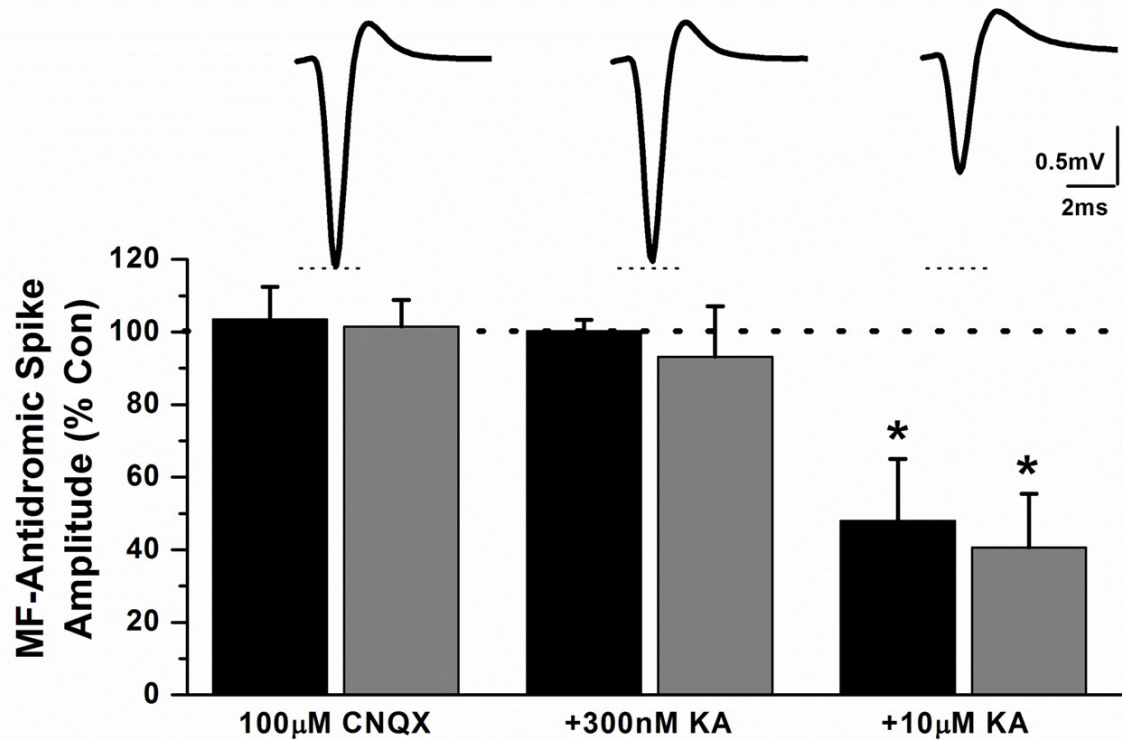
Figure 6.5. CNQX blocks KA-induced facilitation but not depression of mossy fiber excitability. Application of CNQX (100 μM) did not affect the baseline amplitude of the mossy fiber antidromic spike, but was able to prevent the facilitation of low KA (0.3 μM) in sham-treated animals. In the presence of 100 μM CNQX, high KA (10 μM) continued to significantly reduce axon excitability in both sham and pilo-treated animals. Representative waveforms from a sham-treated animal demonstrate the lack of effect of CNQX (100 μM) on the amplitude of the antidromic spike, its blockade of 0.3 μM KA-induced facilitation, and the continued depressive actions of 10 μM KA. Sham-treated group: *black bars* (*n* = 5); pilo-treated group: *gray bars* (*n* = 3). *p < 0.05, t-test.

(0.3 µM) are extremely likely to be occurring through KARs, while the depressive actions by 10 µM KA are likely not ionotropic, or are at least partially mediated through a second-messenger metabotropic function.

6.4.6 KAR-MEDIATED DEPRESSION OF CA3 AXON EXCITABILITY IS SIGNIFICANTLY REDUCED IN EPILEPTIC ANIMALS

In contrast to the bidirectional regulation of MF excitability, KARs localized to the axons of CA3 pyramidal cells reduce excitability in a concentration-dependent manner (Frerking et al., 2001; Mott et al., 2008). Similar to the issues mentioned with alterations in MF excitability, the inability of KARs to properly regulate axon excitability in area CA3 could have huge implications in the epileptic brain and increase hyperexcitability. Antidromic spikes from the axons of CA3 pyramidal cells were evoked by stimulation of CA3 *stratum radiatum* and recorded in the CA3 pyramidal cell layer (Figure 2.2, red electrodes).

In agreement with Mott et al. (2008) we observed concentration-dependent inhibition of CA3 axons with bath applied kainate (Figure 6.6A). However, in pilo-treated animals we found that while depression remained intact it was significantly less than that observed in sham-treated animals at 0.1 µM (p < 0.01), 1 µM (p < 0.001) and 10 µM (p < 0.001). We found that 0.1 µM KA significantly reduces the amplitude (59.9 ± 8.7% of control, n = 6; p < 0.001; Figure 6.6B) and area (87.6 ± 5.0% of control, n = 5; p > 0.05; Figure 6.6C) of the CA3 axon antidromic spike in sham-treated animals. However in pilo-treated animals, while 0.1 µM KA did significantly reduce the amplitude, it was minimal
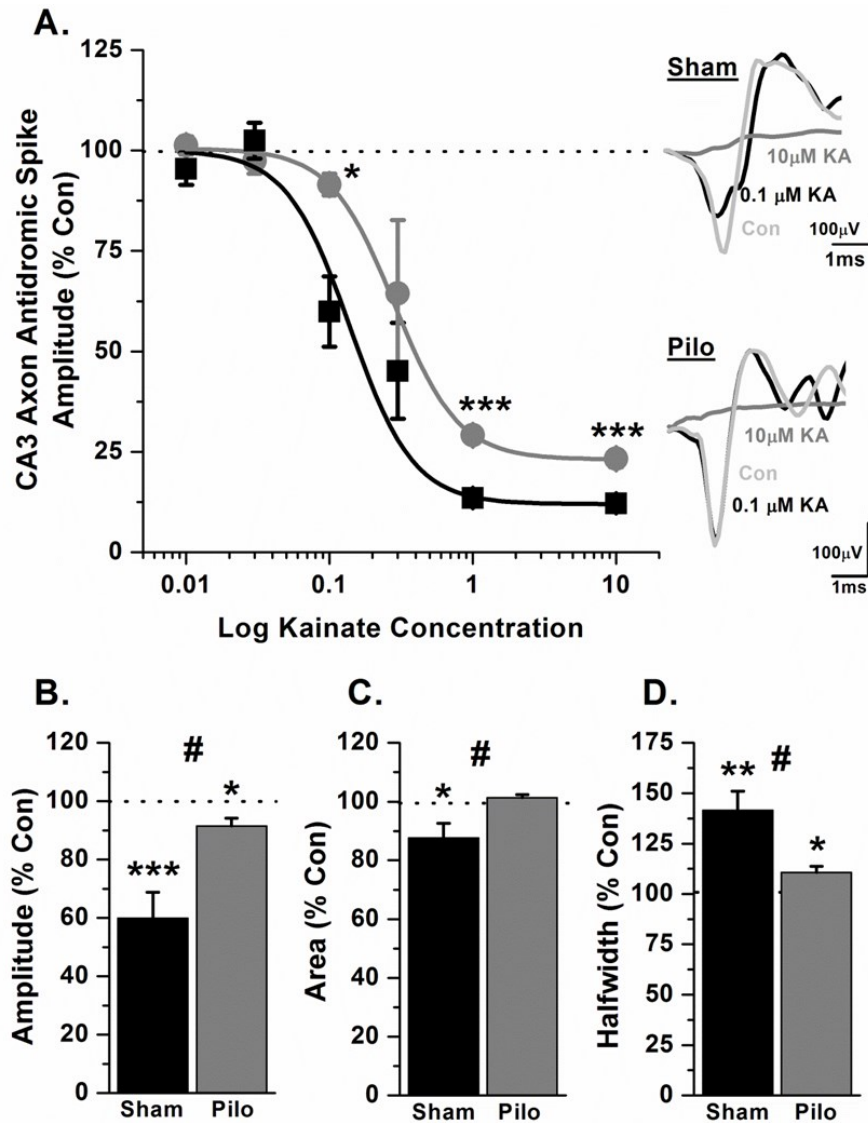
Figure 6.6. KAR-mediated depression of CA3 axon excitability is impaired in epileptic animals. **A.** Kainate concentration response curves from sham-treated (*black squares, n* = 4 – 6) and pilo-treated animals (*gray circles, n* = 2 – 6). Depression produced by low KA was significantly reduced in pilo-treated animals. *Right,* Waveforms from sham and pilo-treated animals. Baseline antidromic spikes (*Con, light gray traces*) were depressed by 0.1 µM KA (black *traces)*, and further depression induced by 10 µM KA (*gray traces).* Depression by 0.1 µM and 10 µM KA was significantly reduced in pilo-treated animals. Application of 0.1 µM significantly decreased the amplitude **(B.)** of both sham- and pilo-treated animals. KA significantly reduced the area **(C.)** of sham-treated, but not pilo-treated animals. KA (0.1 µM) significantly increased the half-width in sham- and pilo-treated animals **(D.)** *p < 0.05; **p < 0.01; ***p < 0.001; #p < 0.05, t-test.

and was significantly different than that observed in sham-treated animals (91.5 ± 2.7% of control, n = 5; $p < 0.05$; Figure 6.6B). In addition, we did not observe any change in the area of the CA3 axon antidromic spike with 0.1 μM, a finding also different from sham-treated animals (101.3 ± 1.2% of control, n = 5; $p < 0.01$; Figure 6.6C). We also examined possible alterations in the half width of the CA3 antidromic spike and determined that it was significantly increased in both sham-treated (141.6 ± 9.5% of control, n = 6; $p < 0.01$) and pilo-treated animals (110.6 ± 3.1% of control, n = 4; $p < 0.05$; Figure 6.6D), but to significantly different levels ($p < 0.05$; Figure 6.6D). This finding of an increase in the half width of the CA3 antidromic spike by KA is in agreement with Mott et al. (2008). Overall these findings demonstrate that KA-induced depression of CA3 axon excitability is significantly impaired in epileptic animals. Thus we suggest that in the epileptic brain the inhibitory tone of CA3 axonal projections is reduced, and may lead to increased hyperexcitability and potentially increased transmission into area CA1.

6.4.7 UBP310 BLOCKS KA-INDUCED DEPRESSION OF CA3 AXON EXCITABILITY

Similar to previous experiments with MF excitability, we examined the ability of the KAR antagonist UBP310 to block the KA-induced depression in sham- and pilo-treated animals (Figure 6.7). We found that in both sham (102.6 ± 9.0% of control, n = 3; $p > 0.05$; Figure 6.7) and pilo-treated animals (102.0 ± 2.2% of control, n = 2; $p > 0.05$; Figure 6.7) bath application of UBP310 (3 μM) had no effect on the amplitude of the CA3 antidromic spike. However, we determined that in sham-treated animals pre-application of UBP310 significantly
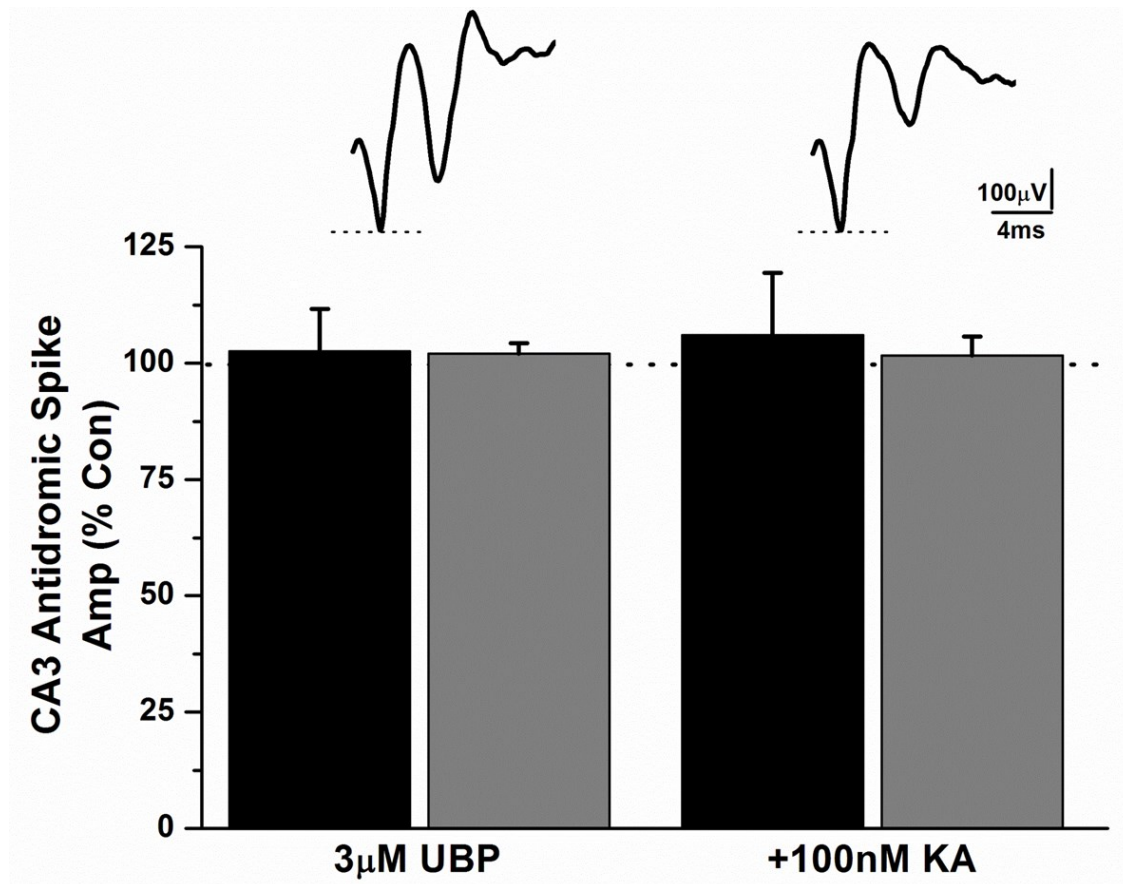
Figure 6.7. UBP310 blocks KA-induced depression of CA3 axon excitability. Application of UBP310 (3 µM) did not affect the baseline amplitude of the CA3 axon antidromic spike in sham or pilo-treated animals. The presence of UBP310 was sufficient to block the depression by low 0.1 µM KA observed in sham-treated animals. Representative waveforms from a sham-treated animal demonstrate the lack of effect of UBP310 (3 µM) on the amplitude of the CA3 antidromic spike as well its blockade of 0.1 µM KA-induced depression. Sham-treated group: *black bars* (n = 3); pilo-treated group: *gray bars* (n = 2).

blocked the anticipated depression by 0.1 µM KA (without UBP310: 59.9 ± 8.7% of control, n = 6; with UBP310: 106.0 ± 13.4% of control, n = 3; p < 0.05; Figure 6.7). In pilo-treated animals the presence of UBP310 appeared to block the depressive actions of 0.1 µM KA, however the difference was not significant likely due to variability and a low n-value (without UBP: 91.5 ± 2.7% of control, n = 2; with UBP310: 101.6 ± 4.1% of control, n = 2; p > 0.05; Figure 6.7). The selectively of UBP310 for KARs demonstrates that the depression of CA3 axon excitability is mediated by KARs. In Chapter 4 we observed a significant reduction in KAR subunit expression in area CA3 at both 60d and 200d post-SE. This reduction in KAR subunit expression likely serves as a rationale for the observed decrease in KAR-regulation of CA3 axon excitability.

6.5 DISCUSSION

Together the results of this study provide the first known examination of alterations in KAR-mediated regulation of hippocampal axon excitability in epileptic animals. In agreement with previous studies we determined concentration-dependent actions of KA on both MF and CA3 axon excitability in sham-treated animals (Kamiya and Ozawa, 2000; Schmitz et al., 2000; Mott et al., 2008). These results are in line with the observation that low concentrations of KA induce opposing actions on MF and CA3 axons, as low KA facilitates MF excitability and reduces CA3 axon excitability (Mott et al., 2008). In epileptic animals we observed that the facilitation of MF excitability by low KA (0.3 µM) and depression of CA3 axon excitability by low KA (0.1 µM) were both

significantly impaired. This finding suggests the possibility that synaptic transmission, including into areas CA3 and CA1, may be critically impaired in epileptic animals. In addition these changes may reflect compensatory changes to limit network excitability in the epileptic brain.

6.5.1 POSSIBLE ROLE OF METABOTROPIC KARs

KARs localized to presynaptic mossy fiber terminals have been reported to regulate transmission in a bidirectional, concentration-dependent manner, where low concentrations of KA facilitate, and high concentrations of KA depress the EPSC (Contractor et al., 2000; Kamiya and Ozawa, 2000; Schmitz et al., 2000; Schmitz et al., 2001a). In agreement with these findings KA has also been shown to have the same bidirectional control on axon excitability, with low KA facilitating and high KA depressing MF excitability (Kamiya and Ozawa, 2000; Schmitz et al., 2000). While these two functions of KARs are distinct from one another, KA does appear to have the same effect. Therefore, alterations in KAR-regulation of axon excitability can likely be extrapolated to suggest similar findings in transmitter release.

While the actions of KA on axon excitability and presynaptic transmitter release are well documented, the mechanism regulating these actions is not as clear. The facilitatory effects of KA on axon excitability are likely due to a slight depolarization of the axon with serves to increase excitability (Schmitz et al., 2000). This is observed as a potentiation of the antidromic spike or orthodromic fiber volley, as well as an increase in transmitter release and potentiation of the

EPSC. In contrast, the decrease in axon excitability by high concentrations is not as straight-forward. Some studies have suggested that the depression is due to a substantial increase in depolarization resulting in the inactivation of Na^+ channels and a subsequent blockade of action potentials (Contractor et al., 2000; Kamiya and Ozawa, 2000; Schmitz et al., 2000). In support of this line of reasoning is that the effects of KA on transmitter release can be mimicked by raising extracellular K^+ levels to increase depolarization (Schmitz et al., 2000). These findings support an ionotropic mechanism of KA-induced facilitation and depression of axon excitability as well as presynaptic regulation of transmitter release.

A metabotropic mechanism has also been identified as a possible explanation of high KA-induced depression of presynaptic transmitter release, and likely also explains the decrease in axon excitability observed in the current study. Negrete-Diaz et al. (2006) reported that KA-induced depression of transmitter release could be blocked by application of the G-protein inhibitor pertussis-toxin (PTX), a finding indicative of a metabotropic action. In support of this mechanism, while high K^+ does in fact produce significant depression of transmitter release, it recovers to baseline fairly quickly, while the depressive actions of KA are significantly longer lasting.

While Negrete-Diaz et al. (2006) demonstrated a metabotropic mechanism of high KA-induced depression of transmitter release, we believe that these findings can be generalized to our examination of axon excitability. We found that while facilitation induced by low concentrations of KA was blocked by all

antagonists examined, the depressive actions were much more complicated. Most surprising was the inability of the glutamate receptor antagonist CNQX to block the depression. While we did not directly explore a metabotropic action, we feel that our findings are suggestive of this mechanism. Therefore, we propose that while facilitation of axon excitability is mediated by ionotropic KARs, the depression induced by high KA is likely through a metabotropic action.

6.5.2 ACTIONS OF KYN ON MF EXCITABILITY

We observed that facilitation of MF excitability by low concentrations of KA was readily blocked by all antagonists we examined. In contrast, the KA-induced depression of MF excitability by high KA was only inhibited by KYN. As previously mentioned, KYN is commonly used as a more global glutamate antagonist and at high concentrations inhibits AMPA, NMDA, and KA-receptors (Ganong et al., 1983; Alt et al., 2004). While KYN does demonstrate some KAR subunit selectively, by preferentially blocking the GluK2 subunit, its most notable action at KARs is potentiating current from GluK2/5-containing KARs (Fisher and Mott, 2011). Regardless of these actions of KYN the ability of this compound to not only block but potentiate axon excitability with 10 μM KA is perplexing. One possible explanation, although improbable, could be that if depression is mediated through a metabotropic action, which we believe is likely, that perhaps the high concentration of KYN (3 mM) used in the current study had inhibitory effects on metabotropic glutamate receptors. This would explain the ability of KYN, but not CNQX, UBP or ACET to inhibit the KA-induced depression of MF

excitability. It should also be noted that many of the studies examining antagonistic properties of not only KYN, but most compounds, have utilized glutamate as the agonist (Ganong et al., 1983; Alt et al., 2004; Fisher and Mott, 2011). It is entirely possible, and quite likely, that the antagonistic actions of KYN are significantly different when kainate is used as the agonist.

6.5.3 ROLE OF ZINC ON MOSSY FIBER KARs

High quantities of zinc are found within the hippocampus, particularly within the hippocampal mossy fibers (Maske, 1955; McLardy, 1962). Within the mossy fiber terminals zinc is actually co-localized with glutamate and is released in a Ca^{2+}-dependent manner (Assaf and Chung, 1984; Howell et al., 1984). Zinc has also been reported to play critical roles in cognition and aging (Szewczyk, 2013). Furthermore, synaptically released zinc has been shown to inhibit both NMDA and $GABA_A$ receptors (Vogt et al., 2000; Ueno et al., 2002; Ruiz et al., 2004), but seemingly has minimal effects on AMPA receptors (Mayer et al., 1989; Dreixler and Leonard, 1994; Lin et al., 2001; Mott et al., 2008). More recently it was demonstrated that synaptically released zinc inhibits postsynaptic KARs localized to the mossy fiber – CA3 synapse, and preferentially acts on GluK4, or GluK5-contaiing KARs (Mott et al., 2008).

In addition to a significant role in regulating synaptic transmission at the mossy fiber – CA3 synapse, zinc has also been shown to regulate axon excitability. Mott et al. (2008) demonstrated that facilitation of MF excitability produced by low concentrations of KA could be prevented by exogenously

applied zinc. In contrast, zinc did not appear to effect the depressive actions of KA on CA3 axons. Increases in synaptic activity, as noted in epilepsy, as well as the aberrant sprouting of mossy fibers, have been shown to increase levels of zinc (Assaf and Chung, 1984; Howell et al., 1984; Li et al., 2001a; Li et al., 2001b; Lin et al., 2001).

In the current study we determined that facilitation, but not depression of MF excitability is significantly reduced in epileptic animals. In light of the current studies, we suggest that this may be due to the apparent increase in the concentration of zinc in the MFs, which serves to block the facilitatory effects of KA. In regards to CA3 axons, we observed that KA-induced depression is significantly reduced in epileptic animals. While Mott et al. (2008) demonstrated that zinc was not able to block KA-induced depression of CA3 axons in control animals, we cannot rule out the possibility that the increased levels of zinc in epilepsy may now function to inhibit KA-induced depression. To explore these possibilities experiments utilizing a zinc chelator would be beneficial, and allow us to essentially lower the levels of zinc in epileptic slices back to baseline levels and determine if we see the appropriate KA-induced facilitation of MF excitability and KA-induced depression of CA3 axon excitability.

6.5.4 POSSIBLE CHANGES IN KAR SUBUNIT COMPOSITION

While the possibility that increased levels of zinc may be responsible for the lack of observed KA-induced facilitation of MFs and depression of CA3 axons in epileptic animals, based on our previous data, we suggest that alterations in

KAR subunit expression may also serve as an explanation. In regards to MF excitability we determined that facilitation, but not depression was absent in epileptic animals. We also observed that expression of the GluK4 subunit was significantly reduced in area CA3 of animals 60 and 200d post-SE (Chapter 4), suggesting that the lack of facilitation may be due to the reduction in GluK4 subunit expression. In support of this proposition, studies utilizing knockout animals have demonstrated that animals lacking the high affinity GluK4 and/or GluK5 subunits display impaired facilitation, but not depression (Contractor et al., 2003; Fernandes et al., 2009; Catches et al., 2012). Therefore, we believe that the loss of KA-induced facilitation of MF axon excitability is most likely due to the significant reduction in GluK4 subunit expression.

While many studies have explored the KAR subunit composition responsible for MF facilitation and depression, few studies have examined KAR-regulation of CA3 axon excitability. Mott et al. (2008), reported that zinc preferentially inhibits GluK4 or GluK5-containing KARs and that zinc did not block the KA-induced depression of CA3 axon excitability. This suggests that KARs localized to CA3 axons are likely GluK2/3-containing KARs. Our anatomical data from epileptic animals (Chapter 4), demonstrated a significant decrease in GluK2/3 expression in area CA3. If KA-induced depression of CA3 axons is in fact mediated by GluK2/3-containing KARs, our observation of reduced inhibition could be explained via alterations in subunit expression. However, we still cannot rule out the possibility that increased levels of zinc may contribute to these findings. In addition, it should be noted that auxiliary proteins, such as Neto1 and

Neto2, have been demonstrated to dramatically impact KAR trafficking and pharmacology. As the current study did not directly explore the interaction between Neto1 and/or Neto2 on KARs, we cannot rule out the possibility that observed alterations are not due to changes in expression of the Neto proteins.

6.6 SIGNIFICANCE

The findings of this study provide the first look at alterations in KAR-mediated regulation of hippocampal axon excitability in epileptic animals. We demonstrated that facilitation of MF excitability is absent in epileptic animals, likely due to alterations in KAR subunit expression. This reduction in facilitatory drive into area CA3 of epileptic animals could serve as a compensatory mechanism to limit epileptogenic activity. Alternatively, it also reveals that the mossy fiber pathway, the main source of communication between the dentate gyrus and area CA3, is significantly impaired in epileptic animals. In addition this study suggests that the depressive actions of high KA on MF excitability are likely through a metabotropic action, a finding supported by previous studies (Cunha et al., 1999; Negrete-Diaz et al., 2006). Furthermore, we report that KA-induced depression of CA3 axon excitability is significantly impaired in epileptic animals, likely due to alterations in KAR subunit expression. This reduced inhibition of CA3 axons would likely contribute to bursting within the highly interconnected CA3 network and would most likely increase synaptic input into area CA1 in the epileptic brain. Overall we suggest that the observed changes in axon excitability in both the mossy fibers and area CA3 are due to the reduction in KAR subunit

expression in the epileptic brain. Further studies would need to be conducted to solidify the impact that the observed changes would have on network excitability within the epileptic brain.

CHAPTER 7

GENERAL DISCUSSION AND SIGNIFICANCE

7.1 DISCUSSION

The data collected from these studies significantly contribute to the scientific literature regarding the impact of status epilepticus and the development of epilepsy on alterations in both inhibitory and excitatory networks. We have demonstrated that while $GABA_AR$ subunit composition is dramatically altered following a prolonged SE event, agents such as stiripentol remain effective in terminating SE, and thus may serve as potential therapeutic targets. We also examined changes in excitatory neurotransmission mediated by the kainate receptor and demonstrated a significant decrease in receptor subunit expression and a subsequent decrease in KAR-mediated transmission in the epileptic brain. We feel that these studies are extremely valuable to our understanding and knowledge of SE and epilepsy, as well as anatomical changes that occur in the brain. These studies may serve as building blocks for potential therapeutic targets for the treatment of epilepsy and/or associated comorbidities. The findings revealed in this document are summarized in Tables 7.1 – 7.4.

SE is a neurologic emergency which requires prompt medical attention and immediate therapeutic intervention. As nearly 50% of SE cases occur in children under the age of 2, our ability to develop safe and effective therapeutics

Table 7.1 Summary of results detailed in Chapter 3

Question	Hypothesis	Findings	Impact/Significance
• BZDs require γ2-containing GABA$_A$Rs to terminate SE • As SE progresses γ2-containing GABA$_A$Rs are internalized • Actions of STP do not require γ2-containing GABA$_A$Rs ➢ *Is STP effective in terminating SE when resistance to BZDs has developed?*	• STP will remain effective in a model of BZD-resistant SE	• STP terminates brief and prolonged SE • Less pharmacoresistance develops to STP than to DZP • STP potentiates GABAergic transmission in an age-dependent manner • STP does not act through the BZD-binding site • STP, not DZP, potentiates phasic and tonic GABAergic transmission after prolonged SE • STP, not DZP, potentiates mIPSCs after prolonged SE	• STP remains effective in terminating BZD-resistant prolonged SE • STP is effective in all age groups examined but is most effective in younger animals • STP alone, or in conjunction with other AEDs would be effective in the treatment of brief and prolonged SE • Development of additional therapeutics with pharmacological profiles similar to STP

Table 7.2 Summary of results detailed in Chapter 4

Question	Hypothesis	Findings	Impact/Significance
• KARs have been implicated in seizure disorders as well as their comorbidities • KARs are widely expressed throughout the hippocampus, a region prone to seizure generation and propagation ➢ *How does the progression of epilepsy alter the expression of KAR subunits throughout the hippocampus?*	• Distinct alterations in KAR subunit expression will be observed in hippocampal regions DG, CA3 and CA1 • We anticipate that the progression of epilepsy will differentially alter KAR subunits	*DG:* 5d- ↓ GluK2/3 & GluK5 60d- ↓ GluK2/3 200d- ↓ GluK2/3, GluK4 & GluK5 *CA3:* 5d- ↓ GluK2/3 & GluK5 60d- ↓ GluK2/3 & GluK4 200d- ↓ GluK2/3 & GluK4 *CA1:* 5d- ↓ GluK2/3 & GluK5 60d- ↓ GluK2/3 200d- ↓ GluK2/3 • GFAP expression increased at all time-points in all regions • Reduced GluK2/3 expression is restricted to limbic regions	• Pilo-induced SE produces significant alterations in KAR subunit expression • Alterations are independent of region and duration of epilepsy • Likely precede changes in KAR-mediated transmission throughout hippocampus • Likely correlated to observed comorbidities of epilepsy i.e., cognition, anxiety and depression

Table 7.3 Summary of results detailed in Chapter 5

Question	Hypothesis	Findings	Impact/Significance
• KAR-mediated transmission is increased in DG of epileptic animals • KARs regulate transmission in area CA3 • KAR subunit expression is significantly reduced in area CA3 of epileptic animals ➢ *Is KAR-mediated transmission at the mossy fiber – CA3 synapse altered in the epileptic brain?*	• Neurotransmission mediated by KARs will be significantly reduced at the mossy – fiber CA3 synapse of epileptic animals	*In epileptic animals:* • KAR-mediated transmission at mossy fiber – CA3 synapse is significantly reduced • Synaptic integration at this synapse is impaired • Reduction is specific to KARs- no change in AMPAR-mediated transmission • Presynaptic regulation of PPF was not different	• Epilepsy generates opposing alterations in KAR-mediated transmission at two different synapses of the same axon • Observed reduction is likely a compensatory mechanism resulting from the increased excitatory circuit in DG • Reduction may contribute to observed comorbidities in epilepsy

Table 7.4 Summary of results detailed in Chapter 6

Question	Hypothesis	Findings	Impact/Significance
• Axon excitability influences synaptic transmission • KAR-mediated axon excitability is subunit dependent • KAR subunit expression is significantly reduced in epileptic animals ➤ *Is KAR-mediated regulation of axon excitability altered in epileptic animals?*	• The ability of axonal KARs to regulate excitability will be reduced in the epileptic brain	*Mossy fibers:* • Facilitation but not depression is absent in epileptic animals • Facilitation is blocked by KAR antagonists • Depression is likely regulated by metabotropic KARs *CA3 axons:* • Depression is significantly reduced in epileptic animals • Depression is blocked by a KAR antagonist	• KAR regulation of axon excitability is impaired in epileptic animals • In MFs, reduction may supplement compensatory downregulation of excitatory transmission • In CA3 axons, reduction likely contributes to epileptogenic activity • Alterations likely influence synaptic transmission into CA3 and CA1

is essential. Our data demonstrates that in a model of prolonged SE, when resistance to BZDs develops, STP continues to terminate SE, in an age-dependent manner. Of note from this study was the observation that the actions of STP are independent of the BZD-binding site of the $GABA_A$ receptor, a finding in agreement with a previous study (Quilichini et al., 2006). While this allows for STP to work in conjunction with additional AEDs, such as clobazam, the exact mechanism of STP remains elusive. It remains a possibility that STP may act on a separate and distinct site of the $GABA_A$ receptor. In addition we feel that the ability of STP to potentiate tonic GABAergic transmission, a mechanism which is maintained following prolonged SE, greatly contributes to its anticonvulsant effects. As extrasynaptic δ-containing receptors are not altered during prolonged SE (Goodkin et al., 2008), this population of $GABA_A$ receptors provide a unique target for the development of new AEDs. The findings provided in Chapter 3 are the first examination of the ability of STP to terminate BZD-resistant SE.

KARs have been implicated in several disease states, specifically epilepsy, where systemic administration of kainate induces SE and the development of spontaneously occurring seizures through activation of GluK2-containing KARs (Mulle et al., 1998; Fritsch et al., 2014). KAR subunits GluK1 – 5 are expressed throughout the brain and hippocampus, specifically at the mossy fiber – CA3 synapse, a region considered to be highly epileptogenic and sensitive to excitotoxicity. Despite the relationship between KARs and seizure disorders, few studies have explored KARs in the epileptic brain. Furthermore, the studies that have explored this interaction have focused on the DG, a region

characterized by aberrant mossy fiber sprouting in epilepsy. As a whole, the experiments discussed in Chapters 4 – 6 provide the first in-depth examination of KAR subunit expression and neurotransmission in epilepsy.

One aspect of KAR-mediated neurotransmission that we did not explore in the current study is the role of the auxiliary transmembrane proteins Neto1 and Neto2. While both proteins are expressed in the hippocampus, data suggests that expression of Neto1 is more abundant and appears restricted to area CA3 and the DG (Copits et al., 2011; Straub et al., 2011b). In addition, studies utilizing Neto1$^{-/-}$ animals have demonstrated that KAR-mediated ionotropic and metabotropic transmission is significantly impaired at the mossy fiber – CA3 synapse (Straub et al., 2011b; Tang et al., 2011; Wyeth et al., 2014). Reportedly this reduction in KAR-mediated transmission is due to a decrease in surface trafficking of the GluK2/3 subunit to the postsynaptic CA3 cell (Wyeth et al., 2014). In the current study we observed a rapid and sustained decrease in GluK2/3 expression in hippocampal regions, specifically area CA3 which was also accompanied by a reduction in KAR-mediated transmission. Due to limitations in antibody availability and selectivity we did not explore alterations in Neto1 or Neto2 expression in epileptic animals. It is possible that Neto1 may be significantly impaired in animals following SE and this reduction impairs proper trafficking of the GluK2/3 subunit and thus reduces transmission.

The current study focused predominately on the ionotropic actions of KARs. While we suggest that the depression of mossy fiber excitability induced by high KA (10 µM) is likely through a metabotropic action we did not specifically

explore this possibility. Nevertheless, the concept that KARs mediate both ionotropic and metabotropic transmission in the hippocampus should be appreciated. In addition to their role in regulating excitatory transmission, activation of KARs by kainate has been demonstrated to depress GABA release at inhibitory synapses (Rodriguez-Moreno et al., 1997). This inhibition was determined to occur through a presynaptic activation of metabotropic KARs linked to the inhibitory $G_{i/o}$ protein, representing the first identification of metabotropic KARs (Rodriguez-Moreno and Lerma, 1998). The ability of KARs to regulate metabotropic transmission prompted additional studies which eventually established that glutamate release at the Schaffer collateral – CA1 synapse is regulated by metabotropic KARs (Frerking et al., 2001), and that the depression of axon excitability and glutamate release observed at the mossy fiber – CA3 synapse likely involves metabotropic KARs (Negrete-Diaz et al., 2006). In addition, it is well documented that KARs mediate postsynaptic excitability through metabotropic regulation of the slow and medium AHP (Melyan et al., 2002; Melyan et al., 2004; Fisahn et al., 2005; Grabauskas et al., 2007). Overall these studies emphasize the diverse role of KARs within the hippocampus and demonstrate their ability to regulate both excitatory and inhibitory transmission through ionotropic and metabotropic mechanisms.

7.2 IMPACT OF REDUCED KAR-MEDIATED TRANSMISSION ON HIPPOCAMPAL NETWORK EXCITABILITY

Our data concerning altered expression of KARs likely has significant

implications for hippocampal network activity. SE and the progression to spontaneous seizures is associated with an increase in network excitability within the DG due to sprouting of aberrant mossy fibers and the subsequent development of an excitatory circuit partially mediated by KARs (Cronin et al., 1992; Wuarin and Dudek, 1996; Sutula et al., 1998; Epsztein et al., 2005). To compensate for this increase in excitability, we suggest that KAR subunit expression is reduced in area CA3 leading to a decrease in excitatory transmission in this region. This decrease in excitatory transmission in area CA3 was specific to KARs, as AMPAR-mediated transmission was unaltered. In addition, facilitation but not depression of MF excitability, which likely influences presynaptic glutamate release was also reduced in epileptic animals. These studies suggest that the reduction in KAR subunit expression and function serves to limit network excitability in area CA3 of epileptic animals.

At the Schaffer collateral – CA1 synapse, activation of KARs by kainate or domoate has been reported to depress glutamate release in area CA1, thus reducing excitatory transmission (Chittajallu et al., 1996; Kamiya and Ozawa, 1998; Vignes et al., 1998; Frerking et al., 2001). Our observation that KAR-mediated depression of CA3 axon excitability is reduced in epilepsy suggests that excitatory transmission into area CA1 may be increased. Thus while the reduction in KAR subunit expression and function at the mossy fiber – CA3 synapse may help reduce network excitability, the reduction in KAR regulation of CA3 axons may increase network excitability at the Schaffer collateral – CA1 synapse. However, this concept is based solely on our studies of CA3 axon

excitability, and thus additional studies would need to be conducted to further determine the impact of this alteration on CA1 network excitability.

7.3 FUTURE DIRECTIONS

Our data demonstrating that STP is effective in terminating BZD-resistant status epilepticus could potentially impact the treatment algorithms currently used for refractory SE. However, in order for STP to be used clinically to treat SE additional studies need to be performed. While our data clearly demonstrates that STP effectively terminates both brief and prolonged SE, these findings were based purely on the appearance of behavioral convulsions. While these behavioral affects are correlated to electrographic activity (Racine, 1972), we did not specifically explore this relationship. In addition, the ability to demonstrate that STP is effective over several animal models of SE would add tremendous validity to our findings. Therefore, future studies would need to determine the impact of STP on EEG activity as well as determine the effectiveness of STP in additional models of SE. Furthermore, STP is commonly utilized in conjunction with other compounds such as clobazam (CLB) (Chiron et al., 2000; Ng and Collins, 2007). While the current study did explore the ability of STP and CLB to potentiate GABAergic transmission in SE-experienced animals, we did not examine the impact of these compounds together on behavioral seizures, something that would provide direct clinical relevance to the method in which STP is currently utilized.

Our anatomical data demonstrates that KAR subunit expression is

significantly reduced in all brain regions post-SE. The electrophysiological data collected from area CA3 is in agreement with these anatomical alterations, particularly the observed decrease in GluK2/3 and GluK4 subunit expression. However, in the DG we observed a significant increase in KAR-mediated transmission, a finding in agreement with a previous study (Epsztein et al., 2005). However, this does not correlate with the decreases in KAR subunit expression in the DG. Future experiments should be aimed at understanding this discrepancy, which likely involves the process of neurogenesis which is unique to the DG. We feel that anatomical data collected from epileptic animals may be hindered by the massive increase, and subsequent decrease, in neurogenesis that takes place post-SE (Parent et al., 1997; Gray and Sundstrom, 1998; Hattiangady et al., 2004). Future studies should be aimed at unraveling the impact of neurogenesis on KAR subunit expression. Similarly, a previous study has suggested that KARs are colocalized with astrocytes (Vargas et al., 2013). While the current study did not observe the appearance of any astrocytic labeling in immunohistochemical analysis of KAR subunit expression this possibility should be explored further, as we did find significant increases in GFAP expression in all brain regions, and all time-points explored. As previously discussed the current study did not explore potential alterations in Neto1 and Neto2 expression in the epileptic brain. Given the apparent role of Neto1 in trafficking of KAR subunits, particularly in area CA3 (Copits et al., 2011; Straub et al., 2011b; Wyeth et al., 2014), future experiments should unquestionably explore

the impact of SE and epilepsy on expression of the auxiliary proteins Neto1 and Neto2 in regulating KAR subunit expression and function.

We concluded that the apparent decrease in KAR-mediated excitatory neurotransmission in area CA3 may be a compensatory modification. This notion was based on our finding, in agreement with other studies, that KAR-mediated transmission is increased in the DG, and that this increase significantly impairs the ability of the DG to properly integrate transmission entering the hippocampus (Epsztein et al., 2005; Artinian et al., 2011). Thus KARs in area CA3 are reduced in an attempt to limit transmission into this hyperexcitable region. Future experiments should be specifically aimed at determining if transmission entering the DG via the perforant path produces a weakened KAR-mediated current in area CA3. This finding would add support to the idea of a compensatory downregulation of KARs in epileptic animals. Additionally, we made several claims that the reduction in KAR subunit expression and the functional impact of these changes could precede the development of comorbid conditions common in epilepsy, such as anxiety, depression and cognitive impairment. A recent study has concluded that the GluK4 subunit may play a role in anxiety and depressive-phenotypes in animals (Catches et al., 2012). However, future experiments would need to focus on specifically addressing this as of yet unclear, but likely relationship.

7.4 HYPOTHESIS REVISITED

The overarching hypothesis that served as the catalyst for the studies outlined in this manuscript was that *status epilepticus and the development of epilepsy produce striking alterations in GABA$_A$ and kainate receptor populations. Furthermore, that these alterations significantly impact receptor pharmacology and neurotransmission in the SE-experienced brain.*

The findings detailed in Chapters 3 – 6 of this document support this hypothesis. Briefly, previous studies had demonstrated that SE resulted in subunit selective internalization of the GABA$_A$ receptor resulting in the development of pharmacoresistance to commonly prescribed BZDs (Walton and Treiman, 1988; Kapur and Macdonald, 1997; Jones et al., 2002; Goodkin et al., 2008). In conjunction with these studies we demonstrated that the pharmacological profile of STP, a compound utilized as add-on therapy for treatment of Dravet syndrome, was effective in terminating both brief and prolonged SE by sustaining its potentiation of GABAergic transmission. In addition we studied alterations in excitatory neurotransmission mediated by the KAR following induction of SE. We demonstrated that SE produces significant and region specific reductions in the expression of KAR subunits, and that these alterations yield a significant decrease in KAR-mediated transmission at the mossy fiber – CA3 synapse, as well as reducing the ability of KARs to properly regulate axon excitability. Together these studies support our hypothesis that SE and the development of epilepsy, significantly alters both the pharmacology and physiology of hippocampal receptor populations.

7.5 SIGNIFICANCE REVISITED

Status epilepticus, characterized as a prolonged seizure, is an extremely serious event that requires immediate medical attention. Nearly 40% of people who experience a single SE event go on to develop epilepsy, making it the most challenging long-term consequence associated with SE (Hesdorffer et al., 1998). While several lines of treatment options for SE are available, approximately 20 – 40% of people prove refractory to these agents, significantly increasing their risk for development of epilepsy, associated comorbidities and death (French, 2007). In light of the staggering impact that SE and seizure disorders can impart, the development of more effective therapeutics is essential. However, in order to develop these drugs we must better understand the impact that SE and the development of epilepsy have on the brain, particularly how receptor populations are altered and the impact of those alterations on neurotransmission.

The studies in this document provide new and essential information regarding how both GABAergic and glutamatergic receptor populations are altered following SE. While BZDs are considered the first-line of therapy for treatment of SE, they develop significant pharmacoresistance that can put patients in extreme danger as the seizures progress. We have demonstrated that STP, a compound already clinically used for Dravet syndrome, is highly effective in terminating SE and develops less pharmacoresistance. This finding will hopefully lead to further exploration of STP as a potential treatment method in the patient population. In addition, our findings demonstrate the types of compounds and their pharmacological profiles that are effective in treating BZD-

resistant SE, and suggest that the development of additional compounds with similar profiles may be advantageous.

The final set of studies explored alterations in KAR subunit expression and neurotransmission. Our understanding of KARs and their role in hippocampal neurotransmission has dramatically increased over the past couple decades. Emerging from these studies was the relationship between KARs and epilepsy. The studies outlined in this document further this field by demonstrating that SE and the progression of epilepsy significantly alter the expression of KAR subunits throughout the three central regions of the hippocampus- DG, CA3 and CA1. In support of the reduction in KAR subunit expression we observed a significant and selective decrease in KAR-mediated transmission, as transmission mediated by AMPA receptors was unaltered. These findings likely demonstrate a compensatory downregulation of KAR-mediated excitatory neurotransmission in the epileptic brain. However, a consequence of this reduction to limit epileptogenic activity may be the development of comorbidities associated with epilepsy. In addition these studies could potentially serve as a rationale for the selective targeting of KARs for therapeutic agents. Together these studies contribute new and exciting information to our knowledge concerning the impact of SE and the development of epilepsy on the inhibitory and excitatory systems of the brain.

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APPENDIX A

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