# TRANSLATIONAL STUDIES OF DISEASE-MODIFYING INTERVENTIONS

# FOR LIMBIC EPILEPTOGENESIS

A Dissertation

by

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# ABSTRACT

Epilepsy is characterized by the occurrence of repeated unprovoked seizures. Currently there is little understanding of the pathophysiological changes in epileptogenesis, the process by which a normal brain is turned into an epileptic one. Therefore, research providing further insight to mechanistic changes observed in epilepsy and epileptogenesis is a critical step in formulating new therapeutic strategies. Temporal lobe epilepsy, arising from insult or injury to the limbic system, is one of the most common types of epilepsy. The hippocampus is a critical structure for epileptogenesis. Epilepsy is associated with marked alterations in the structure and function of GABA-A receptors in the hippocampus. Neurosteroids are endogenous steroids present in the brain that modulate neuronal excitability through interaction with GABA-A receptors. This class of neurosteroids includes allopregnanolone, THDOC, and related 5α-pregnane derivatives. Although neurosteroids are potent anticonvulsants, the precise role of neurosteroids in epileptogenesis remains poorly understood.

The main objective of this dissertation was to understand the role of endogenous neurosteroids and their synthetic analogs in limbic epileptogenesis using a combination of pharmacological, behavioral, and morphological techniques. We utilized two distinct models involving electrical kindling and chemoconvulsant-induced epileptogenesis, utilizing transgenic mouse strains such as GABA δ-subunit knockout mice (δKO). Our studies suggest δKO mice are prone to faster epileptogenesis, increased seizure susceptibility, and altered sensitivity to antiepileptic drugs (AEDs). Our studies provide evidence for alterations in extrasynaptic δGABA-A receptors and increased neurosteroid sensitivity in a mouse catamenial epilepsy model. Neurosteroids may exhibit disease-modification in halting the epileptogenesis. We used a δKO mouse model and uncovered the extrasynaptic GABA-A receptor mechanisms in the antiepileptogenic potential of natural and synthetic neurosteroids. Overall, we demonstrated that a targeted increase in neurosteroid levels elevates synaptic and tonic inhibition within the limbic areas, leading to network shunting and reduction in epileptogenesis.

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# CONTRIBUTORS AND FUNDING SOURCES

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# NOMENCLATURE

| | |
|---|---|
| 3β-AP | 3β-hydroxy-5α-pregnan-20-one |
| δKO | $GABA_A$ receptor δ-subunit knockout |
| AD | Afterdischarge |
| AED | Antiepileptic Drugs |
| AN | Androsterone |
| AP | Allopregnanolone (3α-hydroxy-5α-pregnan-20-one) |
| CA1 | Cornu Ammonis area 1 |
| CA3 | Cornu Ammonis area 3 |
| CCI | Controlled Cortical Impact |
| CNS | Central nervous system |
| DGGC | Dentate gyrus granule cell |
| EEG | Electroencephalogram |
| FN | Finasteride |
| GABA | γ-aminobutyric acid |
| GABAAR | γ-aminobutyric acid type A receptor |
| δ-GABAAR | δ-subunit containing γ-aminobutyric acid type A receptor |
| GN | Gonadotropin |
| GX | Ganaxolone (3α-hydroxy-3β-methyl-5α-pregnan-20-one) |
| mTOR | Mammalian target of rapamycin |
| NMDA | *N*-Methyl-D-aspartate |
| NSW | Neurosteroid Withdrawal |
| PTE | Post-traumatic Epilepsy |
| SE | Status-Epilepticus |
| SRS | Spontaenous Recurrent Seizures |
| TBI | Traumatic Brain Injury |
| THDOC | Allotetrahydrodeoxycorticosterone (3α,21-dihydroxy-5α-pregnan-20-one) |
| TLE | Temporal lobe epilepsy |

# CHAPTER I*
## BACKGROUND AND INTRODUCTION

Epilepsy is among the most prevalent neurological disorders. The epileptic disease state is characterized by recurrent spontaneous abnormal electrical discharges in the brain (seizures) which alter perception, consciousness, and motor activity. Common symptoms of a seizure can vary with the brain regions affected, but often include sudden unusual feelings, uncontrollable twitching and even unconsciousness. Epilepsy affects around 1% of the world population, [Hesdorffer et al., 2013], with 3 million current cases and nearly 150,000 new diagnoses per year in the United States alone [Jacobs et al., 2009]. Although a significant number of antiepileptic drugs (AEDs) are used for controlling seizures, these therapies target only symptoms of the disease, and fail to adequately prevent seizure development, or permanently halt the occurrence of seizures. The goal of the therapy is to eliminate seizures without interfering with normal function [Glauser et al., 2006; 2013]. Despite many advances in epilepsy research, an estimated 30% of people with epilepsy have "intractable seizures" that do not respond to even the best available medication. There is renewed focus on the pathophysiology of epileptogenesis, the process whereby a brain becomes progressively epileptic due to an initial precipitating event. This review of literature highlights the recent advances in novel therapeutic strategies that inhibit epileptogenesis and the potential targets for developing targeted approaches for curing epilepsy.
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## I.1 Common features of epileptogenesis from human and animal studies

A turning point in epilepsy research came in 2000, when the NINDS and multiple research and advocacy groups formed the first "Curing Epilepsy" meeting, designed to bring together ideas and shift the focus of research as whole toward investigations aimed

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unveiling mechanistic basis of epilepsy development, and eventually, a cure for epilepsy [Jacobs et al., 2001]. The following decade brought about a significant increase in research emphasizing disease-modification and prevention for translation to clinically applicable therapies [Jacobs et al., 2009; Simonato et al., 2012]. The Institute of Medicine (IOM) released a consensus report in 2012 on public health dimensions of the epilepsies focusing on promoting health and understanding epilepsy [Austin et al., 2012; Hesdorffer et al., 2013]. The IOM report, *Epilepsy across the Spectrum: Promoting Health and Understanding* provided 13 recommendations for future work in the field of epilepsy, especially one key recommendation on prevention of epilepsy.

'Epileptogenesis' describes the multifaceted alterations in brain structure and physiology responsible for creating a condition characterized by spontaneous recurrent seizures [Pitkanen et al., 2009; Pitkanen and Lukasuik, 2011]. These changes may precipitate from aberrant neural connectivity, an interruption of neurotransmitter balance leading to hyperexcitability, or a combination of these conditions. To this end, epilepsy is categorized as idiopathic Primary Epilepsy, or Secondary Epilepsy, where seizures are the pathophysiological manifestations of one or more of many conditions such as neuronal damage from hypoxia or trauma, infection, cancer/tumor growth, drug withdrawal, or glial dysfunction [Engel et al., 2007]. Of note, exposure to cholinergic neurotoxins such as chemical nerve agents or organophosphorous pesticides can cause epilepsy as a result of status epilepticus [de Araujo Furtado et al., 2012]. While it is accepted that specific types of epilepsy may arise (at least in part) from unique pathophysiological conditions- a number of convergent processes are common across many types of acquired epilepsy. Loss of balance in neuronal excitation/inhibition, inflammation, cell death, and aberrant network plasticity are changes implicated in wide number of epileptic conditions. The historically accepted hypothesis regarding the development of epilepsy (epileptogenesis) includes three phases: (i) an initial precipitating condition or event; (ii) a seizure free latent period; and (iii) chronic epilepsy with spontaneous seizures (**Fig.1**). However, recent investigations of major epileptogenic cascades have provided support for the view of epileptogenesis as an

extended and continuous process beyond the initial expression of spontaneous seizures [Dudek and Staley, 2011].

Temporal lobe epilepsy (TLE) is the most common form of epilepsy with focal seizures, typified by a progressive expansion of unprovoked seizures, which originate in the limbic system [Wieser, 2004]. A hallmark of TLE pathology is the hippocampal sclerosis (HS), a condition of considerable neuronal death and extensive mossy fiber sprouting in the dentate hilus and CA1 and CA3 areas. [Sutula et al., 1989; Nadler, 2003; Buckmaster et al., 2002; Morimoto et al., 2004]. There is still much debate regarding the nature of hippocampal sclerosis; it is not yet known whether this condition contributes to seizure pathology, or is an outcome of the recurring seizure events observed in TLE patients. Several mechanisms have been identified, including loss of interneurons [Van Vliet et al., 2004; Sloviter and Bumanglag, 2013] and neuroinflammation [Vezzani et al., 2007]. Of note, there is great variation in the onset of spontaneous seizures following a precipitating factor [Rao et al., 2006; Norwood et al., 2010]. Thus, the critical window for effective "antiepileptogenic" interventions remains poorly defined for treating epilepsy in people at risk.

Studies in animal models have provided improved understanding of neurophysiological basis of epileptic seizures [Simonato et al., 2012; O'Dell et al., 2012]. Spontaneous seizures arise from hyperexcitable and hypersynchronous neuronal networks and involve both cortical and several key subcortical structures. The general cellular pathways underlying occurrence of epileptic seizures is apparent in three phases: (a) focal epileptogenicity (initiation); (b) synchronization of the surrounding neurons (sync); and (c) propagation of the seizure discharge to other areas of the brain (spread). Electrophysiological recordings in isolated brain sections of epileptic animals have provided critical insights to the molecular and cellular changes underlying epileptic brain regions. A paroxysmal depolarization shift (PDS) is a primary hallmark activity-driven response often observed in individual epileptic neurons. This phenomenon involves

abnormally long depolarization of neurons, manifesting as hypersynchronous sharp wave interictal spike observed via EEG in epileptic neuronal networks.

In 1881 William Gover proposed the notion that *seizures beget seizures*, and nearly a century later the development of the kindling model provided a proof-of-concept for this idea, and has been instrumental in identifying potential molecular targets for modulating or halting epileptogenesis [Goddard et al., 1969; McNamara et al., 1992]. The kindling model utilizes repeated stimulation (either chemical or electrical) to produce a condition reflecting increased seizure activity over time. Another class of models, the post-status epilepticus paradigms, are predominantly utilized for modeling the epileptogenic processes following an acute episode of prolonged seizures. These are experimentally produced by application of pilocarpine, kainate or electrical stimulation [Rao et al., 2006; Buckmaster and Dudek, 1997; Hellier et al., 1999; Glien et al., 2001; Loscher, 2002]. Most importantly, these models display a number of similarities to human limbic epilepsy not observed in the kindling model [Loscher, 2002; Stables et al., 2002]. Application of chemoconvulsants or perforant path stimulation induce SE, which if left unchecked, leads to widespread neuronal injury, the extent of which may be correlated with the duration and severity of SE experienced.  This is followed by a silent "latent period," thought to be similar to that observed in human limbic epilepsy. As observed in clinical TLE patients, brains from post-status animals display stereotypical cell death, aberrant mossy fiber sprouting, and ectopic granule cell proliferation following a seizure free latent period [Rao et al., 2006; Loscher, 2002; Hester and Danzer, 2013]. Although a battery of interventions have been tested in diverse animal models of epileptogenesis, there is yet no FDA-approved drug or therapy that effectively prevents or halts acquired epileptogenesis in those patients at risk [Acharya et al, 2008; Pitkänen and Lukasiuk, 2011]. Clinical trials testing a number of AEDs for antiepileptogenic efficacy have shown no viable protection in high-risk patient populations [Temkin, 2001; Mani et al., 2011]. Thus, there is great need for effective antiepileptogenic agents or disease-modifying therapies that may stem the growing population and societal impact of epileptic patients worldwide.

**Antiepileptogenic drugs**   **Antiepileptic drugs**

epileptogenesis progression

Epileptogenic event

50

40   spontaneous recurrent seizures

30

20

10

0

Minutes/ hours | Days | Weeks | Months

**1** | **2** | **3**

Initial Epileptogenic trigger | Latent Period | Emergence of Chronic Epilepsy

few months or years

−Ion channel activation
−BBB damage
−Excitotoxic apoptosis
−Inflammation and glial reaction
−Changes in synaptic/ signaling/ structural proteins
−Epigenetic dysregulation

−Neurodegeneration and axonal sprouting
−Inflammatory reactions
−Structural/ functional plasticity
−Reorganiztion of neuronal networks
−High synchronous activity

−Hyperexcitabel networks
−Spontaneous EEG and spontanous seizures
−Fibrillary gliosis
−Hippocampus sclerosis
−Mossy fiber sprouting

**Figure 1. An overview of the pathophysiology of epileptogenesis.** Epileptogenesis is the process whereby a normal brain becomes progressively epileptic because of precipitating injury or risk factors such as TBI, stroke, brain infections or prolonged seizures. Epilepsy development can be described in three stages: (1) the initial injury (epileptogenic event); (2) the latent period (silent period with no seizure activity); and (3) chronic period with spontaneous recurrent seizures. Although the precise mechanisms underlying spatial and temporal events remain unclear, epileptogenesis may involve an interaction of acute and delayed anatomic, molecular, and physiological events that are both complex and multifaceted. The initial precipitating factor activates diverse signaling events, such as inflammation, oxidation, apoptosis, neurogenesis and synaptic plasticity, which eventually lead to structural and functional changes in neurons. These changes are eventually manifested as abnormal hyperexcitability and spontaneous seizures. Around two-dozen *antiepileptic drugs* are available for treating seizures by blocking ion channels. Current drug therapy is symptomatic in that available drugs inhibit seizures, but neither effective prophylaxis nor cure is available. Presently, there is no "*antiepileptogenic*" drug for cure of epilepsy. Thus, newer drugs that can better prevent and modify the disease are needed for curing epilepsy.

**I.2 Experimental aspects of epileptogenesis and outcome analysis**

Rodent models of epileptogenesis have contributed to our understanding of the progression from initial injury to onset of epileptic seizures in the mammalian brain. To better understand the manner in which precipitating events lead to a chronic epileptic phenotype more closely resembling human epilepsy with recurrent seizures, a variety of models emerged, such as kindling, pilocarpine or kainic acid-induced SE, electrically-induced SE and organophosphate intoxication [Reddy and Kuruba, 2013]. These models are suitable for studying mechanisms, biomarkers, and intervention of epileptogenesis or optimizing novel drug discoveries. However, modeling of epileptogenesis is tedious, often highly variable, and currently lacks translational validation. However, work in these models has led to increased understanding of epileptogenic processes and have been utilized for identifying antiepileptic drugs. A brief description of three commonly used epileptogenic models follows as a prelude to our studies of experimental therapeutics.

The kindling model of epilepsy was first described 1969 to understand epileptogenesis as a process of secondary focal epilepsy acquired over time in response to primary brain excitation/stimulation [Goddard et al., 1969]. Kindling involves repeated excitatory stimuli, which induce seizures of gradually increasing intensity that permanently alter the neuronal network and persist upon successive trials of excitation. Initially stimuli induce only subconvulsive or partial seizures. Standard kindling stimuli consists of electrical stimulation of the hippocampus or amygdala through permanently implanted electrodes. [McIntyre et al., 2002; Morimoto et al., 2004] Additionally, certain chemical agents such as pentylenetetrazol (PTZ), a $GABA_A$ receptor antagonist, can be used in a similar manner to produce chemical kindling in mice or rats. Repeated stimulus results in a progressively stronger response in terms of seizure severity and duration. Fully kindled animals exhibit level 5 (Racine scale 0 to 5; Racine, 1972) seizures that are representative of complex partial and secondary generalized seizures, making for a model of TLE [Loscher et al, 1998; McIntyre et al., 2002; Morimoto et al., 2004]. Once

an animal reaches this fully kindled state, the robust response to stimuli appears to be permanent. The persistence of this state has led many groups to investigate the mechanistic steps that underlie the transformative process of epileptogenesis [Stables et al., 2002]. In addition to the daily stimulation-until-kindled-state method of kindling, variants exist such as the modification of the "rapid kindling" protocol originally developed by Lothman et al. [1989] as proposed by Sankar and colleagues [Mazarati et al., 2007 ]. Rapid kindling greatly reduces the time necessary to elicit kindled seizures, but produces a transient kindled state. Extended kindling stimulations leads to a state known as "overkindled," which is marked by neuronal damage and the emergence of spontaneous seizures. This produces epileptgoenesis in a manner more similar to the post-status epilepticus models introduced in the following paragraph, but is incredibly time-consuming [Loscher and Brandt, 2010]. Several important questions have surfaced from the conglomerate of kindling studies. Primarily, does kindling truly reproduce human epileptogenesis? An important aspect to note is that spontaneous seizures are only observed in kindling methods which induce neuronal damage, and most studies do not utilize these for due to the aforementioned time and labor requirements. As such, major questions remain regarding the reflection of neurobiological alterations in animals that develop spontaneous seizures and those changes observed in kindled animals [Pitkanen and Halonen, 1998]. Finally, what type of epileptogenic etiology, if any, is recreated in the kindling model? Despite these questions, kindling remains the most extensively used model of TLE. However, the introduction and optimization of post-status epilepticus models has shifted much of the focus on understanding epileptogenesis towards those models, which involve a clinically-relevant latent period as well as spontaneous seizures [Pitkanen and Halonen, 1998].

Status epilepticus (SE) is, potentially life-threatening, emergency neurological condition characterized by a continuous seizure state [Lowenstein, 1999]. Epidemiologic reviews have shown that epilepsy develops in 40% or more of patients who undergo SE [Hesdorffer et al., 1998]. Although SE may be driven via a number of mechanisms, most post SE rodent models rely on chemoconvulsants to induce SE. The most popular

systemic chemoconvulsants are kainate and pilocarpine, which have been thoroughly characterized in regards to the progression of physical and electrographic seizure events, neuropathologies, and disease outcome. In these models, SE is usually terminated with diazepam (or related AEDs) after a pre-determined period, typically 30, 60, 90, or 120-minutes. However, refractoriness to benzodiazepines develops early on in SE, and higher concentrations or follow-up doses of the drug must be used to eliminate SE after prolonged exposure [Jones et al., 2002]. EEG verification is critical for determining the end of SE, as shown by studies in which even early diazepam treatment (10 min post-SE) terminated convulsions but not electrographic seizures [Gualtieri et al., 2012]. Emerging reports have also found increased success in terminating SE using poly-drug treatments, and a recent review by Loscher (2002) covers this subject in great detail. Without pharmacological intervention, SE most frequently results in mortality. SE duration should be limited to the shortest time possible while still inducing epilepsy in most of the animals. Following terminations of SE, a latent period of days to weeks precedes the manifestation of spontaneous recurrent seizures [Goodman, 1998; Stables et al., 2002]. Following SE, most animals develop spontaneous recurrent partial and secondarily generalized seizures (SRS), damage to the limbic system and surrounding structures, molecular level changes in signaling and structure, as well as behavioral and cognitive deficits that closely reflect many of the clinical characteristics of TLE [Morimoto et al., 2004].

Various protocol changes have been implemented over time, aimed at altering dose structures, or pre-treating animals with lithium, in order to increase the number of animals achieving SE while simultaneous reducing overall mortality [Hellier et al., 1999; Glien et al., 2001]. Lithium application induces systemic inflammation and increases blood-brain barrier permeability in rats prior to the administration of pilocarpine, increasing the efficacy and reducing the dose of drug required to induce SE. Although exposure to pilocarpine, as well as SE itself, induce both extended and local inflammatory responses [Voutsinos-Porche et al., 2004], the BBB-modifying adaptive immune reaction induced by lithium pretreatment serves to distinguish the lithium-

pilocarpine-SE from other post-SE models of TLE. Of note, several inflammatory pretreatments, including injection of bacterial lipopolysaccharide (LPS) do not potentiate the effects of pilocarpine in the post-SE model of TLE [Dmowska et al., 2010]. Focal injection is another technique that has been utilized to avoid the systemic administration of chemoconvulsants, which can lead to widespread neuronal injury and extraneous confounds [Cavalheiro et al., 2008].

Some experimental characteristics of post-SE epileptogenesis are shared with other chronic epilepsy models, such as post-traumatic epilepsy (PTE) or post-stroke epileptogenesis [Pitkänen et al., 2007; Losher and Brandt, 2010; Reddy et al., 2016]. SE-induced neuronal damage provides the initial precipitating injury, comparable with a mild TBI or ischemic event. Animals enter an extended (weeks to months) monitoring period following SE, where recordings of electrographic abnormalities, behavioral seizures, and other epileptiform activities are taken. Outcome parameters in these studies most often include: (i) duration of latent period prior to SRS occurrence; (ii) proportion of cohort affected by seizures; (iii) timing or frequency of SRS; and (iv) severity and duration of seizures exhibited. Additional measurements may include quantification of pre- and interictal spikes, fast/slow ripples, or threshold to seizure. The secondary outcomes of major translational interest are cognitive deficits and the evaluation of neuropathology in brain slices. The major advantage of post-SE models is the latent period exhibited prior to the emergence of SRS. Latent period duration is correlated with SE duration and severity. This latent period offers a window for therapeutic intervention and investigation into the step-by –step epileptogenic changes underway. Post-SE models have proven a valuable tool in the search for understanding and intervention in epileptogenesis. Such paradigms continue to provide investigators the ability to analyze a wide range of experimental factors responsible for not only the disease state, but also of disease progression and outcome modification.

Post-traumatic epilepsy (PTE) is defined as the progressive occurrence of unprovoked seizures following a traumatic brain injury (TBI). TBI may be divided into primary and

secondary injuries: the primary injury which occurs upon impact (typically not subject to therapy), and secondary injuries that persist after the initial injury, and are subject to clinical intervention [Werner and Englehard, 2007]. In humans, PTE typically presents itself after a long latent period, the duration of which is critically linked to the nature and severity of the injury. For patients suffering a mild to moderate TBI, the five-year incidence of PTE is roughly 1% [Annegers et al., 1998]. However, 1 in 10 patients with severe TBI display some form of PTE within 5 years, and this number rises to 1 in 6 within thirty years of the initial TBI [Annegers et al., 1998]. There are currently no accepted/effective treatment routes for the prevention of PTE. A meta-analysis of randomized controlled trials observed prophylactic use of anti-epileptic drugs could be used to effectively prevent early seizures in some cases, but there is no evidence for prevention of late seizures or the development of PTE [Schierhout and Roberts, 2001]. Understanding and treating PTE involves a particular complexity due to its position at the meeting point of epileptology and neurotrauma. Post-injury pathologic risk factors for PTE vary by brain region affected, and include sustained intracranial abnormalities such as subdural hematoma or cerebral contusion [Messori et al., 2005]. The nature of the injury is also strongly correlated with development of PTE, with penetrating (and generally more severe) injuries significantly increasing the risk of PTE [Englander et al., 2003]. When the full scope of epileptogenesis is considered, it is very likely that PTE might be alleviated or eliminated by treatments that would not normally be classified as antiepileptics.

The development of relevant animal models has been critical in studying the pathology of PTE, as well as for investigating and validating new treatment methods. Many models of TBI exist, though only a select few are used to study PTE, as the majority of models do not reliably produce spontaneous recurrent seizures, nor other hallmarks of epilepsy pathology, and thus have little relevance to human PTE. The three models used most frequently in studies investigating PTE are fluid percussion (FP), weight-drop, and controlled cortical injury (CCI). The fluid percussion model reproduces TBI via a brief piston-driven fluid pressure pulse on to the intact dura. As in CCI, a craniectomy is

created to allow for consistent and direct application of noxious force to the brain. The percussion produces brief displacement and deformation of neural tissue [Cernak, 2005]. Due to the high level of standardization and broad reflection of human post-TBI pathology, FP has become one of the most widely used model of PTE. The CCI model differs from FP by the means in which the noxious force is delivered. CCI utilizes a controlled mechanical force, delivered by a programmable actuator/piston that may be positioned at different locations and/or angles around the stereotaxic surgery rig. The mechanism of impact allows for a very high degree of precision for impact site, depth, and duration of displacement- making CCI an ideal model for investigating varying impact parameters on injury severity and following pathology.

In stark contrast to the open-skull methods just introduced, the weight drop model utilizes a closed-head impact design. A rod or weight of specified mass is dropped from a specified height, with the animal's head placed directly along the impact path. While this method does not provide the same degree of precision of CCI or FP, it carries several advantages of its own. Mild-TBI injuries may be delivered without the extensive anesthesia or confounds of invasive surgery, and can be carried out rapidly and inexpensively. Furthermore, the lack of restraint and post-injury rotational motion induced more closely resemble sports and automobile related injuries that have become an intense focus within the TBI field. Finally, the closed-head nature allows for the study of repeated injury, a common occurrence in patients with mild TBIs. Neither FP or CCI are capable of reproducing a repeated-injury model of PTE. However, there is currently no animal model that completely recreates the wide spectrum of pathological events by TBI. As the nature and etiology of TBI injuries often varies from patient to patient, continued research will be required to fully elucidate and characterize the broad range of acute and chronic changes underway after TBI.

Electrophysiological events play a key role in the progression of brain injury from persistent seizures, excitotoxicity or traumatic insult to many brain regions. EEG seizure activity can occur in the absence of overt clinical manifestations of SE-induced seizures.

Indeed, it is evident that distinct episodes of generalized seizure discharges occur in a high proportion of SE-injured animals, but without overt manifestations or disturbance of normal motor behavior. Therefore, continuous EEG monitoring is a critical component of the overall experimental design for epileptogenesis, although 24-hour video monitoring adds a powerful means of confirmation for behavioral manifestations of epileptiform activity detected on the EEG. Various video-EEG montages are used to acquire EEG signals depending on the model and study design. Electrodes are typically implanted immediately following the induction of stroke, though chronic observation studies may call for implantation several days after the ischemic event, particularly if implantation could interfere with immediate interventions. Post-stroke monitoring is divided into acute and chronic stages. Acute stage observations begin immediately following stroke and consist of continuous EEG/Video recording for motor abnormalities or electrographic signs of epileptiform activity. These acute observations may continue up to a week after injury. Chronic recording studies utilize continuous recordings for up to 1 year following the ischemic event. Advances in technology have increased ease of long-term continuous seizure monitoring through commercially available wireless-EEG-video systems, as well as conventional tethered systems.

The monitoring provided through these systems overcomes a number of confounds present in data from intermittent recordings; primarily the determination of false negatives, or overestimation of ictal event frequency due to the sporadic and unpredictable nature of seizure occurrence. Spontaneous seizure counts are generated from analysis of EEG recordings, based on predetermined spike parameters. These seizures usually last between 20 and 50 s and are characterized by a pattern of hypersynchronous high-amplitude firing. As in post-SE studies of epileptogenesis, primary outcomes are measured by the frequency, duration, and severity of spontaneous seizures. Evaluation of related neuropathology is assessed through histological analysis.

**I.3 Rapamycin and mTOR intervention for epileptogenesis**

Rapamycin is a naturally occurring antifungal macrolide antibiotic that exerts powerful immunosuppressant effects. Rapamycin (more appropriately- sirolimus) is a specific inhibitor of the serine/threonine protein kinase mammalian target of rapamycin (mTOR). mTOR mediates a number of developmental processes including neurogenesis and cellular proliferation and migration. It is also involved in several other processes related to epilepsy, such as axonal sprouting, axonal regeneration, dendritic development, and structural microtubule dynamics [Watanabe et al., 2011]. mTOR signaling indirectly influences neuronal excitability through modulation of synaptic structure and plasticity (**Fig.2**). Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways maintain a critical role in the regulating the neural connectivity and network dynamics by affecting the arborization and spine formation of dendrites [Kumar et al., 2005, Jaworski et al., 2005]. A secondary route by which mTOR may affect neuronal excitability is through modulation of neuronal ion channels/receptors [Huang et al., 2012].

Disruption of mTOR signaling has been implicated in a number of human disease states. Of particular interest for this review is mTOR regulation of neuronal excitability and its involvement in epilepsy and epileptogenic processes [Jones and Thompson, 2009; Sarbassov et al., 2005]. Aberrant activation of mTOR has been investigated as pathogenic mechanism underlying epileptogenesis and remains a key target for interventions aimed at modifying disease state or preventing epilepsy altogether [Meng et al., 2013]. Currently, rapamycin and related analogs ("rapalogs") are being investigated for potential therapeutic benefit (**Table 1**). Tuberous sclerosis complex (TSC) is perhaps the most recognized of mTOR-related epileptic conditions. TSC is a genetic condition in which benign tumors develop in the CNS and other organs, causing seizures/focal epilepsy along with a number of cognitive and physiological defects including cardiac, renal, and retinal disorders, and encephalitis [Smalley, 1998]. These tumors (a condition known as subependymal giant cell astrocytomas (SEGA)) arise from defective genes coding for hamartin (TSC1) and tuberin (TSC2), which regulate cell

proliferation and inhibit mTOR signaling [Moavero et al., 2013]. Indeed, when TSC patients were treated with rapamycin, cognitive deficits can be alleviated and seizures transiently decreased in both frequency and severity, but the protective effect was reversed following discontinuation of treatment [Moavero et al., 2013].
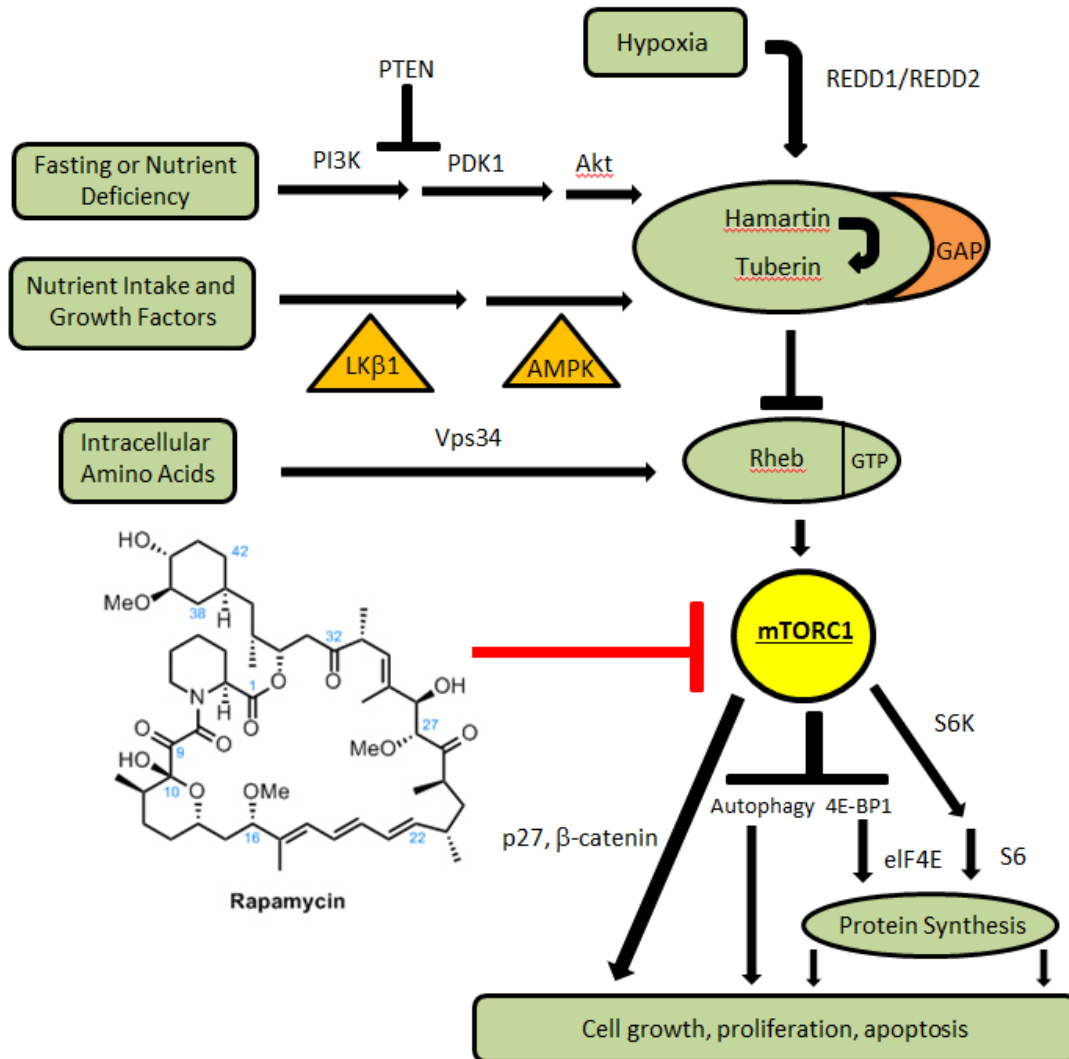


**Figure 2. The mTOR pathway in epileptogenesis.** mTOR mediates a number of developmental processes including neurogenesis and cellular proliferation and migration. It is also involved in a handful of more specific processes related to epilepsy, such as axonal sprouting, axonal regeneration, dendritic development, and structural microtubule dynamics. mTOR signaling indirectly influences neuronal excitability through modulation of synaptic structure and plasticity.

Many studies have observed increased mTOR signaling following induction of seizures and/or SE [Zeng et al., 2009; Buckmaster et al., 2009; Huang et al., 2010]. Further studies demonstrated that pretreatment with mTOR inhibitors can confer antiepileptogenic effects by slowing the development of epilepsy [Zeng et al., 2009]. Additionally, post-treatment with mTOR inhibitors can exert antiepileptic properties by decreasing seizures severity and frequency [Zeng et al., 2008]. Reduction of mossy fiber sprouting following SE is correlated with lower SRS frequency, and disease modification as a whole. Investigation of inhibition of the mTOR pathway for modification of epilepsy is still in early stages. In the pilocarpine-SE model, epileptic rats post-treated with rapamycin displayed significantly reduced seizure frequency, demonstrating what appears to be a direct antiseizure effect [Huang et al., 2010]. In contrast, other studies utilizing rapamycin treatment in the status-epilepticus model in mice do not demonstrate an antiepileptogenic effect in preventing SRS occurrence [Buckmaster and Lew, 2011]. Additionally, in the kainate-status epilepticus model, rats treated with rapamycin either shortly before or shortly after status epilepticus displayed decreased SRS occurrence and reduced cell death compared to control SE groups, bolstering the notion that mTOR inhibition may be an effective antiepileptogenic therapy [Yang et al., 2017].

Still, other studies in the amygdala electric stimulation model provide conflicting data: animals treated with rapamycin displayed no changes in epileptogenic outcome measures [Sliwa et al., 2012]. An explanation for the variance in results may be found within differences in strain/species, dosing regimens, and methods of monitoring. Interestingly, Huang et al [2012] reported a detrimental and epileptogenic effect of rapamycin treatment in immature rats. Young rats pretreated with rapamycin displayed lower seizure threshold and more severe seizure in response to PTZ, kainate, and pilocarpine. At minimum, data from continuing status epilepticus models clearly highlight the complexity and double-edged nature of mTORs involvement in epileptogenesis and hyperexcitability. It remains to be determined whether the effects of

mTOR inhibition are truly antiepileptogenic or anticonvulsant, or perhaps both [Wong, 2011].

Due to a number of factors, including increased societal awareness, TBI and PTE are rapidly becoming a focus for clinicians and researchers seeking to understand and treat human epilepsy. Combining aspects of acute neuronal injury with those of chronic inflammation and hyperexcitability, PTE often encompasses a variety of cognitive and physiological deficits. Early experiments using the weight-drop model of TBI demonstrated that extended treatment with rapamycin following TBI lead to decreased cell death and increased cognitive/functional performance [Erlich et al., 2007]. Other reports from the CCI model of TBI in mice also suggest that post-TBI treatment with rapamycin retards epileptogenic processes such as mossy fiber sprouting and cell death [Guo et al., 2011]. Recently, several studies have demonstrated disease modifying and neuroprotective effects of rapamycin treatment following TBI [Butler, 2015; Nicolaeva, 2015; Song, 2015),], lending more evidence to support a true antiepileptogenic role in PTE. However, when examining the scope and diversity of epileptogenic forces, it seems likely that mTOR inhibitors may elicit a number of inter-related actions that fall within one or both of the classifications. If investigations into mTOR's involvement in epileptogenesis continue to provide evidence supporting these early studies, it could have profound clinical implications for future TBI patients, as well as those currently suffering from or at risk for developing epilepsy.

**Table 1. Rapamycin and mTOR-based intervention of epileptogenesis in rodents.**

| Model | Treatment | Duration | Parameters & Outcome | Author |
|---|---|---|---|---|
| Kainate SE - Rat | 24Hr Post-SE | 7 weeks | ↓ Mossy Fiber Sprouting & SRS | Zheng et al. (2009) |
| Kainate SE - Rat | 24-72Hr Pre-SE | | ↓ Mossy Fiber Sprouting, Cell Death, SRS | |
| Kainate SE - Rat | 4Hr Post-Status | 1-7 weeks | ↓ SRS, BBB Leakage, Gliosis | Vliet et al. (2015) |
| Kainate SE – Young Rat | 24-72 Hr Pre-SE | 1 dose | ↓ Seizure Threshold, ↑ Severity | Huang et al. (2012) |
| Pilocarpine – Young Rat | 24-72 Hr Pre-SE | Pre-SE | ↓ Seizure Threshold, ↑ Severity | Huang et al. (2012) |
| Pilocarpine - Rat | 10week Post SE | Pre-SE | ↓ Mossy Fiber Sprouting & SRS | Huang et al. (2010) |
| Pilocarpine - Mice | 24Hr Post-SE | 6 days | ↓ Mossy Fiber Sprouting, DG hypertrophy, SRS | Buckmaster and Lew (2011) |
| Pilocarpine - Mice | 24Hr Post-SE | 8 weeks | ↓Mossy Fiber Sprouting, SRS present | Heng et al. (2013) |
| Amygdala Stimulation | 24Hr Post-SE | 2 weeks | No Inhibition of epileptogenesis | Sliwa et al. (2012) |
| Hippocampus Kindling -Mice | During Kindling | | Delayed Kindling Epileptogenesis | Sankar (2010) |
| PTZ – Young Rat | 24-72 Hr Pre-SE | Pre-SE | ↓Seizure Threshold, ↑ Severity | Huang et al. (2012) |
| CCI–TBI - Mice | 1Hr Post-impact | 4 weeks | ↓Mossy fiber sprouting, Cell Death, SRS | Guo et al. (2011) |
| Weight Drop-TBI - Mice | 4Hr Post impact | 4 weeks | ↓Cell Death, ↑Cognitive Scores | Erlich et al. (2007) |
| CCI – TBI - Mice | Once daily following CCI | Continuous | ↓Mossy fiber sprouting, SRS | Butler et al. (2015) |
| NSE-PTEN cKO-Mice | 6 weeks old | Daily, continuous | ↓ SRS Frequency and Severity | Zhou et al. (2009) |
| Pilocarpine | At start of SE | 1 dose | ↓Cell Death | Chwiej et al. (2010) |

## I.4 Neuroinflammation intervention of epileptogenesis

Brain inflammation is known to play a critical role in epileptogenesis [Vezzani et al., 2011]. While local inflammation is meant to protect tissue after insult, aberrant inflammatory responses alter normal cell function, and can lead to serious consequences such as blood brain barrier disruption [Vezzani et al., 2012], which can ignite the development of seizures and lead to intractable epilepsy [Oby and Janigro, 2006]. The pathogenic mechanisms operant in this process are partially-mediated by inflammatory cytokines (including IL-10, IL-1B, IL-1Ra) and include disruption in ion channels function, and abnormal uptake and release of excitatory neurotransmitters such as glutamate [Vezzani et al., 2011, Viviani et al., 2007; Wetherington et al., 2008; Friedman et al., 2009; Xu et al., 2013]. Brain cyclooxygenases (COX1 and COX2), termed prostaglandin-endoperoxide synthase (PTGS), serve as the rate-limiting enzymes which catalyze the metabolism of arachidonic acid to prostaglandins, are upregulated and induced by seizures. Manifestations of chronic inflammation in the form of abnormal leukocyte infiltration, increased cytokine expression, and reactive gliosis have been observed in brain tissue from human patients suffering from refractory focal epilepsy [Choi et al., 2009]. Just as the implications for inflammation in epileptogenesis are broad, so too are the routes by which inflammation may be targeted in therapeutic attempts aimed at disease prevention and modification. Anti-inflammatory medications, such as corticosteroids and adrenocorticotropic hormone, have been used in pediatric treatment of severe epilepsies including infantile spasms. Several cytokines have been found to be synthesized during seizures including interleukin 1-$\beta$ (IL-1$\beta$), transforming growth factor beta 1 (TGF-$\beta$1) and cyclooxygenase (COX-2) [Vezzani et al., 2012] which have been considered to have proconvulsant properties and to be associated with epileptogenesis [Friedman et al., 2009].

Experimental studies have demonstrated anticonvulsant activity of specific anti-inflammatory drugs, such as COX-2 inhibitors and inhibitors of IL-1-converting enzyme/caspase-1 and antagonists of IL-1$\beta$ receptors [Shafiq et al., 2003; Dhir et al.,

2005; Vezzani et al., 2010]. Therefore, it is likely that these drugs may have clinical potential in epilepsies associated with pro-inflammatory processes in the brain. Of particular interest is IL-1β's reduction of synapse-mediated GABA inhibition in the CA3 area of the hippocampus [Zeise et al., 1997]. Inhibiting IL-1 synthesis reduces seizure-induced neurodegeneration, though the extent and nature of this effect may vary, as blocking IL-1 synthesis significantly reduced seizure intensity as well [Maroso et al., 2011]. There is evidence of increased levels of IL-1β and IL-1R1, and ICE/caspase-1 activation in resected epileptogenic tissue from patients with pharmacoresistant TLE, and to contribute to experimentally-induced acute seizures [Vezzani, 2007]. The experimental agent VX-765, a selective inhibitor of ICE, has been successfully demonstrated to inhibit acute- and chronic refractory partial seizures in preclinical trials [Maroso et al., 2011].

Celecoxib, a non-steroidal anti-inflammatory drug (NSAID) has been tested in animal models of epileptogenesis. In the lithium-pilocarpine SE model, 42 days of celecoxib administration starting one day post-SE reduced seizure frequency and duration compared to control animals. Of note, treatment resulted in disease modification, but did not prevent the development of epilepsy. Other work has shown treatment with celecoxib ameliorated pilocarpine-SE-induced hippocampal neurodegeneration and other tell-tale signs of epilepsy pathology in rats [Jung et al., 2006]. Both increased seizure threshold and modification of PTZ-induced kindling epileptogenesis has been observed in rodents after administration of COX2 inhibitors prior to seizure induction [Dhir, 2007]. However, other groups that found that in the KA-SE model, mice pretreated with the COX2 inhibitor nimesulide displayed increased seizure severity and duration, and higher mortality, when compared to control animals [Kunz and Oliw, 2001].

A number of subsequent studies have shown that COX2 activation may convey some neuroprotective effects in specific rodent models of TLE [Manabe, 2004; Takemija, 2006], although previous investigations found that overexpression of COX2 increased susceptibility to glutamatergic excitotoxicity in vivo and in vitro [Kelley et al., 1999].

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Parecoxib is another NSAID of particular interest to epilepsy research, and is classified as a second generation selective inhibitor of COX2. The administration of this drug 18 days after pilocarpine-induced SE in adult rats failed to prevent the development of epilepsy or decrease the frequency or duration of seizures, but yielded a mild improvement in their severity [Polascheck et al., 2010].

**Table 2. Neuroinflammation-based strategies for epileptogenesis.**

| Drug | Target | Model | Outcome | Author |
|------|--------|-------|---------|--------|
| Celecoxib | COX-2 | Pilocarpine-SE | ↓ SRS Frequency and Duration, cell death | Jung et al. (2006) |
| Parecoxib | COX-2 | Pilocarpine-SE | ↓cell death | Polascheck (2010) |
| Aspirin | COX-1 & COX-2 | Pilocarpine-SE | ↓SRS, cell death, Mossy fiber sprouting | Ma et al. (2012) |
| Erythropoietin | Intrahippocampal | Li-Pilocarpine SE | ↑Neurogenesis | Paradiso et al. (2009) |
| Curcumin | Oxidative stress | Kainate SE | ↓ SRS Frequency and Duration, cell death | Kiasalari et al. 2013). |
| Resveratrol | Oxidative stress | PTZ model | ↓ Seizures, cell death | Saha and Chakrabarti, 2014 |

COX-2 manipulation can affect learning and other functions in the brain. In rats, COX-2 inhibitors induce behavioral deficits, disrupt hippocampal learning function, and disrupt Morris water maze acquisition under control conditions [Cowley et al., 2008]. Similar studies have also shed light on a modulatory role in neurogenesis: treatment reduced the number of proliferating neurons in the adult hippocampus by 30-90% in control/intact groups [Goncalves et al., 2010], and when administered immediately following

pilocarpine-induced SE [Jung et al., 2006]. Mice with global COX-2 knockout and wild-type mice treated with COX-2 inhibitors displayed higher mortality and more severe seizures after chemoconvulsant insults [Toscano et al., 2008; Baik et al., 1999]. The dualistic nature of this effect is based on timing and has the potential to complicate research considerably. To overcome these issues, a conditional knockout mouse was developed in which the COX-2 gene is selectively ablated postnatally in forebrain neurons [Serrano et al., 2011]. Surprisingly, conditional COX-2 knockout mice do not differ in their response to pilocarpine-induced SE. These findings suggest that COX2 does not affect neuronal seizure susceptibility in these areas, adding more confounding evidence to the COX2 investigation. In summary, these reports show stark inconsistencies in regards to COX2's role in seizures and epileptogenesis. However, to date, no selective COX2 inhibitor has demonstrated a clear, clinically-viable therapeutic option for preventing epileptogenesis.

## I.5 Modification of adenosine pathway in epileptogenesis

Adenosine is an endogenous neuromodulator with many physiological effects by interacting with four adenosine receptor subtypes (A1, A2A, A2B, and A3). Adenosine has an inhibitory effect in the central nervous system. Adenosine serves as a potent anticonvulsant that effectively inhibits excitatory transmission in the brain [Boison, 2016a;b]. In epilepsy, the release of adenosine is suggested to mediate cessation of seizure activity and the postictal refractory period. Indeed, intrahippocampal infusion of adenosine reduced the frequency of spontaneous seizures in chronic epileptic rats, an effect further confirmed by the inhibition of spontaneous seizures following selective activation of adenosine A1 receptors [During and Spencer, 1992]. However, serious adverse effects of adenosine on peripheral systems preclude its clinical therapeutic utility by traditional administration methods. Thus, strategies that increase local adenosine levels in the brain might represent viable approach to inhibit epileptogenesis. Alternate strategies are investigated such as adenosine-based gene therapy to knockdown adenosine kinase, a key enzyme responsible for adenosine degradation. The

antiepileptogenic potential of transient focal adenosine-delivery was tested in a rat model of systemic kainic acid (KA)-induced progressive TLE, and was found to significantly reduce epileptogenesis [Williams-Karnesky et al., 2013; Szybala et al., 2009]. Other studies have demonstrated adenosine-augmentation based therapies' prevention of epileptogenesis and related cognitive deficits and are described previously [Boison, 2016a;b].

**I.6 Stem cell therapy for epileptogenesis**

Network disruption stemming from cell death and aberrant reorganization are thought to play a major contributory role in epileptogenesis. With this in mind, augmentation and modulation of network reorganization following epileptogenic insult provides an attractive route for potential therapies aimed at curing epilepsy. Neurons and glial cells intended for therapeutic transplantation can be experimentally produced from a range of stem cells, such as neural stem cells (NSC's) and induced pluripotent stem (iPS) cells. The successful implantation and potential network integration of these cells may provide for functional improvements in many neurodegenerative disorders [Lindvall et al., 2012]. TLE is characterized by multiple hippocampal abnormalities, including reduced subclasses of GABA-ergic interneurons, cell death, aberrant dendritic growth, synaptic reorganization, and hyperexcitability. These features may be both causal and resultant of the spontaneous seizures characteristic of TLE [de Lanerolle et al., 1989; Engel, 2001]. Therefore, interventions using NSC grafting should aim to alter these changes or improve the balance between inhibitory and excitatory signaling by implanting cells that develop to GABA synthesizing-neurons and neuroprotective astrocytes into the hippocampus. A study investigated the potential of intravenous administration (24 h after SE) of beta galactosidase-encoded human NSCs on seizure development in a rat pilocarpine-SE model of TLE [Chu et al., 2004]. About 87% of control animals exhibited SRS, while only 13% of NSC-grafted rats displayed SRS. Additionally, seizure severity was reduced in the NSC-treated rats. Jing et al. [2009] transplanted a suspension of cultured adult subventricular zone (SVZ) neurosphere cells into the rat

hippocampus at one week after a unilateral intracerebroventricular (ICV) administration of a small dose of kainate. Grafted rats indeed displayed reduced abnormal spike frequencies, and the extent of reduction was correlated to the number of surviving graft-derived cells, suggesting a disease-modifying effect.

Another study investigated the antiepileptogenic efficacy of transplanting fetal hippocampal NSCs into the hippocampi of adult rats one week following SE [Kuruba et al., 2009]. At 3 months post-grafting, significant reductions in seizure severity, seizure duration, and seizure frequency were observed [Kuruba et al., 2009]. Assessments of sham graft-surgeries have shown no effect on epileptogenesis [Waldau et al., 2010]. Hippocampal neural stem cell grafting following a precipitating insult has demonstrated considerable effects on ameliorating the extent of epileptogenesis and SRS in animal models of TLE [Jing et al., 2009; Kuruba et al., 2009; Hattiangady, 2010]. However, the effectiveness of these pioneering transplant strategies for alleviating cognitive deficits have not yet been well-studied. Although the precise mechanisms underlying NSC graft-mediated seizure suppression are still under investigation, a number of analyses support the notion that addition of substantial numbers of the GABAergic neurons and neuroprotective cells into the epileptic host hippocampus may retard the development and reduce the severity of epileptogenic processes [Waldau et al., 2010]. Future studies are needed to confirm the hypothesized post-implant synaptic integration of NSC graft cells in host, but NSC-grafting nonetheless present an intriguing route for interrupting or reversing epileptogenesis.

## I.7 Interrupting the TRK pathway in epileptogenesis

Significant seizure-induced increases in brain-derived neurotrophic factor expression (BDNF) and tyrosine receptor kinase B (TrkB) activation have been observed in human patients and in a number of animals of TLE [Yan et al., 1997; Westmore et al., 1994; Murray et al., 2000; Takahashi et al. 1998]. BDNF is a small protein whose binding to the ectodomain of TrkB induces phosphorylation of select neuronal-activity-modulating

intracellular signaling pathways. Additionally, immunohistochemical evidence of increased TrkB activation, evident as increased pTrk immunoreactivity in the mossy fiber pathway of hippocampus, has been shown following induction of seizures by diverse patterns of electrical stimulation and diverse chemoconvulsants in rats and mice [He et al., 2004; 2010]. Moreover, activation of TrkB by BDNF produces structural changes in the hippocampal dentate granule cells [Danzer et al., 2002] similar to those identified in the epileptic brain [Houser, 1992]. BDNF promotes increased efficacy of excitatory synapses connecting principal neurons, a form of long term potentiation [Muller et al., 2000; Minichiello et al., 2002; Xu et al., 2000]. BDNF-mediated activation of TrkB can also compromise GABA-mediated inhibition [Tanaka et al.,1997]. Intraventricular infusion of TrkB receptor bodies markedly inhibited the development of kindling in adult rats, while infusion of TrkA or TrkC receptor bodies had no effect [Binder et al., 1999, a;b]. These findings demonstrate that scavenging BDNF *de novo* in the adult brain inhibits kindling development. Moreover, these findings support the idea that the neurotrophin receptor critical for progressive seizure development in this model is exclusively TrkB. This notion was further reinforced by the observation that conditional knock-out of TrkB from specific subsets of CNS neurons inhibited all behavioral seizure progression in the mouse kindling model [He et al., 2004]. This conditional deletion of TrkB is the only alteration known to fully eliminate all behavioral manifestations of kindling development. In whole, these studies provide a compelling argument for BDNF-activated TrkB as a necessary role in epileptogenesis in the kindling model.

Recent studies have investigated the downstream signaling pathways mediated by TrkB activation. Activation of TrkB induces two major signaling pathways: phosphorylation of Y515 leading to binding of the adaptor protein, SHC, and phosphorylation of Y816 leading to binding and activation of the enzyme PLCγ1 [He et al., 2014]. Consequent studies in mutant mice heterozygous for PLCγ1 observed impaired rates of kindling compared to WT controls, and implicated PLCγ1 signaling as the dominant pathway by

which TrkB activation promotes limbic epileptogenesis [He et al., 2014]. Furthermore, recent studies demonstrated that uncoupling TrkB from PLCγ1prevents post-SE epileptogenesis [Gu et al., 2015]. This highlights PLCγ1 as a novel target for potential therapies aimed at preventing epileptogenesis, particularly following status epilepticus.

## I.8 Interruption of JAK-STAT pathway in epileptogenesis

Epileptogenesis is associated with a number of changes in gene expression. Dysregulation of genes involved in inhibitory neurotransmission can be a powerful force driving hyperexcitability. The GABA-A receptor (GABA-AR) is the primary source of fast synaptic inhibition in brain, and not surprisingly, aberrant changes in GABA-AR subunit expression are thought to be direct contributors to epileptogenesis. Studies in post-SE animals have shown significant changes in subunit composition of inhibitory GABA-ARs in the DG. Recent investigations into the signaling pathways that underlie these post-SE changes have identified several promising molecular targets for therapies aimed at augmenting inhibitory signaling to halt epileptogenesis or for functional improvement in epilepsy [Grabenstatter et al., 2014].

The JAK-STAT pathway is activated by a variety of methods, including cytokines binding to their specific receptors, resulting in transphosphorylation of Janus kinases (JAKs) that then lead to phosphorylation of STAT proteins. Protein and/or mRNA levels for pSTAT3, and STAT3-regulated genes were evaluated in WP1066 (STAT3 inhibitor) and vehicle-treated rats during stages of epileptogenesis to determine the acute effects of STAT3 inhibition on chronic epilepsy [Grabenstatter et al., 2014]. Early WP1066 administration reduces known downstream targets of STAT3 transcription, known for their roles in cell-cycle progression and cell survival. [Grabenstatter et al., 2014]. Following TBI, WP1066 treatment improved the degree of recovery of vestibular motor function after injury. In addition, reducing JAK/STAT pathway activation after severe experimental TBI reversed the decrease in the GABA-AR α1 protein levels and

improved motor recovery [Raible et al., 2015]. BDNF- and seizure-dependent phosphorylation of STAT3 stimulates the binding of inducible cAMP early repressor protein (ICER) to phosphorylated CREB at the Gabra1:CRE site. Inhibiting JAK/STAT signaling in mice ameliorated seizure-induced decreases in GABAAR alpha1 expression, suggesting that BDNF can modulate GABA-AR abundance through at least two discrete pathways [Lund et al., 2008]. This line of evidence further supports the notion that STAT inhibition may be a therapeutically viable route for increasing inhibitory signaling in epilepsy. However, as practical as this approach may be, further investigation into the extended dynamics of these pathways, as well as the mechanisms by which they affect epileptogenesis are needed to develop translational therapies.

## I.9 Antiepileptic interventions for epileptogenesis

Antiepileptic drugs (AEDs) are the first line and primary treatment options for the symptomatic relief of seizures and other comorbidities in epileptic patients. As the focus of epilepsy research turns more towards the understanding of epileptogenesis and potential means by which that process may be stopped or reversed, the historical term AED is being replaced by ASD- antiseizure drug, a more fitting description for the manner in which these therapies work. Although dozens of approved, clinically-efficacious agents have been developed, the potential of these drugs to *prevent* epilepsy has historically been of secondary concern.  Indeed, despite the broad group of available ASDs, no accepted intervention strategy (pharmaceutical or otherwise) has been shown to effectively prevent or halt epileptogenesis in individuals at risk. The studies investigating the antiepileptogenic potential of conventional anticonvulsants have nonetheless provided intriguing evidence for potential disease-modification, serving as critical steps in evaluating therapeutic strategies. Studies on valproate [Silver, 1991] and levetiracetam [Loscher, 2012], which are widely prescribed ASD's, suggest that chronic treatment with either may delay (but not prevent) kindling acquisition, even after stopping drug treatment. Long-term treatment with ethosuximide and levetiracetam has also been shown to confer a compelling reduction in seizure development in genetic

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animal models of absence epilepsy [Blumenfeld, 2008]. This effect too, persists beyond the drug-treatment time table. Contrasting these results, studies in SE models have shown unclear, and at times, conflicting results with respect to levetiracetam's antiepileptogenic potential. Studies in rat-SE models did not find chronic post-treatment with levetiracetam to be effective in providing neuroprotection nor in inhibiting the development of spontaneous seizures [Brandt, 2007]. Further SE studies investigated phenobarbital as a potential antiepileptogenic agent, but results appear to in conflict, based on the timing of the treatment after E and the SE-inducing agent itself [Brandt, 2010]. Phenytoin post-treatment was shown to provide neuroprotective effects in rat models of SE, but did not affect the development of seizures [Cuhna, 2009]. Investigations utilizing the kindling model [Silver, 1991] have shown progression-retarding effects from treatment with valproate, carbamazepine, and phenobarbital.

However, alongside these results it should be noted that reducing the intensity or duration of the stimulation-induced electrographic seizure following drug pre-treatment may reduce the ability of the kindling stimulus to trigger proximal discharges sufficiently strong enough to drive the consequent epileptogenic process in the kindling model. Simply put, this notion suggests that an acute anticonvulsant effect present during repeated kindling stimulations may reduce or prevent the stimuli from triggering epileptogenic processes necessary for kindled-state development, and thus would appear to have a true epileptogenic effect when in fact the phenomena observed is the product of a weakened epileptogenic stimulus. Topiramate has consistently been shown to reduce cognitive impairment and cell death in rat SE models. These reports are supported by others showing that topiramate treatment improves recovery in rats following lateral fluid percussion TBI [Hoover, 2004]. Of particular interest in these cases is topiramate's ability to reduce glutamate release and potential excitotoxicty following TBI [Alves, 2003]. As of yet, neither phenytoin, phenobarbital, carbamazepine, valproate, nor magnesium have been thoroughly investigated in clinical trials aimed at preventing the development of post-traumatic epilepsy [Temkin, 2009].

**Table 3. Lack of antiepileptogenic effects with antiepileptic drug therapy.**

| AEDs | Model | Treatment | Outcome | Author |
|---|---|---|---|---|
| Carbamazepine | Pilocarpine-SE | 4 days, 1 hr after SE | ↓cell death | Cunha (2009) |
| | Kainate-SE | 8 weeks, 24hr Post SE | ↓SRS, cell death | Capella and Lemos, (2002) |
| | Electrical kindling | Prior to kindling stimulations | Not effective | Schmutz et al. (1988); Silver et al. (1991) |
| Phenytoin | Pilocarpine-SE | 4 days, 1hr after SE | ↓CA1, CA3, hilus degen. | Cunha (2009) |
| | Electrical kindling | Prior to kindling stimulations | Not effective | Turner et al. (1977); Ebert et al. (1997) |
| Phenobarbital | Li-Pilocarpine-SE | 2 weeks, 90min after SE | ↑Latency to SRS, ↓SRS Frq/Severity | Brandt et al (2010) |
| | Kainate SE | 40 or 97 days, 24hr after SE | Not effective | Bolanos (1998) |
| | Electrical kindling | Prior to kindling stimulations | ↑time to kindling | Turner et al. (1977); Silver et al. (1991) |
| Valproate | Pilocarpine-SE | Weeks after SE | Not effective | Brandt et al. (2006) |
| | Hippocampal Kindling | Prior to kindling stimulations | ↑time to kindling | Silver (1991) |
| Levetiracetam | Electrical kindling | Prior to kindling stimulations | ↑time to kindling | Löscher et al. (1998); Stratton et al. (2003) |
| | Electrical-SE | Weeks after SE | Not effective | |
| | Pilocarpine-SE | weeks after SE | Not effective | Brandt et al. (2007) |
| Topimirate | Electrical kindling | Prior to kindling stimulations | ↑time to kindling | Amano et al. (1998); Mazarati et al. (2007) |
| | Pilocarpine-SE | Weeks after SE | Not effective | Frisch et al. (2007) |
| Vigabatrin | Electrical kindling | Prior to kindling stimulations | ↑time to kindling | Shin et al. (1986) |
| Diazepam | Hippocampus kindling | Prior to kindling stimulations | ↑time to kindling | Schmutz et al. (1988) |

**I.10 Progesterone interruption of epileptogenesis**

Progesterone is an endogenous hormone that has shown powerful anticonvulsant action in clinical studies [(Bäckström et al., 1984; Herzog, 1995; 1999], and animal trials [Selye 1942; Craig, 1966; Kokate et al., 1999; Frye and Scalise, 2000; Reddy et al., 2004] Furthermore, men are more likely to develop epilepsy than women [Hauser et al., 1993; Christensen et al., 2005; McHugh and Delanty, 2008], and women with epilepsy become more susceptible to seizures in conjunction with reduction in progesterone levels around menstruation [Herzog et al., 1997; Reddy, 2009a; Wu et al., 2013; Carver et al., 2014]. These observations have made progesterone an appealing candidate for intervention in epileptogenesis. Kindling studies have shown that progesterone can acutely inhibit evoked seizures [Holmes and Weber, 1984; Mohammad et al., 1998; Lonsdale et al., 2003], as well as hinder the progression of epileptogenesis [Holmes and Weber, 1984; Edwards et al., 2001; Reddy et al, 2010]. Despite these successes, progesterone has not been extensively investigated in models of epileptogenesis.

While an NIH-sponsored clinical trial investigating progesterone as a treatment for epilepsy in women found no significant difference in progesterone and placebo groups, further analysis of the data showed a significantly greater success rate in women suffering from increased perimenstrual seizure severity [Herzog et al., 2012]. Previous studies have shown that progesterone supports the normal development of neurons, and that it reduces the extent of brain damage after TBI [Roof et al., 1994; Cutler et al., 2005; 2007]. It has been observed in animal models that females have reduced susceptibility to TBI and this protective effect has been hypothesized to be caused by increased circulating levels of progesterone in females [Roof and Hall, 2000; Meffre et al., 2007]. A number of additional studies have confirmed that progesterone has neuroprotective effects [Gibson et al., 2008]. Promising results have also been reported in human clinical trials. Recently, two clinical studies have evaluated progesterone as a treatment for moderate to severe TBI [Wright et al., 2007; Xiao et al., 2008]. These studies demonstrated the efficacy of progesterone as a neuroprotective agent in TBI.

Progesterone is highly efficacious in reducing disability and death in TBI. Progesterone has neuroprotective properties in acute models of ischemic injury, stroke, and astroglial dysfunction [Koenig et al., 1995; Jiang et al., 1996; He et al., 2004], further evidencing its beneficial effects after brain injury.

Progesterone also modulates inflammation, an appealing characteristic for potential therapies in a number of inflammation-associated neuronal disorders, including epilepsy [Vezzani, 2005]. Indeed, multiple preclinical trials investigating progesterone have demonstrated neuroprotection after neuronal injury [Roof et al., 1994; Koenig et al., 1995; Jiang et al., 1996; Cutler et al., 2007]. Progesterone exerts modulatory effects on multiple mechanisms underlying epileptogenesis. Progesterone treatments has been shown to reduce inflammatory signaling through the inhibition of secreted phospholase A2 enzyme, which stimulates pro-excitatory actions downstream [Yagami et al., 2002; DeCoster et al., 2002]. The cellular effects of progesterone are mediated through progesterone receptors (PRs), which are nuclear receptors found in the limbic system, neocortex, and hypothalamus [Brinton et al., 2008]. However, a number of studies utilizing rodent models of limbic epileptogenesis have highlighted an interesting dynamic regarding the nature of the anticonvulsant effects of progesterone treatment [Reddy et al., 2010; Reddy and Mohan, 2011; Reddy and Ramanathan, 2012]. Concurrent progesterone treatment significantly decreased the progression of stimulation-evoked seizures in mice undergoing hippocampal kindling [Reddy et al., 2010]. Subsequent studies in PRKO mice showed that progesterone treatment exerts an attenuating effect on the initial stages of kindling epileptogenesis without the presence of PRs. Late-stage kindling progression was not affected by progesterone treatment, suggesting that the role of PRs in kindling epileptogenesis may be multifaceted and temporally dictated [Reddy and Mohan, 2011].

Progesterone is rapidly metabolized into neurosteroids pregnanolone and allopregnanolone, which could mediate progesterone's attenuating effects on kindling epileptogenesis. This possibility is supported by emerging evidence that neurosteroids

can retard the development of spontaneous seizures in post-SE models of epileptogenesis [Biagini et al., 2006, 2009]. Further support for this hypothesis can be found from observing the effects of finasteride (a 5α-reductase and neurosteroid synthesis inhibitor that prevents the conversion of progesterone and other hormones to neurosteroid derivatives) treatment in the mouse hippocampal kindling model [Reddy and Ramanathan, 2012]. In adult mice, pre-treatment with finasteride led to complete inhibition of the progesterone-induced retardation of limbic epileptogenesis in mice [Reddy and Ramanathan, 2012]. These observations strongly suggest that progesterone derived neurosteroids likely provide a high degree of mediation to the antiepileptogenic properties observed in kindling studies. However, neurosteroids have been shown to modulate PR activity by intracellular conversion to progesterone receptor-binding products [Rupprecht et al., 1993], and as such the overall disease-modifying capacity of progesterone likely occurs through a combination of mechanisms in both PR-dependent and PR-independent signaling pathways.

## I.11 Neurosteroids as potential endogenous antiepileptogenic agents

Neurosteroids are produced from hormone precursors within the brain, and are able to exert unique and rapid modulation of neuronal excitability. A wide variety of neurosteroids is known to be produced within the brain from estrogen and androgen precursors [Baulieu, 1981; Kulkarni and Reddy, 1995]. Allopregnanolone (3α-hydroxy-5α-pregnane-20-one, AP), allotetrahydrodeoxycorticosterone (3α,21-dihydroxy-5α-pregnan-20-one; THDOC), and androstanediol (5α-androstane-3α,17β-diol) are produced via sequential A-ring reduction, and are under investigation for treatment of epilepsy and other neuronal disorders [Reddy, 2009a].

Neurosteroids induce changes in neuronal excitability through the binding of GABA-ARs (**Fig. 3**), which are pentameric chloride channels that interact with a varied number and type of allosteric modulators [Harrison et al., 1984; 1987; Majewska et al., 1986; Gee et al., 1988; Purdy et al., 1990; Hosie et al., 2007; 2009]. When activated, these

receptors permit an influx of chloride ions into the cell, hyperpolarizing the membrane, leading to reduced excitability on a cellular and systems level. The nature of interaction between allopregnanolone (and related neurosteroids) varies with concentration and receptor subunit type. At low concentrations, they act as positive allosteric modulators, potentiating chloride currents by increasing the duration and frequency of channel opening [Twyman and Macdonald, 1992; Lambert et al., 2009; Ramakrishnan and Hess, 2010]. At higher concentrations, they can directly activate the GABA-ARs [Harrison et al., 1987; Reddy and Rogawski, 2002; Carver and Reddy, 2016]. The inhibitory effects from GABA-AR activation are divided into two classes based on the nature and location of the receptors. Synaptic GABA-ARs generate phasic inhibition via swift activation by presynaptic GABA release. Extrasynaptic GABA-ARs generate tonic inhibition through continuous activation by low concentrations of ambient GABA. Synaptic and extrasynaptic GABA-ARs vary in subunit make up and physiological distribution. These subunit differences convey distinct pharmacological profiles to various subtypes of GABA-ARs. Of primary interest in epilepsy is the low-efficacy activity of extrasynaptic receptors in the dentate gyrus, as the tonic currents generated by these receptors play an important role in modulating excitability in the hippocampus.

The effect of neurosteroids on GABA-ARs occurs by binding to discrete sites on the receptor-channel complex that are located within the transmembrane domains of the α- and β-subunits [Hosie et al., 2006; 2007], which they access by lateral membrane diffusion [Chisari et al., 2009; 2010]. The binding sites for neurosteroids are distinct from the recognition sites for GABA, benzodiazepines, and barbiturates [Hosie et al., 2009]. Androgenic neurosteroids such as androstanediol may interact with these sites, and a recent study indicates that this agent is a positive allosteric modulator of GABA-ARs, but they are less potent and efficacious than progesterone-derived neurosteroids [Reddy and Jian, 2010; Carver and Reddy, 2016]. Although neurosteroids act on all GABA-AR isoforms, they have large effects on extrasynaptic δ-subunit containing GABA-ARs that mediate tonic currents [Wohlfarth et al., 2002; Belelli et al., 2002;

Carver et al., 2014]. This δ-specific potentiation by THDOC and related neurosteroids is selective for receptors with low-efficacy gating at high or low concentrations of GABA [Bianchi and Macdonald, 2003]. This means that even at receptor-saturating concentrations of GABA, neurosteroids can still significantly enhance inhibitory currents generated by δ-containing receptors. Not surprisingly, δ-containing receptors are extremely responsive to neurosteroid potentiation, and δ-subunit knock-out mice display greatly diminished sensitivity to neurosteroids [Mihalek et al., 1999; Spigelman et al., 2002].

Neurosteroids may play a role in chronic epilepsy. Neurosteroid modulation of tonic activation of extrasynaptic GABA-ARs can regulate excitability during epileptogenesis [208]. Given the complex plasticity in GABA-ARs in epilepsy, it is difficult to predict the functional outcome of altered subunit compositions. A consistent finding from studies that have used various models of chronic epilepsy is that tonic conductances are largely preserved in epileptic brain around the time when synaptic inhibition is reduced [Mtchedlishvili et al., 2001; Sun et al., 2007; Zhang et al., 2007]. Studies in a status epilepticus rodent model of TLE have shown a striking reduction in δ-subunit containing GABA-ARs in the dentate gyrus [Peng et al., 2004; Zhang et al., 2007], suggesting that neurosteroid effects on nonsynaptic GABA-ARs may be reduced. A reduced effect of neurosteroids on synaptic currents in dentate gyrus granule cells was observed post status epilepticus animals [Sun et al., 2007]. Reduced potency (but not efficacy) of inhibitory neurosteroids [Stoffel-Wagner et al., 2000; 2003; Biagini et al., 2009] has also been observed in post-SE animals. P450scc (cholesterol side-chain cleaving enzyme) is vital part of endogenous neurosteroid synthesis. Its expression may be linked with neurosteroid production, as it has been observed to be upregulated in the hippocampus of TLE patients and in animal models of TLE [Biagini et al., 2009]. Several observations support this notion. In the pilocarpine model, rats develop seizures after a latent period that mirrors that of P450scc elevation, and treatment with finasteride (a neurosteroid synthesis inhibitor) is capable of reducing latency-to-seizure expression after SE, as well as exacerbating seizure intensity [Biagini et al., 2006].
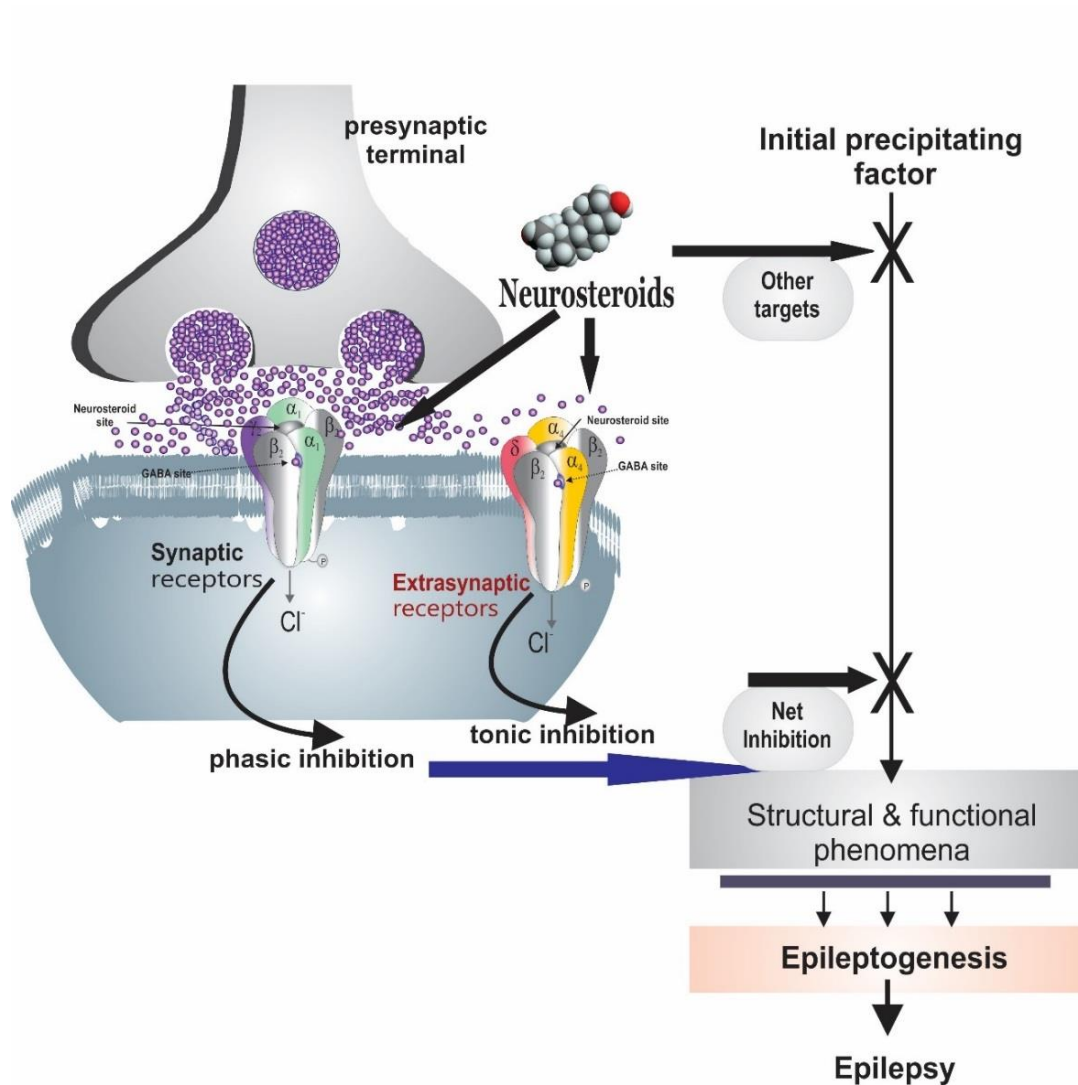
**Figure 3. Potential molecular mechanisms of neurosteroid interruption of epileptogenesis.** In the brain, allopregnanolone and related neurosteroids may retard epileptogenesis by the interruption of one or more of the pathways leading to development of epilepsy, which generally occurs following an initial precipitating event. Neurosteroids such as allopregnanolone binds to synaptic and extrasynaptic GABA-A receptors and enhances phasic and tonic inhibition within the brain. Synaptic GABA-A receptors, which are pentameric chloride channels composed of $2\alpha2\beta\gamma$ subunits, largely mediate the phasic portion of GABAergic inhibition, while extra-synaptic GABA-A receptors, pentamers composed of $2\alpha2\beta\delta$ subunits, primarily contribute to tonic inhibition in the hippocampus. Neurosteroids binds to the "neurosteroid site(s)" on GABA-A receptors, which are distinct from sites for GABA, benzodiazepines, and barbiturates. Neurosteroids activate both synaptic and extrasynaptic receptors, enhance the phasic and tonic inhibition, and promote maximal net inhibition, and thereby may affect epileptogenesis.

Neurosteroid administration has been shown to exert powerful anticonvulsant action in a number of animal seizure models [Reddy, 2010], and ongoing research has provided evidence of endogenous neurosteroids' potential role in modulating epileptogenesis [Edwards et al., 2001; Biagini et al., 2006; 2009a; 2010; Reddy et al., 2010]. Neurosteroids have been used to successfully inhibit seizures induced by PTZ, bicuculline, pilocarpine, and evoked seizures in kindling paradigms [Kokate et al., 1994; Belelli et al., 1989; Frye, 1995; Wieland et al., 1995; Reddy and Rogawski, 2001; 2010; Reddy et al., 2004; Kaminiski et al., 2004; 2005]. However, neurosteroids have the ability to potentiate generalized absence seizures in a manner similar to other GABAergic agents [Snead, 1998; Citraro et al., 2006].

Neurosteroids increase threshold to seizure in the 6-Hz model, a paradigm in which trans-corneal stimulation is used to evoke generalized limbic-like seizures [Kaminiski et al., 2004; Carver and Reddy, 2016]. Additionally, neurosteroids have been shown to be highly efficacious in inhibiting seizures caused by withdrawal from GABA-modulating drugs, as well as cocaine and others [Reddy and Rogawski, 2001; Tsuda et al., 1997; Devaud et al., 1996; Carver et al., 2014]. Unlike benzodiazepines, chronic treatment with neurosteroids has not been associated with anticonvulsant tolerance, and important aspect for viable therapeutics that suggests neurosteroid therapy may be especially suited for long-term treatment [Kokate et al., 1998; Reddy and Rogawski, 2000a]. The development of epilepsy is linked to complex alterations in neuroplastic mechanisms. Dysregulation of neurosteroid synthesis may also play a role. This premise is being tested in various epileptogenic models [Reddy and Mohan, 2011]. Treatment with finasteride resulted in a significant increase in epileptogenesis in the hippocampus-kindling model, thought to be due to a reduction in allopregnanolone and other neurosteroids [Ramanathan and Reddy, 2011]. Furthermore, progesterone treatment significantly decreased the rate of kindling epileptogenesis, and effect that was completely blocked by administration of finasteride [Reddy and Ramanathan, 2012]. Neurosteroid-mediated increase in tonic inhibition in the hippocampus could inhibit the spread of the seizure discharge from the hippocampal

focus and thereby suppress the rate of development of behavioral kindled seizure activity without affecting the focal electrographic discharges. Although the exact mechanisms are unclear, that data from these studies suggests that neurosteroid therapies present a number of unique opportunities for intervening in epileptogenesis.

## I.12 Epigenetic inhibition of epileptogenesis

The heterogeneous and often complex nature of epileptic conditions has remained a significant obstacle to our understanding of the true contribution of genetic and environmental factors in the initiation and development of disease state. While great progress has been made in linking specific genes or genetic conditions with various forms of epilepsy, there remains great difficulty in elucidating these roles in discrete epileptic conditions. Recently, studies aiming at understanding the complex nature of these interactions have highlighted specific epigenetic mechanisms (DNA methylation, micro-RNA signaling, and histone modifications) for involvement in epileptogenesis [Saugstad, 2015]. Methylation of DNA's cytosine residue is carried out by DNA methyltransferases (DNMT), resulting in a silencing effect on gene expression. Deregulation of DNA methylation has been observed in a number of neural disorders such as stroke, cancer, and epilepsy [Hwang et al., 2013]. Of particular interest to this report are the discrete epigenetic changes observed within the pilocarpine, kainate, and kindling models of epilepsy [Huang, 2002; Jia, 2006; Garriga-Canut, 2006].

In normal conditions, epigenetic modifications are essential for growth and development (e.g. X-chromosome inactivation in females), as control of gene expression is carried out primarily by gene silencing [Henshall and Kobow, 2015]. Investigations into epigenetic processes have revealed significant alterations to the epigenome of an epileptic brain [Graff et al., 2011; Sweatt, 2013]. This 'epigenome' encompasses the physical environment and changes in gene-supporting proteins such as histones- maintaining the three-dimensional structure of DNA [Graff et al., 2011]. These alterations can cause dramatic shifts in gene expression, potentially conferring dramatic effects on disease

state. Therefore, a variety of potential treatments have been proposed to target the various neurotrophic, anti-inflammatory, neuroprotective, and epigenetic pathways [Acharya et al., 2008; Pitkanen and Lukasiuk, 2011; Ravizza et al., 2011]. However, the translational potential of treatments based on epigenetic pathways is only recently being openly explored in epilepsy and epileptogenic models.

miRNAs represent a class of 19-24 nucleotide sequence of noncoding RNAs that regulate gene expression by targeting messenger RNAs for cleavage or translational repression by binding complementary bases of the target messenger RNA in the 3' region. More than 2500 miRNAs have been identified in humans, each holding some degree of influence on gene transcription at multiple messenger RNAs. miRNAs regulate a range of important steps in gene expression including RNA transcription, translation, and expression of key epigenetic enzymes including the aforementioned DNMTs and HDACs [Sato et al., 2011]. Indeed, a number of groups have identified pro-inflammatory and/or pro-epileptogenic miRNAs, such as miR-155 [Lee et al, 2014] and miR-134 [Gaughwin, 2011]. Emerging evidence on these and other miRNAs point to a potential role in mediating epileptogenesis [Selvamani et al., 2012; Yin et al., 2014; Bake et al., 2014], with some studies demonstrating long-lasting pro-excitatory effects from even transient miRNA blockade, as Lippi et al. did with miR-101 [2016]. Antagomirs, mi-RNA-silencing oligonucleotides, represent a potentially viable route for disease-intervention or modulation, as they have the capability to potently inhibit specific miRNAs. This technique might be utilized for reduction of pro-inflammatory or epileptogenic signaling. Application of antagomirs targeting miR-134 has been shown to protect against pilocarpine-induced status epilepticus [Jimenez-Mateos et al., 2012]. Additionally, selective silencing of miR-134 after SE reduces the extent of spontaneous seizure expression and neuronal death in mice [Jimenez-Mateos et al., 2014]. Previous work from the same group also demonstrated that reducing hippocampal levels of miR-132 reduces the extent of seizure-induced degeneration of neurons [Jimenez-Mateos et al., 2011]. Hu et al. (2012) observed neuroprotective effects after targeting miR-34a (previously observed to be upregulated) following SE in rats [Hu et al., 2011; 2012]. It is

important to note that not all miRNAs upregulated during epileptogenesis are pro-epileptogenic. Indeed, miR-146a expression is increased in rats following SE, however, it was found that silencing miR-146a promotes an increased inflammatory response associated with epileptiform activity in neurons [Gorter et al., 2012; Iyer et al. 2014]. Still other miRNA's have been observed to be downregulated during epileptogenesis, of note are miR-128 and miR-137, which are associated with regulation of cell proliferation and migration, respectively [Tan et al., 2013; Risbud et al., 2013].

The complex nature of these antagomir-mi-RNA interactions is still being explored, but preliminary interventions with specific antagomirs have provided evidence for protection against excitotoxicity in animal models [Jimenez-Mateos et al., 2012; 2014]. Furthermore, inhibiting methylation of miRNA transcription sites can inhibit transcription of several miRNAs [Bhadra et al., 2013]. miRNA profile studies of resected human epileptic brain tissue observed localization of a discrete group of miRNAs in the nucleus of these neurons [Kan et al., 2012]. Based on these reports, miRNAs may serve as a potential target for antiepileptogenic therapies, with the potential of serving as biomarkers for neuronal populations undergoing epileptogenesis prior to the onset of seizures. Moreover, identifying the direct epigenetic functions of miRNAs that are upregulated in epileptic brain samples will lead to a better understanding of the complex mechanisms that underlie epileptogenesis.

**Table 4. miRNAs identified in human tissue & experimental models of epileptogenesis.**

| miRNA | Change in Regulation | Model | Author |
|---|---|---|---|
| miR-34a | Upregulated | Pilocarpine SE - Rats | Hu et al., 2011; 2012 |
| miR-132 | Upregulated | Pilocarpine SE - Mice | Jimenez-Mateos et al., 2011 |
| miR-134 | Upregulated | Pilocarpine SE – Mice Pilocarpine SE - Rats | Jimenez-Mateos et al., 2012;2014; Song et al., 2011 |
| miR-184 | Upregulated | Resected Human Tissue | McKiernan et al., 2012 |
| miR-146a | Upregulated | Electric Stimulation SE | Gorter et al., 2014 |
| miR-155 | Upregulated | Pilocarpine SE - Rats | Asshab et al., 2013 |
| miR-128 | Downregulated | Modulation of Inhibition, Mice | Tan et al., 2013 |
| miR-137 | Downregulated | Pilocarpine SE - Rats | Risbud and Porter, 2013 |

DNA contained in a human nucleosome is tightly coiled around a histone octamer that is composed of H2A, H2B, H3, and H4 histone pairs. Epigenetic marks are added to, and removed from these sites through the complex action of enzymes such as HDACs and HATs. HDACs play a crucial role in repressing gene transcription by condensing chromatin structure. This is achieved through subsequent removal of acetyl groups from the lysine residue of core histone proteins. HDACs can also remove acetyl moieties from

transcription factors, thereby further suppressing gene activity [Morris et al., 2010]. There are four classes of HDAC enzymes within the HDAC superfamily (I, II, II, IV), with classes I and II constituting the majority of HDAC in the brain [Gray and Ekstrom, 2001]. In diseases associated with dysregulation of histone modification, HDAC inhibitors can play an important regulatory role in disease state and progression. Of note, HDAC inhibitors exert significant influence on the cell-cycle and proliferation of tumor cells, slowing aberrant growth and inducing apoptosis. These tumor-suppressing properties of HDAC inhibitors have contributed to their use as anti-cancer and neuro-protective agents.

It has been reported that the expression of HDAC II is up-regulated following SE in mice [Jagirdar et al., 2015]. In contrast, mice with a genetic knockout of the HDAC II gene show enhanced cognitive activity and synaptic plasticity [Guan et al., 2009]. Therefore, epigenetic therapies that include HDAC enzyme inhibitors such as sodium butyrate, trichostatin A, and valproic acid (VPA) all have the potential to alter gene expression profiles in epileptic disorders, providing a novel route for disease modification, and potentially- reversal of epileptogenic process [Mehler, 2010]. A promising HDAC inhibitor, sodium butyrate, increases acetylation of histones H3 and H4 in the hippocampus and cerebral cortex of mice. Sodium butyrate has also been shown to modulate the effects of the AEDs dizocilpine and flurazepam to antagonize electrically precipitated seizures [Deutsch et al., 2009].

VPA is a well-established AED, which displays increasing anticonvulsant effects over time [Gottlicher et al. 2001]. One of several mechanisms of action, VPA displays HDAC-inhibiting activity, which as expected, exerts a suppressive effect on gene transcription [Henshall and Kobow, 2015] multiple aspects of the epigenome, promoting increased H3 acetylation in the brain [Eleuteri et al. 2009], while also facilitating the direct or indirect demethylation of DNA [Dong et al. 2007).]. This ability to exert multiple effects on the epigenome likely further contributes to VPA's effectiveness as an anti-epileptic agent. Long-term cognitive impairment linked to abnormal dentate gyrus

neural progenitor cell propagation, which occurs because of kainic-induced seizures, can be blocked by treatment with VPA. This ability to alter histone deacetylase-dependent gene expression in the DG lends protection from seizure induced cognitive impairments [Jessberger et al 2007]. Studies investigating the HDAC inhibitor TSA have found that preventing deacetylation has a neuroprotective effect [Huang et al., 2002], although the underlying mechanisms may be due in part to calcium channels modulation and restriction of calcium permeability.

A group of enzymes called DNMTs, which are essential for normal CNS function, accomplishes DNA methylation. There are three active subfamilies of DNMT enzymes (DNMT1, DNMT3a, and DNMT3b). The latter two enzymes are responsible for methylation of new sites of the genome, while the role of DNMT1 is to maintain DNA methylation tags [Song et al., 2012]. Hypermethylation of certain extracellular matrix proteins such as Reelin is directly implicated in the pathophysiology of temporal lobe epilepsy [Kobow et al., 2009]. Reelin is critical for normal structural dynamics, including dendritogenesis, synaptogenesis, and synapse maturation, in maintaining the correct laminar structure of granule cells in the DG [Herz and Chen, 2006]. Hypermethylation causes low expression of Reelin in the DG and leads to the anatomical epileptic hallmark of altered granule cells, due to deficient Reelin activity [Heinrich et al., 2006]. DNA methylation has also been observed to play critical role in advancing the progression of epileptogenesis [Kobow and Blumcke, 2012]. At the onset of seizure activity, increased levels of epigenetic modifications take place, which are associated with increased seizure activity and contribute to development of the epileptogenic condition [Kobow and Blumcke, 2012]. The increased activity of DNMT enzymes and the subsequent hypermethylation is collectively referred to as the 'methylation hypothesis of epileptogenesis' [Kobow and Blumcke, 2012]. Therefore, developing novel drugs that allow for specific inhibition of these methylating pathways may lead to breakthroughs in retarding the progression of epileptogenesis.

Up-regulation of DNMT activity is well reported in many patients with temporal lobe epilepsy [Kobow and Blumcke, 2012; Zhu et al., 2012]. In vitro studies suggest that DNMT inhibitors such as zebularine result in decreased spontaneous neuro-excitatory transmission in primary hippocampal neurons [Levenson et al., 2006]. The underlying mechanism may influence the genes that are associated with neuronal hyperactivity through DNA methylation. Furthermore, the analysis and comparison of chronic epileptic animals and healthy controls demonstrated that genomic-wide changes in DNA methylation were present following status epilepticus [Kobow et al, 2013]. Specifically, in the pilocarpine-induced rat model of chronic epilepsy, a significant up-regulation of DNA methylation was observed [Kobow et al., 2013]. DNMT inhibitors developed to date function by preventing methylation of neighboring sites on DNA in the brain [Kelly et al., 2010]. In development of DNMT inhibitors that can penetrate the blood-brain barrier, it is vital to consider toxicity, potency, and specificity. In this regard, second generation DNMT inhibitor may provide a preferable route to prevent epilepsy and neurodegeneration, by inhibiting the translation of messenger RNAs that encode for DNMTs, rather than blocking methylation action directly [Hwang et al., 2013].

# CHAPTER II
# AIMS AND OBJECTIVES

The main objective of this dissertation research proposal is to understand the role of endogenous neurosteroids and their synthetic analogs in limbic epileptogenesis using a combination of pharmacological, behavioral, and morphological techniques. We propose to utilize two distinct models involving electrical kindling and chemoconvulsant-induced mouse models of epileptogenesis, utilizing transgenic mouse strains such as GABA δ-subunit knockout mice (δKO). The global δKO model is widely used to study the neurophysiological basis for tonic currents, and our pilot studies suggest δKO mice are prone to faster kindling epileptogenesis, increased seizure susceptibility, and altered sensitivity to antiepileptic drugs (AEDs). The research is organized into three specific aims.

## II.1 Specific Aim 1

The first specific aim investigates role of δ-subunit extrasynaptic GABA-A receptors in a perimenstrual neurosteroid-withdrawal model of catamenial epilepsy.

Neurosteroids play a key role in the pathophysiology of catamenial epilepsy, a menstrual-cycle related disorder characterized by seizures that cluster most often during the perimenstrual or periovulatory period, when progesterone levels are low (Herzog and Frye, 2003; Herzog et al., 2004; 2011; Reddy et al., 2012). Presently there is no approved drug therapy for catamential epilepsy. Progesterone is a precursor for the synthesis of neurosteroids such as allopregnanolone in the brain (Reddy et al., 2004; Tuveri et al., 2008). AP and related neurosteroids have anticonvulsant properties and protect against seizures. Although the exact cause of catamenial epilepsy is poorly understood, there is growing evidence suggesting perimenstrual neurosteroid withdrawal (NSW) may be a key triggering factor for catamenial seizures (Smith et al., 1998ab; Reddy et al., 2001, 2012; Reddy, 2009ab; Gangisetty and Reddy, 2010; Pack et al.,

2011). We previously developed an animal model of catamenial epilepsy (Reddy et al., 2001; 2012; Reddy and Zeng, 2007). In this rodent model, neurosteroids including AP and synthetic analogs such as ganaxolone have enhanced activity in the catamenial epilepsy model (Reddy and Rogawski, 2000; 2001; Reddy et al., 2012). Neurosteroid replacement therapy for prevention of catamenial seizures has been previously proposed (Reddy and Rogawski, 2009; Reddy, 2013). However, the molecular mechanisms underlying enhanced anticonvulsant activity of neurosteroids in catamenial epilepsy remain unclear. There is indication that steroid hormone fluctuations affect $\delta$-subunit plasticity (Smith and Gong, 2005; Reddy et al., 2012). Additonally, there is evidence of reduced sensitivity to GABA-A receptor modulating neurosteroids in mice lacking $\delta$GABA-A receptors. Therefore, we hypothesized that the enhanced potency of neurosteroids in catamenial epilepsy may be due to a relative increase in the expression of extrasynaptic, $\delta$GABA-A receptors in the hippocampus, and that this effect will be diminished in global $\delta$KO mice.

The main objective of this aim is to investigate the system-level impact of $\delta$-subunit GABA-A receptors on susceptibility to catamenial-like seizures using a mouse model of neurosteroid withdrawal. Fully-kindled $\delta$KO and WT mice will be given sequential gonadotropin treatments to increase circulating levels progesterone, as well as brain levels of progesterone-derived steroids. This treatment is followed by finasteride, a $5\alpha$-reductase inhibitor which prevents the conversion of progesterone to allopregnanolone, without affecting circulating hormone levels (Reddy and Gould, 2012). Catamenial epilepsy is a result of perimenstrual decreases to circulating and brain neurosteroid levels (Reddy, 2009; Herzog et al., 2011). Progesterone has protective, anticonvulsant effects, as it is converted into neurosteroids such as allopregnanolone (Reddy et al., 2004). The $\delta$-subunit plasticity in the hippocampus is influenced by epileptiform activity, stress, the menstrual cycle, pregnancy, and parturition (Glykys and Mody, 2007, Maguire and Mody, 2007, 2008; Sanna et al., 2009; Zhang et al., 2007). Large subunit plasticity occurs in response to fluctuations of progesterone and neurosteroids during the

ovarian cycle (Reddy et al., 2001; Maguire et al., 2005; Tuveri et al., 2008; Reddy, 2009). These neuroendocrine changes have been proposed to modulate levels of seizure susceptibility. Exacerbation of seizures has been observed in women upon inhibition of progesterone metabolism with finasteride (Herzog and Frye, 2003). Neurosteroid withdrawal alters GABA-A receptor plasticity and increases seizure susceptibility (Reddy et al., 2012). Neurosteroid withdrawal plays a key role in seizure exacerbations in women with catamenial epilepsy and related conditions. Therefore, observations from viable neurosteroid withdrawal paradigms may play a critical role in advancing therapies for clinical patients.

**II.2 Specific Aim 2**

The second specific aim investigates role of δ-subunit extrasynaptic GABA-A receptors in susceptibility to limbic epileptogenesis in mouse in wildtype condition as well as germline deletion of δ-subunit-containing $GABA_A$ receptors.

To determine the role for δ-subunit extrasynaptic GABA-A receptors in limbic epileptogenesis we utilize two models: (a) hippocampus kindling model of epileptogenesis; and (b) pilocarpine-induced post-SE model of epileptogenesis. Behavioral evidences in whole animal models can aid in confirming the greater role of hippocampal network inhibition effects on the brain. To determine the alterations in susceptibility of δKO mice, they will be subjected to kindling or pilocarpine epileptogenesis. Finally, we will utilize morphological approaches to verify the seizure-induced changes in hippocampal neuronal injury and mossy fiber sprouting.

The main objective of this aim to determine the role for δ-subunit extrasynaptic GABA-A receptors in limbic epileptogenesis. To measure susceptibility to epileptogenesis, we will utilize two mouse models: (a) hippocampus kindling model of epileptogenesis; and (b) pilocarpine-induced post-SE model of epileptogenesis. Behavioral evidences in whole animal models will aid in confirming the greater role of hippocampal network

inhibition effects on the brain. To determine the alterations in susceptibility of δKO mice, they will be subjected to kindling or pilocarpine epileptogenesis. Finally, we will utilize morphological approaches to verify the seizure-induced changes in hippocampal neuronal injury and mossy fiber sprouting.

**II.3 Specific Aim 3**

The objective of the third specific aim is to determine the role of δ-subunit extrasynaptic GABA-A receptors in the antiepileptogenic actions of neurosteroids in mouse models in wildtype condition and with germline deletion of δ-containing GABA-A receptors.

Currently, there are no specific drugs for preventing or curing epilepsy. The mechanisms underlying the development of acquired epilepsy are not well understood. The term "epileptogenesis" is used to describe the complex plastic changes in the brain that, after a precipitating event, convert a normal brain into a brain debilitated by recurrent seizures (Pitkänen et al., 2009). Limbic epilepsy is caused by diverse cascading factors such as brain injury, stroke, infections, or prolonged seizures. The kindling model has provided a conceptual framework for the idea that "seizures beget seizures" and for developing new molecular targets for preventing epilepsy (Goddard et al., 1969; McNamara et al., 1992). The fully kindled state in animals allows studying the persistence of epilepsy weeks or months after development. In addition, therapeutic interventions can be used to understand disease-modifying criteria necessary for impeding, preventing, or protecting against seizures. Using a combination of behavioral and pharmacological studies, we explored the progression of kindling epileptogenesis and developmental defects that may alter brain function within the germline δ-subunit knockout mouse model. We hypothesized that mice with a targeted, germline deletion of δ-subunit in the brain exhibit a markedly increased propensity for the development and persistence of kindling epileptogenesis. We provide a rationale for seeking an improved trangenic mouse model under conditional deletion of δ-subunit for further investigation of the functional role of δ-subunit expresssion in the hippocampus.

The main objective of this aim is to determine the functional role for δ-subunit extrasynaptic GABA-A receptor signaling in the protective effects of neurosteroids in stopping or slowing the progression of epileptogenesis. We will utilize two mouse models to substantiate the disease-modifying effects of neurosteroids in epileptogenesis: (a) hippocampus kindling model of epileptogenesis; and (b) pilocarpine-induced post-SE model of epileptogenesis. The modulatory and anticonvulsant effects of allopregnanolone and ganaxolone, prototypical neurosteroids, will be evaluated in the context of epileptogenesis and δ-specific knockout using δKO mice. In this study, we will investigate the novel role of neurosteroids in epileptogenesis using different approaches for manipulating neurosteroid levels in the brain: (i) gonadotropin-induced elevations in neurosteroids; (ii) exogenous administration of allopregnanolone; and (iii) administration of the synthetic neurosteroid ganaxolone. Together, we hope to generate robust evidence of the involvement of δ-subunit extrasynaptic GABA-A receptors in the protective effects of neurosteroids and their synthetic analogs.

# CHAPTER III
# MATERIALS AND METHODS

## III.1 Experimental animals

Wildtype (WT) adult female C57BL/6 mice, 25 to 30g each were used in this study. GABA$_A$ receptor δ-subunit knockout mice (*Gabrd*$^{-/-}$, δKO) were also used (Mihalek et al., 1999). All strains were maintained on a hybrid C57BL/6-129SV background. All mice were housed four to a cage with access to food and water *ad libitum*. The mice were housed in an environmentally controlled animal facility with a 12 h light/dark cycle. The animals were cared for in strict compliance with the guidelines outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Animal procedures were performed in a protocol approved by the university's Institutional Animal Care and Use Committee.

## III.2 Perimenstrual model of neurosteroid withdrawal

A state of perimenstrual-like neurosteroid withdrawal (NSW) hormonal condition was induced in animals by a standard progesterone-finasteride regimen as described previously (Gangisetty and Reddy, 2010), which was based on published protocols for induction of NSW (Smith et al., 1998ab; Moran et al., 1998; Moran and Smith, 1998). The overall experimental paradigm is illustrated in **Fig.4.** Adult female mice were given subcutaneous injection of progesterone (25 mg/kg) twice-daily for seven days. On the final injection, finasteride (50 mg/kg, i.p.) was administered to block 5α-reductase activity for inhibiting progesterone conversion to AP and related neurosteroids. Progesterone was administered rather than allopregnanolone because circulating levels of progesterone, such as those found during the luteal phase, are readily converted to neurosteroids in the brain regions that express neurosteroid synthesizing enzymes (Mellon et al., 2001; Agís-Balboa et al., 2006). The progesterone administration protocol results in a high physiological concentration of allopregnanolone in plasma, and acute

withdrawal is evident by a nearly complete decline in allopregnanolone 24 hours after finasteride administration (Gangisetty and Reddy, 2010). Control mice were administered 15% β-cyclodextrin vehicle with the same frequency for the seven day injection period. Experimental studies were carried out 24 hours following the final finasteride or vehicle injection.

(A)



(B)
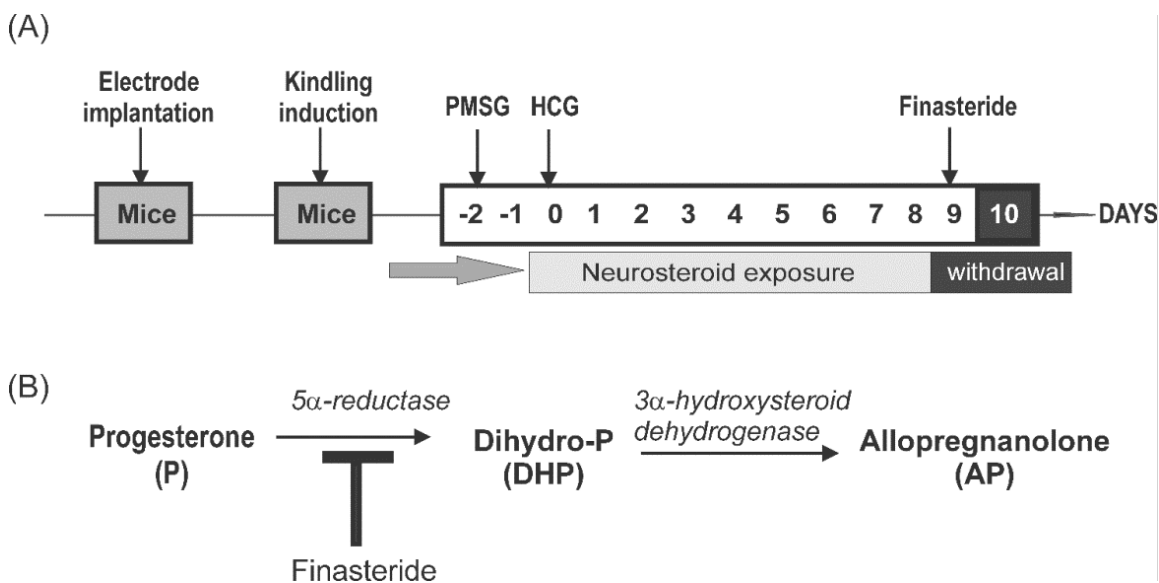


Fig. 4.  **Experimental paradigm of mouse catamenial epilepsy model.**  A: Gonadotropin treatment regimen in mice for creating a perimenstrual-like state of elevated neurosteroids followed by neurosteroid withdrawal (Reddy et al., 2012). **B:** Abrupt inhibition of the neurosteroid allopregnanolone synthesis was accomplished by pharmacological blockade of 5α-reductase activity with finasteride to produce neurosteroid withdrawal (NSW) as reported previously (Reddy et al., 2012).

## III.3 Hippocampus kindling

To determine the effect of neurosteroid modulation and role of extrasynaptic δGABA-A receptors on limbic epileptogenesis, we utilized the hippocampus kindling model of temporal lobe epilepsy (Goddard et al., 1969). Hippocampus kindling models complex partial seizures through a protocol involving repeated subconvulsant stimuli that evoke afterdischarges (ADs) observed via hippocampal recording electrode. These stimulations lead to progressively greater AD's and the development of generalized behavioral seizures which increase in intensity until reaching the fully kindled state- a relatively

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stable condition in which stimuli result in intense electrographic and stage 5 behavioral seizures. Once a fully kindled state is reached, stimuli that were originally subconvulsive will continue to elicit prolonged ADs and behavioral seizures even with a month or more between stimulations (Reddy and Mohan, 2011). Electrode implantation and stimulation procedures for mouse hippocampus kindling were performed as described previously (Gangisetty and Reddy, 2010).

Briefly, anesthetized mice (ketamine-100 mg/kg and xylazine-10 mg/kg) were stereotaxically implanted with a twisted bipolar stainless steel electrode (model MS303/1; Plastic One, Roanoke, VA) in the right hippocampus (2.9 mm posterior, 3.0 mm lateral, and 3.0 mm below dura) (Franklin and Paxinos, 1997), which was secured to the skull using small anchor screws and dental acryllic. After 7-14 days of recovery mice were tested to determine the afterdischarge stimulation threshold. This was carried out by stimulating at 15 min intervals beginning with an intensity of 25 $\mu$A, and increasing in increments of 25 $\mu$A until an afterdischarge of at least 5s was obtained. ADT determination can be carried out any time prior to the beginning of kindling, and is not considered as a kindling stimulation for analysis purposes. For kindling stimulations, intensity was set to 125% of the threshold value for each animal. Behavioral seizures elicited by stimulation were rated according to the Racine (1972) seizure scale, modified for mice: stage 0, no seizures or minor behavior arrest; stage 1, chewing, facial twitches; stage 2, chewing and head nodding; stage 3, forelimb clonus; stage 4, bilateral forelimb clonus and/or rearing; stage 5, falling, jumping, running, full clonus. Kindling stimulations were continued daily within the same 4-hour window (10a-2p) each day, until animals reached fully kindled state indicated by the expression of stage 5 seizures on three consecutive days. Seizure duration was recorded by a trained observer as the total duration of behavioral seizure expression starting from the first signs of post-stimulus freezing and/or twitching through the cessation of convulsions and/or return to normal activity. Afterdischarge duration was recorded as the total duration of hippocampus electrographic spike activity (amplitude $> 2 \times$ baseline) occurring in a rhythmic pattern at a frequency $> 1$Hz.

### III.4 Drug treatment in kindling studies

The overall experimental protocol for drug treatment and kindling stimulations is illustrated in **Fig.5**. In the kindling characterization study, no drug treatments were given. For gonadotropin-induced sustained elevation of neurosteroid level studies, a previously validated (Reddy et al., 2012) sequential injection protocol was followed whereby animals received pregnant mare's serum gonadotropin (PMSG; 5 IU s.c.) at 3:00 PM 2 days prior to the beginning of kindling, followed 46 h later by human chorionic gonadotropin (HCG; 5 IU s.c.) at 1:00 PM. Sequential gonadotropin treatment produces robust increases in circulating levels of progesterone's neurosteroid derivative allopregnanolone by 24 hours after the second injection. These elevated levels persist through day 10, and no further gonadotropin treatments were given during the kindling process. For studies utilizing allopregnanolone (AP; 0.5 mg/kg s.c., Steraloids Inc., Newport, RI) and ganaxolone (GX, 0.5/1.0/3.0 mg/kg s.c.,Steraloids Inc., Newport, RI), injections were given daily 15 minutes prior to kindling stimulation, from the first stimulation until mice had reached fully kindled state. In the 3.0 mg/kg GX group, kindling was stopped after 40 stimulations (~3 times normal kindling duration), with no animals exhibiting kindling progression beyond Racine level 2 seizures. In all studies using finasteride (F, 50 mg/kg i.p., Steraloids Inc, Newport, RI), finasteride injections were given daily 1 hour prior to kindling stimulations. Stock solutions of AP, GX, and F were made in 15% β-cyclodextrin (Captisol, Cydex RC-0C7-100, Lawrence, Kansas) solution. Gonadotropins (Sigma, St. Louis) were dissolved in saline. Control animals received vehicle injections. Prior studies from our group have shown that β-Cyclodextrin alone, even at much higher concentrations than used here, failed to affect kindled seizures (Reddy, 2010). Kindling stimulations were continued daily within the same 4-hour window (10a-2p) each day, until animals reached fully kindled state indicated by the expression of stage 5 seizures on three consecutive days.
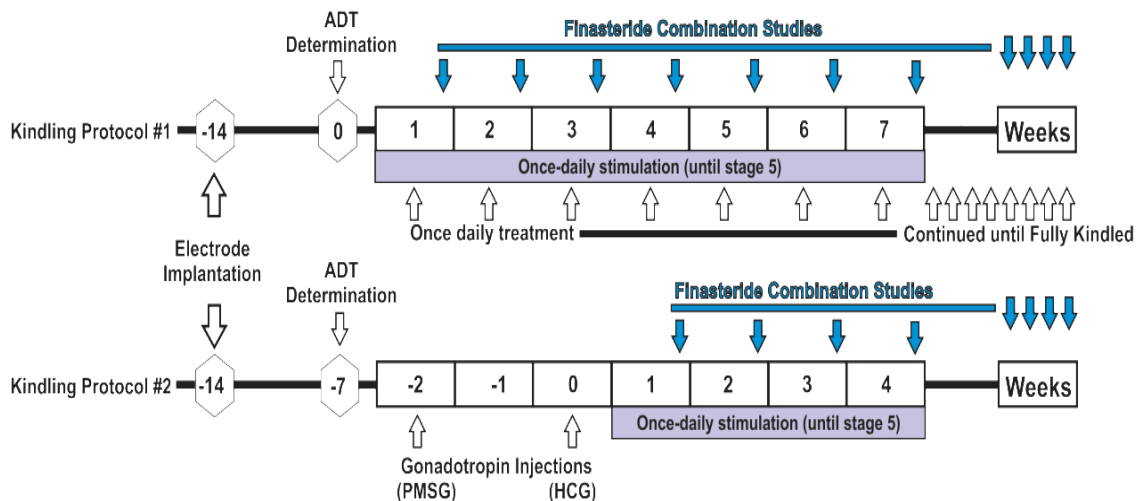
**Figure 5. Experimental protocol for testing interventions in hippocampal kindling.** The diagram illustrates variations of the standard hippocampal kindling protocol utilized for testing the effects of various treatments conditions on the development of hipopcampal kindling, a model of temporal lobe eipleptogenesis. Following electrode implantation and recovery, mice received either daily injections or sequential gonadotropin treatment. Combination treatments followed the dosing schedule outlined above. All studies were carried out until mice reach fully-kindled state.


## III.5 Estimation of neurosteroid levels

Mice were anesthetized with isoflurane, and 0.5 ml carotid blood was collected in heparinized tubes. The plasma was separated by centrifugation at 12,000g for 10 min and stored at −20°C. The concentration of allopregnanolone was quantified by liquid chromatography-mass spectrometry as described previously (Reddy et al., 2004). Briefly, a 200 µl plasma sample was added to a tube containing evaporated internal standard. The steroid and internal standard were extracted with 4 ml of hexane. Each sample was analyzed using the atmospheric pressure chemical ionization technique under acidic conditions. The detection limit of the assay was <5 ng/ml.

**III.6 Pilocarpine and wireless telemetry implantation**

Young adult male C57BL/6 mice, 60-75 d of age, were used in the present studies. GABA$_A$ receptor δ-subunit knockout mice (*Gabrd*$^{-/-}$, δKO) were also used (Mihalek et al., 1999). All animal use protocols conformed to National Institutes of Health guidelines and were approved by the Texas A&M University, College Station, Institutional Animal Care and Use Committee. Two weeks prior to pilocarpine (one week prior to surgery), mice were given ~0.5g Nutrical (Vetoquinol) diet supplement daily in addition to standard pellets to encourage weight gain prior to implantation and habituate the animals to the more palatable and calorically dense food for post-SE recovery. One week prior to the administration of pilocarpine-SE protocol, animals were implanted with DSI TA10-ETA-F20 (Data Sciences Incorporated) wireless telemetry devices. Briefly, mice were anesthetized with ketamine/xylazine (100 & 10 mg/kg, i.p.), and an incision was made down the midline from the coronal suture to the nape of the neck. Devices were implanted in a subcutaneous pocket prepared in the left or right flank. Leads were placed at the surface of the brain, just below the skull at +1mm/-1mm and +3mm/-2.9mm Bregma, and secured with dental acrylic. The incision was closed with wound clips and animals were allowed to recover for one week. Following recovery from surgery, status epilepticus was induced by the administration of pilocarpine, as described previously (Peng et al., 2004). Briefly, mice were treated with the cholinergic antagonist methylscopolamine nitrate (1 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO) to minimize the peripheral cholinergic effects of pilocarpine hydrchloride. 30 minutes after administrtion of methylscopolamine, mice received an injection of pilocarpine hydrochloride (300 mg/kg, i.p.; Sigma-Aldrich) to induce status epilepticus. SE was verified and monitored via EEG and was terminated with diazepam (DZ) (10 mg/kg, i.p.; Abbott Laboratories, Chicago, IL) 1.5 h after the onset. A follow-up dose of DZ (5 mg/kg, i.p.) was given following the initial dose if termination of SE was not verified from EEG. Following SE pilocarpine-treated mice were continuously EEG and video monitored using DSI Acquisition software for the occurrence of spontaneous seizures up

to 90 days after SE. Drug injections for post-SE intervention studies were carried out daily or twice-daily, adherring to a specific schedule for consistent dosing throughout the intervention period.
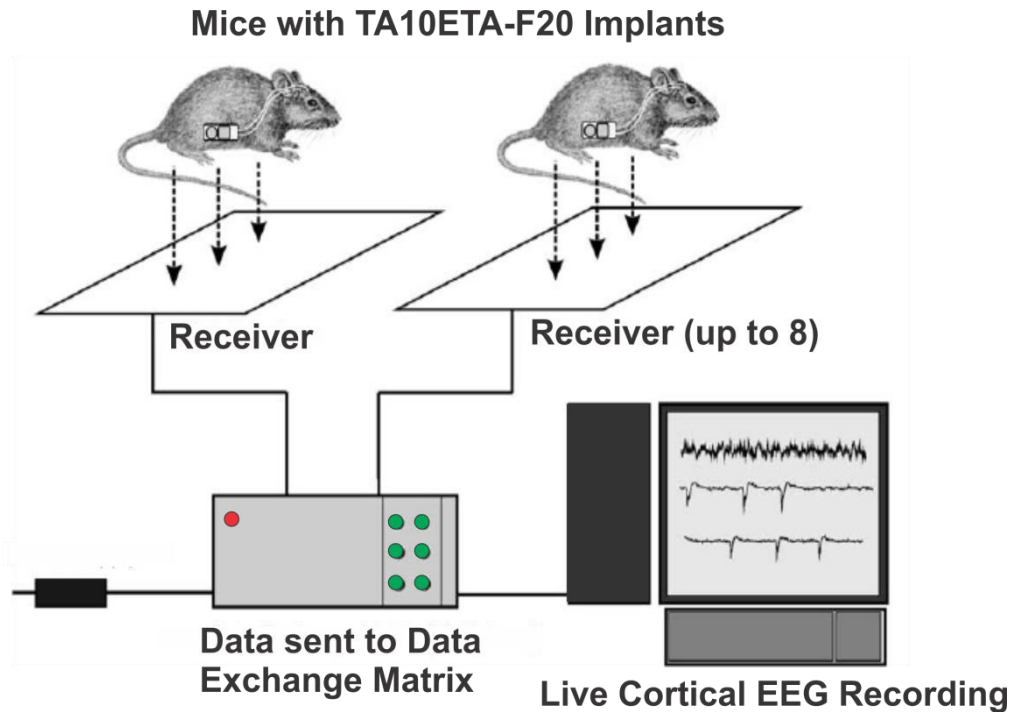


**Fig.6.  A schematic illustration of experimental protocol for wireless EEG recording in mice.**

### III.7 Histology and neuropathology studies

Adult mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Transcardial perfusion of saline followed by 4% paraformaldehyde in sodium phosphate buffer was completed to preserve the rodent brain. Following cryoprotection of brain, the tissue was rapidly frozen in isopentane pre-cooled to -70˚C. The frozen tissue was then cut in 30 μm coronal sections on a cryostat and mounted on gelatin-coated slides. Sections were processed for distribution of GABA-A receptor δ-subunit using a specific Gabrd primary antibody (1:250) (Millipore, Billerica, MA), according to the avidin-biotin-complex method (Hsu et al., 1981). Nissl stained sections of WT and δKO were utilized to study the hippoacmpus morphology.

## III.8 Immunohistochemistry of brain sections

Following is the brief description of the experiments that we carried out for immunohistochemistry with mouse brain sections. Mouse brains were cryoprotected with 0.1 M phosphate buffer (PB, pH 7.4) containing 20% sucrose for 72 hours and rapidly frozen in isopentane pre-cooled to -70ºC with dry ice. All brains were stored in a freezer at -80°C before sectioning. Serial cryostat sections (30 μm) were cut coronally through the cerebrum containing the amygdale and the hippocampus, approximately from bregma -0.58 mm to bregma -4.16 mm (cf. the Mouse Brain in Stereotaxic Coordinates by Paxinos & Franklin, 1997). Every 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th, 10th sections of each series of 10 sections (interval: 300 μm) were collected separately (approximately 12 sections per set per brain). All sections were stored free-floating in FD sections storage solution™ at -20ºC before processing. The sections were mounted on 1"x3" Superfrost Plus microscope slides (3 sections per slide, 12 sections per brain) and stained with FD cresyl violet solution. Sections of the 2nd, 3rd, 4th, 5th, 6th, and 7th sets were processed for NeuN(+)- and PV(+)-immunostaining with specific monoclonal mouse anti-NeuN antibodies (Cat# MAB377, 1:1000; Millipore, Temecula, CA, USA) or mouse anti-parvalbumin (PV, Cat# P3088, 1:2000 in PBS; Sigma-Aldrich, St. Louis, MO, USA). Briefly, after inactivating the endogenous peroxidase activity with hydrogen peroxidase and washes in 0.01 M phosphate-buffered saline (PBS), sections were incubated free-floating in PBS containing the normal blocking serum, Triton X-100, and the specific antibody for 65 hours at 4°C. Subsequently, the immunoreaction products were visualized according to the avidin-biotin complex method of Hsu et al. using the Vectastin elite ABC kit (Vector Lab., Burlingame, CA) and 3', 3'-diaminobenzidine (Sigma, St. Louis, MO) as a chromogen. Following thorough washes in distilled water, all sections were mounted on gelatin-coated slides, dehydrated in ethanol, cleared in xylene, and coverslipped in Permount® (Fisher Scientific, Fair Lawn, NJ).

### III.9 Stereology quanification

Stereology techniques allow for a reliable quantitative description of a 3D object to be made from 2D measurements. Design-based stereology was used to quantify the total number of neurons, percentage of neuroprotection, and tissue volume in various stained sections mentioned above, as previously described (Golub et al., 2015). The stereology system consists of an Olympus BX53 microscope (Olympus, Tokyo, Japan) fixed with a DP73 cooled digital color camera (Model: DP73-1-51, Olympus, Tokyo, Japan) and ORCA-R2 digital CCD camera (Hamamatsu, Hamamatsu City, Japan) for immunofluorescence images. A motorized stage (Model: H101ANNI, Prior Scientific, Rockland, MA) controlled by universal microscope automation controller with encoder (ProScan III, Prior Scientific, Rockland, MA) and Proscan III joystick (Prior Scientific, Rockland, MA) makes the protocol and hardware more user-friendly. The stereology software used in this protocol is newCAST (Version: VIS 4.0, Visiopharm, Denmark). At the end of EEG and behavioral observation period (i.e., at the end of 3 days post-SE), experimental animals were perfused and processed for staining mentioned above.  Serial sections (30 μm thick) were cut coronally through the forebrain containing the amygdala and the hippocampus (Paxinos and Watson, 2007). The sections were collected serially in 24-well plates filled with phosphate buffer (PB, pH 7.4). Every 20th section through the entire hippocampus was then selected from at least five animals. Sections were taken at 300 μm intervals  and processed for immunohistochemistry. Post-sectioning measurements revealed minimal variability in slice thickness. However, sections showed significant thickness shrinkage along the Z-axis following NeuN immunostaining. The average thickness of the sections was reduced to 52–60% of the initial section thickness (n=32). The difference in overall shrinkage of sections from different subjects was insignificant between groups.

To determine the differences in neurodegeneration between these groups with epilepsy, we quantified the number of surviving neurons in the dentate gyrus (DG), dentate hilus (DH), CA1, CA2 and CA3 pyramidal cell layers, via optical fractionator cell counting

with the Visiopharm's stereology system. The disector height is selected when 90% of cells in the field of the slice will be counted in the optical disector cubic. The volume of any specific region of interest in mice can be found by utilizing the 10x objective lens in the Olympus BX53 microscope. To produce accurate volume estimation, at least 200 points were required to be counted in each region of interest. For example, for 10 sections of tissue, on average, 20 crosshairs overlaying the region of interest in each section were counted (West, 2012; 2013a). To obtain neuronal counts in the DG, CA1, CA2, CA3 and DH of each animal, 10-15% of total region area for NeuN(+) cells and 10-15% for PV(+) cells were counted from randomly selected frames, respectively. The frames were placed superficially over each of these cell layers in every twentieth section throughout the hippocampus. The percentage of area was increased from 10% to 15% for NeuN(+) and PV(+) when determining the number of neurons in the CA2 and DH to ensure a large enough sample was being analyzed. All cells present in the optical disector frames (20% of field of view size) at 60x oil objective were counted in each of the regions of the hippocampus. The absolute cell number counts and densities were calculated using the optical fractionator component of the Visiopharm software. The sampling scheme chosen ensured that the sample concentration remained constant for each section. Thus, counting 10% of the DG, CA1 and CA3 or 15% of the CA2 and DH, effectively guaranteed every NeuN(+) neuron within the hippocampal regions had equal odds of being selected and counted (Golub et al., 2015).

**III.10 Timm staining and densitometry analysis**

Timm staining was conducted as described previously (Cavazos et al., 1991). Mice were deeply anesthetized with ketamine/xylazine mix and transcardially perfused with 75 mL 0.9% saline solution followed by 100 mL 1% sodium sulfide solution. Perfusion of 100 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (7.4 pH) followed, and a final perfusion with 50 mL 1% sodium sulfide was carried out. The brain was dissected and post-fixed in 4% paraformaldehyde overnight at 4°C. Brains were then processed with

phosphate buffer and sucrose treatment and cut in 20 μm coronal sections with a cryostat. Slices were then dry mounted onto slides and allowed to dry overnight. Gum arabic, citrate buffer, hydroquinone, and silver lactate reagents were mixed mechanically on the day of staining. Mounted slides were uniformly soaked in the Timm stain in dark for 3.5 hours. Slides were then washed with distilled water and counter-stained with 0.1% cresyl violet, if desired. Slides were permanently fixed with DPX mountant (Sigma) and allowed to dry before imaging and analysis. Staining intensity for Timm and δ-subunit antibody histology was quantitatively measured by densitometric analysis. Densitometry was completed in hippocampus regions of interest with ImageJ software. Mean density of gray-scale staining was normalized to area and white background. Density scores were then non-parametrically graded using a linear scale.

## III.11 Behavioral studies

**Elevated plus-maze**. Anxiety-like behaviors in pilocarpine-epilepsy mice were evaluated in the elevated plus maze (EPM) test, a sensitive behavioral assay of anxiety in rodents (Lister, 1987; Kulkarni and Reddy, 1996). The plus maze test exploits the natural conflict that rodents exhibit between exploration of a novel area and aversion to open areas and height (Kulkarni and Reddy, 1996). The plus maze was constructed of two open arms (16 x 10 cm) and two enclosed arms (16 x 10 cm) elevated to a height of 25 cm from the floor. Mice were tested at 90 days post-SE. For testing, each mouse was placed in the center of the plus maze facing an open arm. During the 5-min test period, the number of entries onto the open arms and the enclosed arms were determined. When a mouse entered an open arm, the time was measured. An arm entry was recorded when all four paws entered into an arm. Mice were examined in a quiet room under normal fluorescent room light. The maze was cleaned thoroughly with 95% ethanol at the end of each test and subsquent tests were performed with a 5 minute delay to allow for drying and airing out. Animals spend more time in the enclosed arms than the open arms and anxiolytic drugs typically increase the proportion of open arm entries in relation to total entries and the time spent on the open arm. In the present study, we calculated the

percent of open arm entries with respect to the total number of arm entries in the 5 min observation period. We also measured the time spent on open arms and determined a percentage of the 5 min period that the mouse spent on the open arms. Increased percent of open arm entries and percent of time on open arms is interpreted as an anxiolytic effect.

**Open field test.** Anxiety-like behavior was assessed in the Open Field Test, an assay used to measure anxiety in rodents. Much like the elevated plus test, the open field utilizes the conflict presented between rodents' natural tendency to explore novel areas and their aversion to open spaces where they would be vulnerable to predation and danger. The open-field test consists of a 60 cm × 60 cm wooden square surrounded by a 50 cm high wall. The area of the maze that was within 15 cm from the wall was considered as peripheral. The rest of the open-field was considered as the central area (Christakis et al., 2012; Hess *et al*., 1992; Takahashi *et al*., 2006). Animals spend more time in the perimeter than in the central area, and anxiolytic drugs typically increase the time spent and /or entries in the open area. Mice were placed in the center of the arena and allowed to move freely around the apparatus to explore the environment for 5 min (Paylor et al., 2006). Between each animal tested, the open-field was cleaned with clothes dampened with 95% ethanol and allowed to dry and air out. In the present study, we calculated the time spent in the center area and number of entries/exits from the central area. Total center entries provided an estimate of overall locomotor activity.

**Novel object recognition.** Non-spatial object memory function was assessed using Novel Object Recognition paradigm, the hallmark method for evaluating memory function in rodents by utilizing their natural tendency for exploring novel objects and environments. Test apparatus consists of the Open Field Test container, which mice are familiarized to in three 5 minutes session prior to introduction of two identical objects spaced equidistant from each other and opposing corners of the apparatus. Mice were then given three 10 minutes sample sessions to explore and become familiar with these objects. 24 hours following the object familiarization sessions, mice were reintroduced to the apparatus, with one of the previous objects changed out for a novel object of similar size. Number of explorations (approaching <1") of each object were recorded

during a 5 minute test session. The ratio of novel/familiar explorations was used to assess the animals' ability to recognize the familiar object from the novel object

## III.12 Drugs and reagents

For all chronic and behavioral experiments involving allopregnanolone (3α-hydroxy-5α-pregnan-20-one, AP) and ganaxolone (3α-hydroxy-3β-methyl-5α-pregnan-20-one, GNX), neurosteroids, progesterone, and finasteride (*N*-(1,1-dimethylethyl)-3-ox-(5α,17β)-4-azaandrost-1-ene-17-carboxamide), drug was made in 20% β-cyclodextrin in sterile saline solution. All drugs (except diezapam, an i.p. injection) were administered to animals subcutaneously in a volume equaling 1% of the animal's body weight. AP, GX, and finaseride were acquired from Steraloids (Newport, RI).

## III.13 Statistical analysis

For behavioral testing, including kindling data, group data were expressed as mean ± S.E.M. The Kruskal-Wallis ANOVA and Mann-Whitney U test were used to analyze significant differences in seizure stages between gender and genotypes. Significant differences in afterdischarge durations between genotype and treatment groups were assessed by the Student's upaired t-test, repeated measures ANOVA, or one-way ANOVA followed by Dunnett's test. Dose-response curves and their $ED_{50}$ values in the 6-Hz test were analyzed for significance using the Litchfield and Wilcoxon test. Differences were considered statistically significant at $p < 0.05$.

## IV.1 The role of δ-subunit extrasynaptic GABA-A receptors in a perimenstrual neurosteroid-withdrawal model of catamenial epilepsy

### IV.1.1 Progression of hippocampus kindling development in δKO mice

To investigate the role of δ-subunit containing GABA-A receptors in catamenial epilepsy, we first used the hippocampus kindling model of complex partial seizures to generate epilepsy in female δKO mice and WT counterparts. As shown in Fig. 7, daily kindling stimulation of δKO mice was associated with a steady progression of behavioral seizures (Fig. 7A) and AD duration (Fig. 7B). Mice were subjected to once-daily kindling via an implanted electrode in the dentate gyrus region until they exhibited stage 5 seizures for 3 consecutive days, which is considered the fully kindled state. WT mice reached the fully-kindled state with consistent stage 5 seizures after 20 stimulations (Fig. 7A). Compared to WT mice, δKO mice exhibited accelerated kindling epileptogenesis, but once at fully-kindled state, stimulation-induced seizures did not significantly differ from WT animals in measures of seizure severity (AD and seizure duration, seizure level). δKO mice did exhibit a significantly lower seizure threshold (ADT- Fig. 7C- data) and reduced number of stimulations to reach fully kindled state (7A) compared to WT mice. Afterdischarge threshold does not change for individual WT or δKO mice during kindling, and AD thresholds were checked and confirmed immediately prior to induction of NSW. Of note, δKO mice also initially exhibited longer AD's than WT counterparts; however, this difference was diminished by the time animals reached a fully-kindled state. Within fully-kindled animals, average seizure and AD duration did not differ between WT and δKO mice (Fig. 7D). Overall, fully kindled

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δKO mice exhibit signs of hyperexcitability and experience accelerated kindling rate, but do not display evoked-seizure exacerbation, when compared to WT mice.
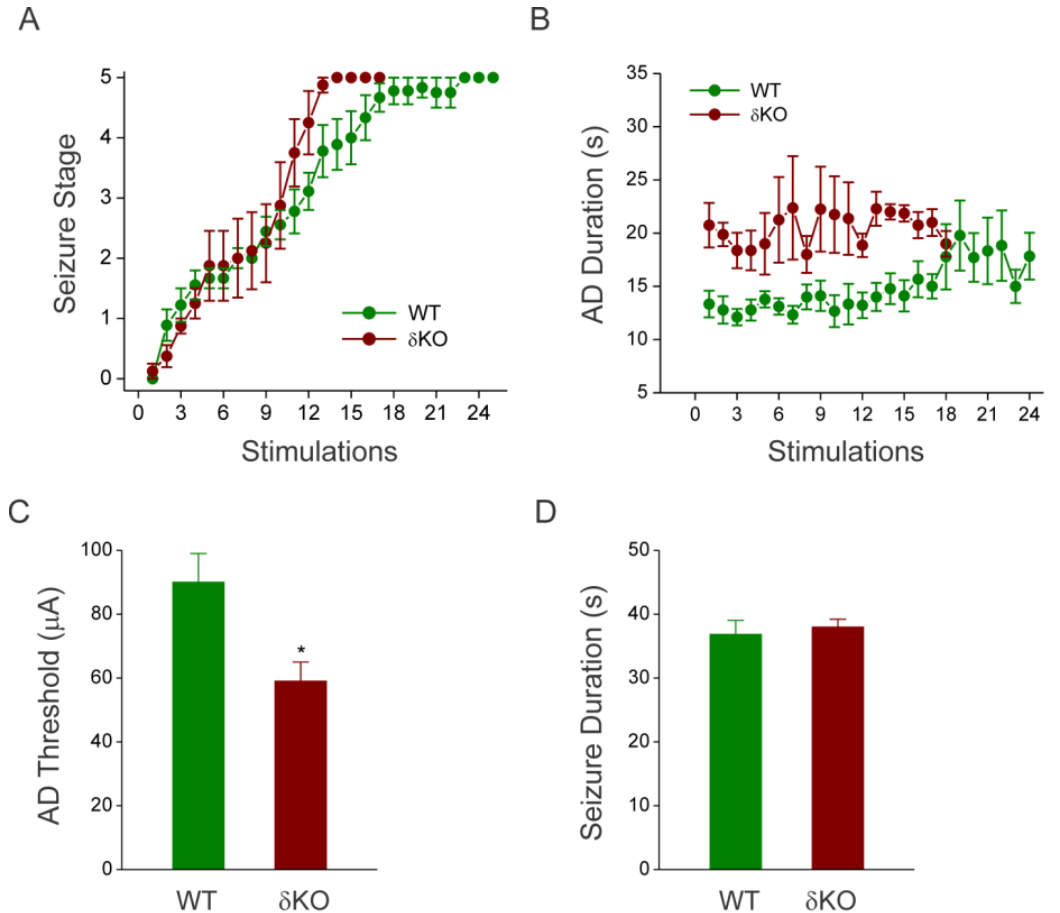


**Fig. 7. Hippocampus kindling epilepsy development in female δKO mice.** Mice were stimulated daily until they exhibit a steady-state, fully-kindled stage 5 (generalized) seizures. **(A)** Behavioral seizure stage during 24-days period associated with kindling stimulations. δKO mice exhibited faster rate of kindling than WT mice, as evidenced by higher seizure scores at corresponding stimulations. **(B)** AD duration during the study period. δKO mice displayed higher AD than WT animals at corresponding stimulations. **(C)** Average AD threshold used for hippocampal kindling stimulation in WT and δKO mice. Overall, δKO had a lower threshold for kindling stimulation. **(D)** Average duration of generalized evoked-seizure activity in individual fully-kindled WT and δKO mice did not differ immediately prior to the induction of NSW. Data was presented as mean ± SEM (N=12 δKO, N= 8 WT mice).

**IV.1.2 NSW exacerbates seizure susceptibility and evoked-seizures in fully-kindled δKO mice**

To create a condition that mimics the hormonal milieu and epileptic state of perimenstrual catamenial epilepsy, fully-kindled female WT and δKO mice were subjected to the NSW paradigm. This model consists of sequential gonadotropin treatments (PMSG and HCG, Day -2 and Day 0), which increase circulating levels of progesterone, and its neurosteroid derivative AP. On day 9, finasteride, a 5α-reductase inhibitor, was given to produce a robust inhibition of endogenous neurosteroid production without interfering with circulating hormone levels (Reddy et al., 2012). Circulating levels of AP decrease rapidly after finasteride treatment, falling to levels below that of pre-gonadotropin treatment within 24 hours, creating a state of neurosteroid withdrawal similar to that which may occur in women following the drop of circulating AP around menstruation. Behavioral tests and observations were performed at 0, 12, 24, and 48 hours following the induction of neurosteroid withdrawal.

To investigate the role of δGABA-A receptors in NSW-associated increase in seizure susceptibility, we analyzed the stimulation-evoked seizure activity in animals undergoing NSW. Fully-kindled adult female WT and δKO mice were subjected to the NSW protocol as described in Fig. 4. Four key parameters were assessed as indices of catamenial-like seizure expression: (i) ADT current, (ii) AD duration, (iii) seizure intensity as per the Racine scale, and (iv) duration of seizures. Consistent with theorized heightened excitability due to sudden reduction of inhibition-augmenting neurosteroids, there was a marked decrease in the ADT current to induce generalized seizures in WT and δKO mice at 24 h after NSW (mean ADT value, 105 and 60 μA for WT control and withdrawal, respectively, and 62 and 30 μA for δKO control and withdrawal, respectively) (Fig. 8A). The mean duration of the individual generalized seizures was similarly increased and longer in withdrawal than in control animals of either genotype (Fig. 8B). Although increases in AD duration were observed in both WT and δKO mice at 12 hr and 24 hr after NSW, δKO mice displayed a significantly greater peak increase

(at 12 hr) in AD duration than WT counterparts (Fig. 8C). This response was significantly higher 12 h and 24 h after NSW and returned to control level by 48 h after withdrawal in both WT and δKO animals (Fig. 8C and Fig. 4), indicating a transitory period for seizure exacerbation after NSW. The number of animals exhibiting generalized seizures at 50% of normal kindling stimulation ADT current (a separate but valuable measure of seizure threshold) was significantly higher after NSW than in the control group (Fig. 8D). We further analyzed the changes in electrographic seizure duration (AD) during NSW through comparison with pre-NSW averages and found that WT animals expressed progressive increases in seizure duration through 12 and 24 hour time points, δKO animals undergoing NSW experience a much more rapid (peaking at 12 hours) and robust (200% peak δKO vs. 140% peak for WT) increase in seizure duration than their WT counterparts. AD duration in NSW-δKO mice decreased between 12 and 24 hours post-NSW, but only to levels consistent with WT counterparts (120%) at 24 hours. AD durations for both genotypes continued a decreasing trend following after 24 hours of NSW, remaining slightly higher than those recorded pre-NSW (baseline) when observed at 48 hours post-NSW. Further analysis of current-threshold changes in WT and δKO mice in NSW, represented as % of pre-NSW AD threshold, revealed similar reductions in threshold in WT and δKO mice at 12 and 24 hour time points (Fig. 8F).
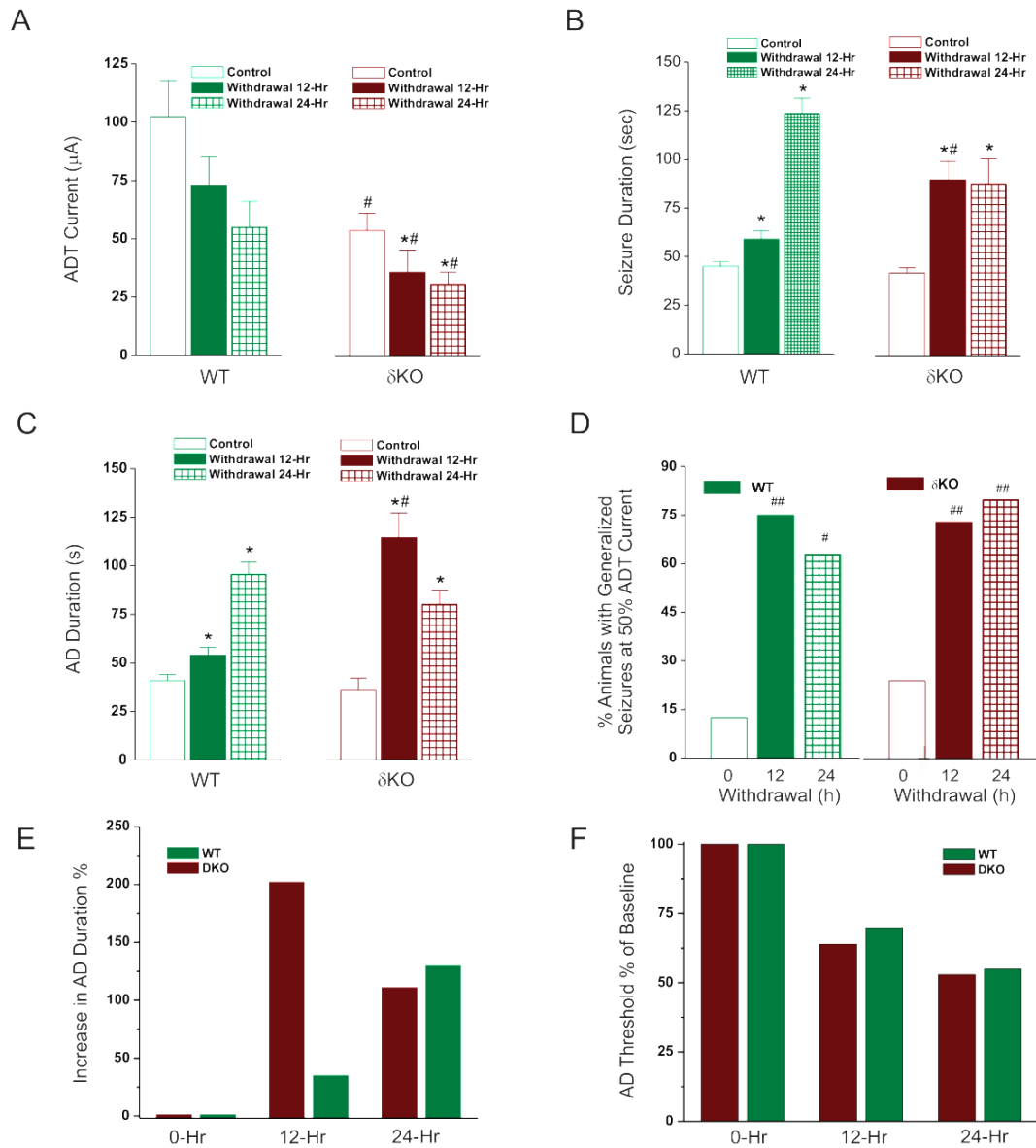
**Fig. 8. Accelerated and augmented catamenial-like evoked-seizure effect during neurosteroid withdrawal in δKO mice. (A)** Intensity of ADT current for eliciting generalized (stage 4/5) behavioral seizures at 12 h and 24 h after neurosteroid withdrawal. ADT decreased in WT and δKO at 12 h and 24 h time points. **(B)** Duration of behavioral (stage 4/5) seizures increased in both genotypes at 12 h and 24 h after neurosteroid withdrawal. **(C)** Duration of AD 24 h after neurosteroid withdrawal. WT mice displayed increasing AD at 12 h and 24 h. Compared to WT mice, δKO mice experienced a more rapid and robust increase in AD by 12 h. **(D)** The time-course of percent of animals exhibiting generalized seizures at ≤50% ADT current. **(E)** Increase in AD duration over 24 h post-NSW, as % of baseline AD for each group. **(F)** AD threshold represented at % of baseline (pre-NSW) threshold for WT and δKO mice during 24 h post-NSW. Data presents the mean ± SEM (N=9 δKO, N= 8 WT mice).

**IV.1.3 Accelerated and augmented NSW-induced exacerbation of electrographic seizures in δKO mice**

Representative electrographic events for each group are illustrated in Fig. 9. Neurosteroid-withdrawn WT & δKO animals showed continuous bursts of spikes that progressively increased in amplitude and duration, indicating heightened epileptiform activity (Fig. 9). In WT mice, average electrographic seizure (AD) duration was increased by 30% over baseline values 12 h after withdrawal, reached maximal levels of 200% over baseline 24 h after withdrawal, and declined to near-control level by 48 h after NSW (Fig. 9). Compared to WT animals, neurosteroid-withdrawn δKO mice displayed an accelerated timeline and augmentation of electrographic seizure exacerbation, with average AD duration peaking at nearly 300% of baseline value at 12h after withdrawal. At 24h after withdrawal, δKO mice continued to exhibit elevated AD duration, which returned to near-control levels at 48 h after withdrawal. Finasteride without gonadotropin pretreatment did not cause exacerbation of evoked-seizures in fully-kindled WT and αKO animals (data not shown), indicating the specificity of NSW on the exacerbation of seizure activity in fully-kindled WT and δKO mice. In summary, fully-kindled δKO mice undergoing the NSW paradigm experience a similar, but significantly more rapid and robust exacerbation of evoked-seizures than WT counterparts.
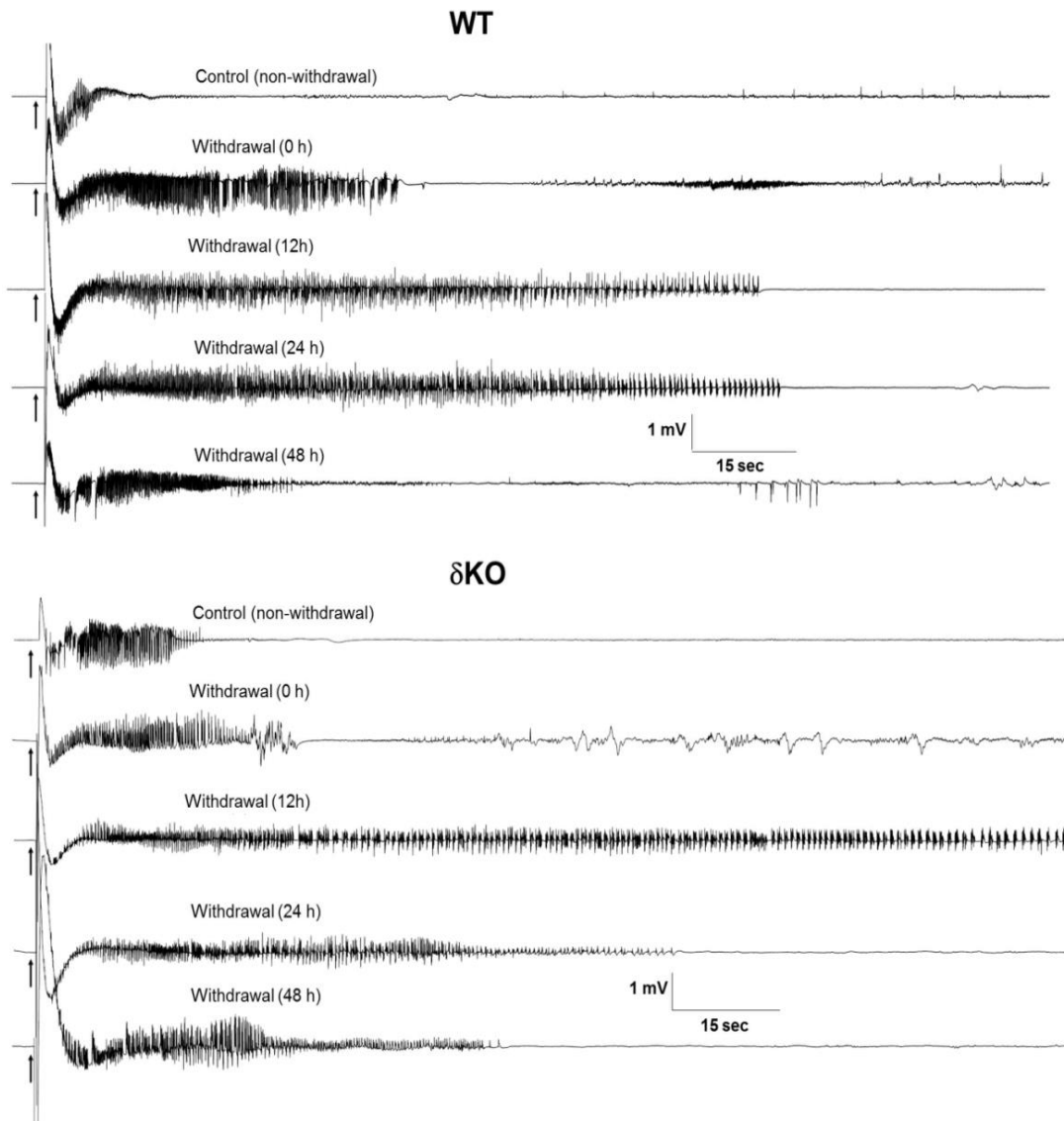
**Fig. 9. Neurosteroid-withdrawal induced exacerbation of electrographic seizure activity in fully-kindled in δKO mice.** Representative traces illustrating exacerbation of stimulation-induced electrographic seizure activity in a fully-kindled WT (top panel) and δKO mouse (bottom panel) during neurosteroid-withdrawal period of 0 to 48 hours. Compared to WT mice, δKO mice exhibited a more rapid increase in AD duration, and well as a greater increase in AD duration at peak effect (12 hr). AD durations are similar at all other time points.

**IV.1.4 Neurosteroid withdrawal induces spontaneous generalized seizures in δKO mice**

To further characterize the possible effects of NSW, animals were placed under continuous video surveillance to monitor for spontaneous generalized seizures and related behavior during the 24 hours following induction of NSW (Fig. 10). This period corresponds with the greatest increase in excitability in both WT and δKO mice, as observed in the exacerbated severity of stimulation-evoked electrographic and behavioral seizures (Fig. 8 & 9). Spontaneous (unprovoked) generalized seizures are not normally observed in fully-kindled, non-NSW animals of either genotype. Videos were analyzed for any mice displaying spontaneous generalized behavioral seizures of Racine level 5 by a trained observer. As shown in Fig. 10, the δKO cohort exhibited significantly increased incidence of spontaneous seizures during 24 h after NSW as compared to their WT counterparts (50% of δKO animals, compared to 20% of WT), indicating the striking increase in seizure exacerbation in δKO mice.
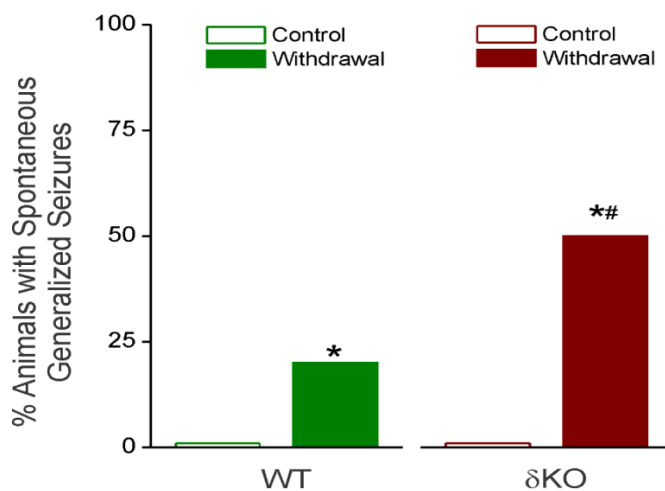


**Fig. 10. Neurosteroid withdrawal-induced increase in spontaneous seizure activity in δKO mice.** Data represents percentage of mice showing spontaneous seizures (without stimulation) from 0-24 h following induction of withdrawal (N=8 DKO, N= 10 WT mice), Wilcoxon Signed Rank Test, $p < 0.05$.

## IV.1.5 Neurosteroid withdrawal induces diazepam insensitivity in fully-kindled δKO mice

To further investigate the role of δGABA-A receptors in NSW-associated alterations in the antiseizure profile of benzodiazepines, diazepam was characterized pharmacologically in fully-kindled δKO mice, as well as 24 h after NSW (Fig. 11AB). In control (non-withdrawal) WT and δKO animals, diazepam produced a dose-dependent suppression of behavioral seizure activity (Fig. 11A) and AD duration (Fig. 11B) with significant effects at 0.1, 0.3, and 1 mg/kg, confirming diazepam protection against hippocampus kindling-induced seizures. In contrast, δKO mice undergoing NSW had significantly decreased seizure protection by diazepam (Fig. 11AB). In control WT and δKO mice, doses of 0.1, 0.3, and 1 mg/kg diazepam produced 40%, 90%, and 95% seizure suppression, respectively (Reddy et al., 2012). However, doses of 0.1, 0.3, and 1 mg/kg produced 5%, 40%, and 85% seizure suppression in neurosteroid-withdrawn δKO mice. These results suggest δKO mice experience similar but reduced shunting effects on diazepam efficacy during NSW compared to WT mice, with greater differentiation of effects at higher doses.
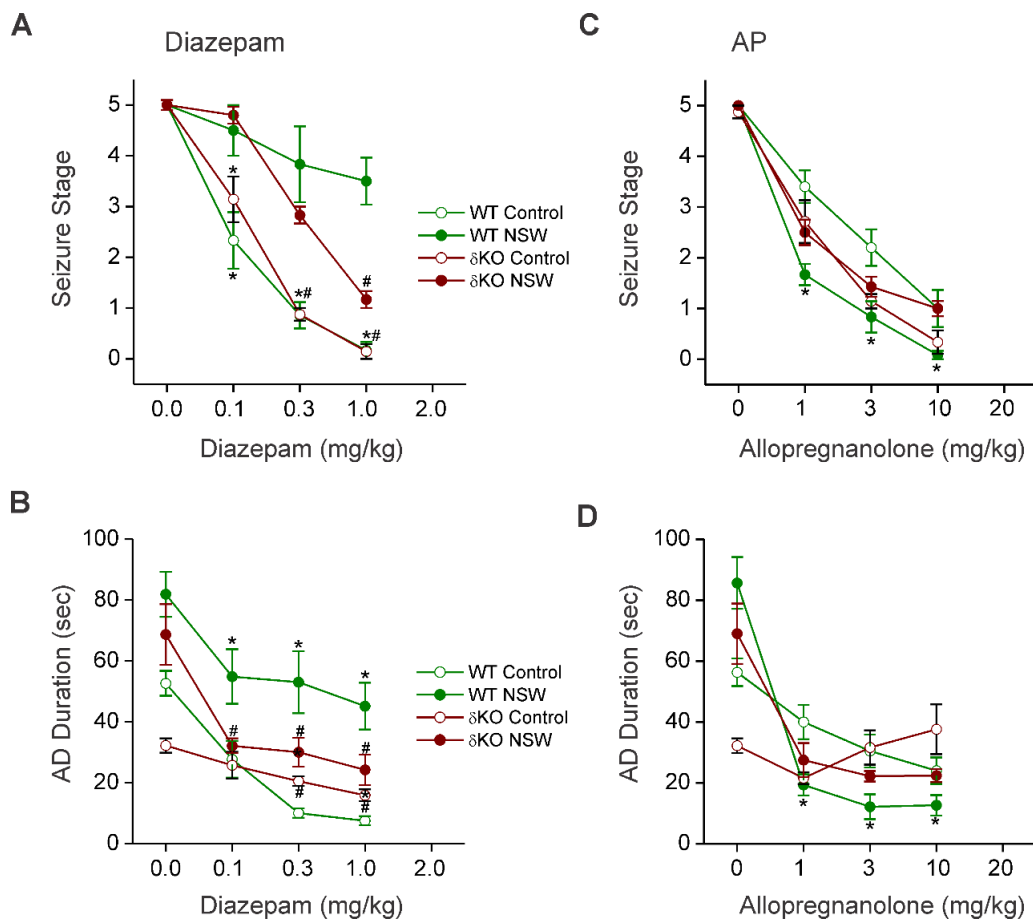
**Fig. 11. Pharmacological evaluation of antiseizure sensitivity of diazepam and allopregnanolone in δKO mice. (AB)** Neurosteroid withdrawal (NSW) diminishes the antiseizure efficacy of diazepam in fully-kindled WT and δKO mice. Dose-response curve for diazepam (0.1-1 mg/kg, i.p.)-induced suppression of behavioral seizure stage (A) and AD duration (B) in WT and δKO groups. **(CD)** NSW diminishes the antiseizure efficacy of allopregnanolone in δKO mice. Dose-response curve for allopregnanolone (1–10 mg/kg, s.c.)-induced suppression of behavioral seizure stage (A) and AD duration (B) in WT and δKO groups. Test drugs were given 15 min before kindling stimulations. Data represents the mean ± SEM (N=6-9 mice group). *p<0.05 vs. control (non-withdrawn) group; p<0.05 vs. WT (genotype) group (WT data adapted from Reddy DS, Gould J, Gangisetty O. (2012). A mouse kindling model of perimenstrual catamenial epilepsy. J Pharmacol Exp Ther 341, 784-793).

**IV.1.6 Unaltered neurosteroid anticonvulsant sensitivity in fully-kindled δKO mice during NSW**

In addition to diazepam, we investigated the efficacy of the prototype neurosteroid AP in δKO mice 24 h after NSW (Fig. 11CD). Fully-kindled control and neurosteroid-withdrawn δKO mice were tested in the hippocampal kindling model with three doses of AP (1, 5, and 10 mg/kg s.c.). At these doses, AP exerted dose-dependent suppression of the behavioral seizures (Fig. 11C) and AD duration (Fig.11D) in fully-kindled control WT and δKO mice. However, in contrast to the enhanced neurosteroid sensitivity observed in fully-kindled WT mice after NSW (Reddy, et al. 2012), no change in AP's suppression of behavioral seizures was observed between control and neurosteroid-withdrawn δKO mice (Fig. 11C), and suppression of AD in neurosteroid-withdrawn δKO mice was not significantly different beyond the 1 mg/kg dose (Fig 11D). Moreover, previous studies showed plasma levels of AP achieved at various doses of AP treatment were similar between control and withdrawn WT mice, especially without significant drug accumulation in withdrawn animals (Reddy et al, 2012), indicating that AP sensitivity was not caused by pharmacokinetic factors. The synthetic neurosteroid ganaxolone (1, 3, and 10 mg/kg) also produced enhanced (60%) efficacy in fully kindled neurosteroid-withdrawn WT animals (data not shown), confirming the enhanced sensitivity to neurosteroids in the NSW model of catamenial epilepsy (Reddy and Rogawski, 2001). Overall, these data provide powerful evidence for potential therapeutic viability of δGABA-AR-targeting drugs for catamenial epilepsy patients suffering from intractable seizures around menses.

**IV.2 The role of δ-subunit extrasynaptic GABA-A receptors in susceptibility to limbic epileptogenesis**

**IV.2.1 Accelerated kindling epileptogenesis in δKO mice**

To investigate the role of δ-subunit extrasynaptic GABAARs in limbic epileptogenesis, we used the hippocampal kindling model of temporal lobe epilepsy in WT and δKO mice. Hippocampal kindling is a well-accepted model of epileptogenesis in which repeated stimulation elicits progressive epileptiform discharges (afterdischarges) that result in a persistent epileptic state. Additionally, behavioral seizures resulting from the kindled-state serve as the basis for many first-line screenings for AEDs in acute seizure reduction (Fig. 5.). As δ-GABA-ARs are major contributors to tonic inhibition in the DG, the "gate-keeper" of the hippocampus (Fig. 3), we hypothesized that δKO mice would display accelerated kindling rates due to reduced ability to inhibit the propagation of stimulus-induced seizures. Indeed, female δKO mice displayed accelerated hippocampus kindling epileptogenesis when compared to WT female (Fig. 12). Female δKO mice displayed significantly higher mean seizure scores than WT counterparts from stimulation session 10 through the end of kindling (12A). Interestingly, δKO mice displayed significantly longer ADs than their WT counterparts throughout the kindling process. Although the difference in AD duration decreased as kindling proceeded, a significant difference was observed until animals began expressing stage 5 seizures and/or reached the fully kindled state. In fully kindled mice, ADT remained significantly different between δKO and WT (figure 8A), but AD duration reached closer to convergence between the two genotypes (AD durations for all).

In comparing overall kindling rates, we examined the average number of stimulations to reach fully kindled state for each group. The increased seizure scores of δKO mice reflected the decreased number of stimulations required to kindle these animals, compared to WT counterparts. WT females averaged 19.2 ± 0.89 stimulations to fully-kindled state vs 15.9 ± 0.64 in δKO females. Further analysis of kindling rate date revealed a biphasic pattern of seizure-stage progression within the kindling process in

both genotypes. To this end, we divided kindling progression into 2 stages: From stage 0 (stimulation 1) to stage 2, and from stage 2 to fully-kindled state. We then examined kindling rates as expressed by seizure-stage/stimulation for each phase. We observed that kindling rates did not differ between WT and $\delta$KO animals during the initial part of kindling. However, kindling rate accelerated during late-phase kindling (2-5), and this effect was significantly more pronounced in $\delta$KO mice (WT ~0.35SS/stimulation, $\delta$KO ~0.50 SS/stimulation). This late-phase acceleration was responsible for the overall differences in kindling rate between WT and $\delta$KO mice. Examination of electrographic seizure traces reflected these findings, and representative traces of each group at key points in kindling are provided following the graphs (Fig. 12E).

We also observed sex-differences in kindling rate, independent of genotype. WT male mice required an average of 16.8 $\pm$ 0.76 to reach full kindled state, around 4 less than WT females. Similarly, $\delta$KO males kindled with an average of 13.5 $\pm$ 0.89 stimulations, compared to 16.9 $\pm$ 0.64 in $\delta$KO females. On average, AD durations were higher in males than in females of the same genotype, though this difference was more pronounced in WT mice. In comparing kindling rates for males and females, we examined overall rates (13C), as well as initial and late-phase kindling (13D). Within each sex, kindling rates did not differ between WT and $\delta$KO animals during the initial part of kindling. However, kindling rate accelerated during late-phase kindling (2-5) in all groups except for WT males, which maintained rates similar to that of WT females in late-phase (~.35 SS/stimulation) throughout the kindling process. This late-phase increase was responsible for the accelerated kindling rates observed in $\delta$KO mice vs WT of the same sex, and was much more pronounced in $\delta$KO females than WT females (WT ~0.35SS/stimulation, $\delta$KO ~0.50 SS/stimulation).
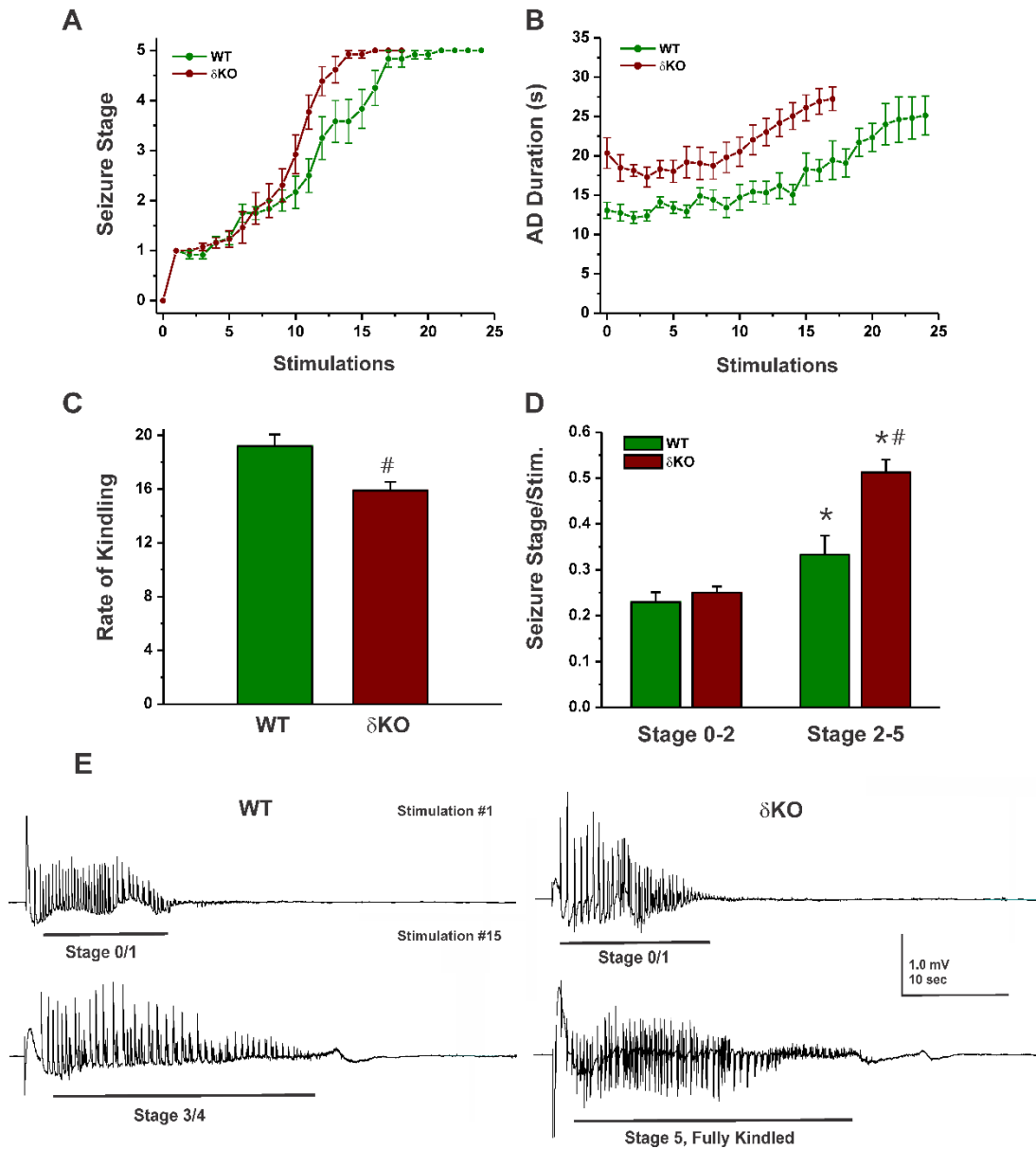
**Fig. 12. Accelerated kindling epileptogenesis in δKO mice. (A)** Female δKO mice displayed accelerated hippocampus kindling epileptogenesis when compared to WT female, as expressed by higher mean seizure scores at corresponding stimulation sessions. **(B)** Comparison of AD durations; δKO mice displayed significantly higher AD durations than WT counterparts at corresponding stimulation sessions. **(C)** Comparison of hippocampus kindling rates between female WT and δKO mice. **(D)** Analysis of Kindling Progression in female WT and δKO mice, as expressed as seizure stage advancement per stimulation. **(E)** Representative traces comparing stimulation-induced electrographic seizures from WT and δKO mice during kindling epileptogenesis. Data represents the mean ± SEM (N=10 mice/group). #p<0.05 vs. WT of same condition; *p<0.05 vs. same genotype of compared condition.
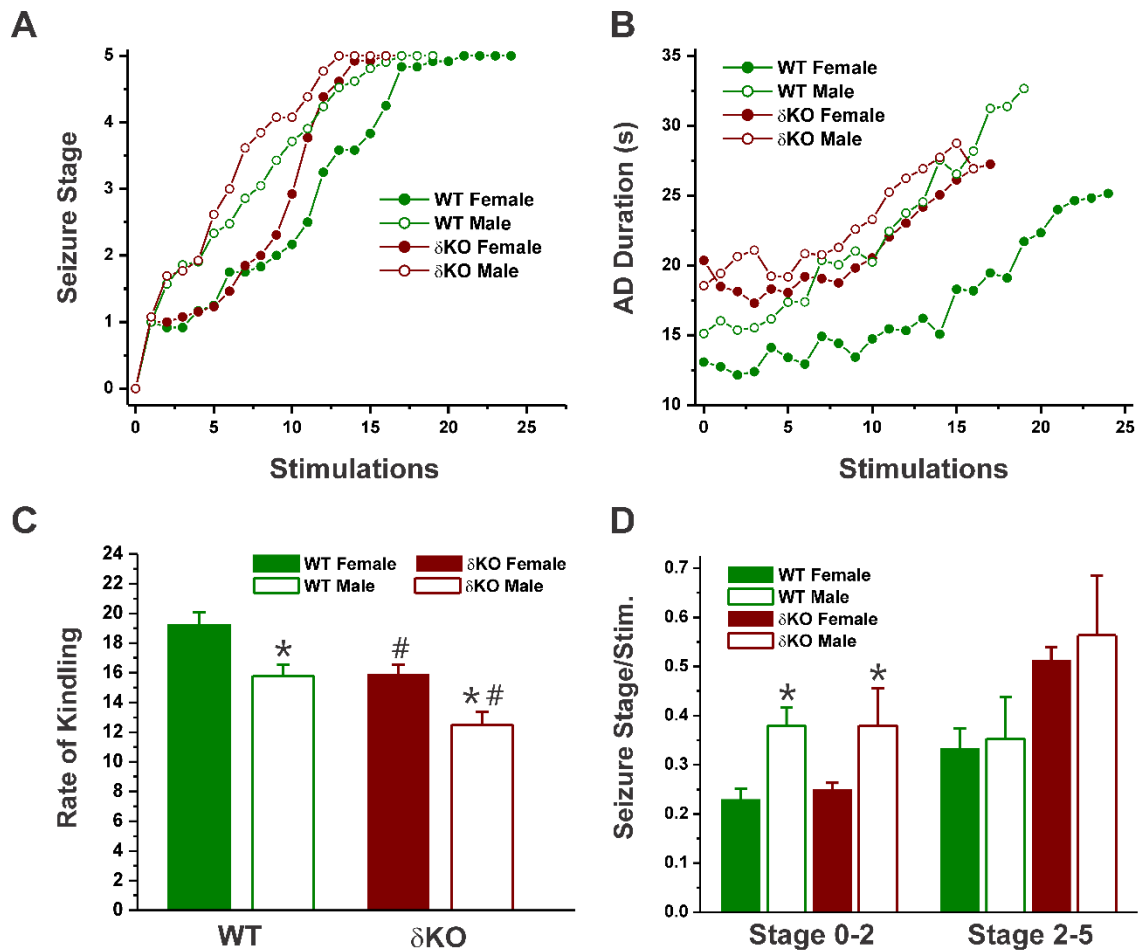
**Fig. 13. Accelerated kindling epileptogenesis in male mice, independent of genotype. (A)** Male WT and δKO mice displayed accelerated hippocampus kindling epileptogenesis when compared to females of the same genotype. **(B)** Afterdischarge duration for WT and δKO. Knockouts display prolonged afterdischarge duration vs. WT. Males display prolonged afterdischarge duration vs. females **(C)** Comparison of hippocampus kindling rates between male and female WT and δKO mice. **(D)** Comparison of Kindling Progression in male and female WT and δKO mice, as expressed as seizure stage advancement per stimulation. Data represents the mean ± SEM (N=10 mice/group). *p<0.05 vs. female of same genotype, #p<0.5 vs. same sex WT mice.

## IV.2.2 Altered Epileptogenesis in δKO mice in the pilocarpine model of temporal lobe epilepsy

We observed accelerated hippocampal kindling rates in δKO mice, and sought to further investigate the role of δ-GABA-ARs in epileptogenesis using a neurodegenerative model of epileptogenesis. Therefore, we used the pilocarpine status epilepticus model of epileptogenesis. Adult male WT and δKO mice were observed for 90 days following termination of SE to detect and quantify the occurrence of spontaneous recurrent seizures (SRS) among experimental groups. Animals were implanted with DSI wireless telemetry devices, and allowed to recover for one week prior to the start of experiments. To induce SE, mice received scopolamine (1 mg/kg, s.c.) 30 minutes prior to administration of pilocarpine (300 mg.kg, i.p.). SE was allowed to continue uninterrupted for 90 minutes before being terminated with diazepam (10 mg/kg, i.p.). Termination of SE was verified by cessation of electrographic seizures via EEG, and a second dose of diazepam (5 mg/kg, i.p.) was given if needed to end SE.

Contrary to the observation of accelerated kindling epileptogenesis in δKO mice, germline deletion of δ-GABA-ARs did not significantly accelerate the course of post-SE epileptogenesis, but resulted in greater variation and a number of alterations of disease-progression. WT and δKO mice displayed similar trends in expression and occurrence of SRS following SE, as displayed in the Kaplan-Meyer curve in Fig. 13A and SRS frequency overlay in Fig. 16. The majority of responding WT and δKO mice displayed latency to first seizure between days 4-10 (WT avg. 6.33 ± .42 days) following SE, although there was greater variation in the δKO mice (δKO avg. 13.46 ± 5.7 days), a portion of which displayed greatly extended latency-to-seizure following SE. Incidence of epilepsy was slightly higher in δKO mice (13D). WT mice averaged slightly higher total seizure expression (49.33±7.38) than δKOs (41.9±8.6), although the difference between groups was not of statistical significance (13C). The average single seizure duration was moderately increased in δKO mice (δKO 25.36 ± 5.97s; WT 22.05 ± 4.4s, 13E), resulting in similar overall average total time seizing between genotypes (δKO

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892.75 ± 268.7s; WT 1102 ± 137.9s, 13F). δKO mice experienced greater mortality (45%, 13G) during SE than their WT counterparts (34%, 13G), and greater overall mortality rates during the 90 day study (δKO 55%; WT 45%, 13H, Timeline 13I). The implications of this observations and the potential to eliminate affected individuals will be addressed in the discussion. Seizure expression is visualized in figures 14-16, which show the clustered nature of seizure occurrence, as well as the heterogeneity within cohorts. Overall, the nature and extent of the epileptogenesis observed here was similar between the genotypes, which was in stark contrast to our hypothesis.
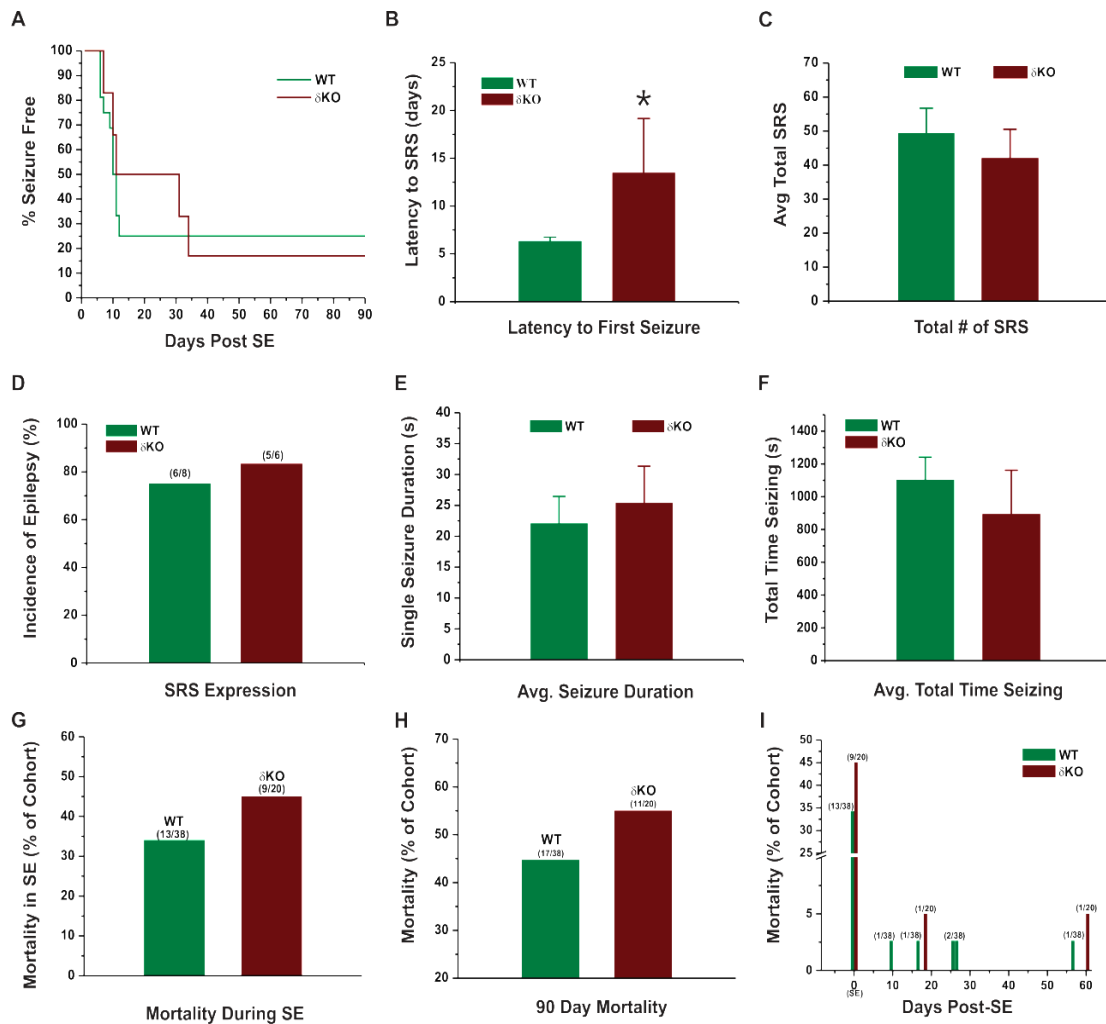
**Figure 14. Progression of epilepsy in WT and δKO mice following pilocarpine-SE. (A)** Seizure expression as percentage of cohort in WT and δKO mice. **(B)** Average latency to first spontaneous recurrent seizure. δKO mice displayed much greater variation in latency than WT mice. **(C)** Graph shows average number of seizures per individual in each cohort. **(D)** Bar graph showing total incidence of epilepsy in each cohort. **(E)** Average duration of individual SRS was not significantly different between WT and δKO mice. **(F)** Average cumulative duration of SRS for individuals in WT and δKO cohorts. **(G)** Mortality rates for WT and δKO mice during SE. **(H)** Overall mortality rates for WT and δKO mice during 90 day study. **(I)** Timeline of mortality events for WT and δKO mice following pilocarpine-SE.
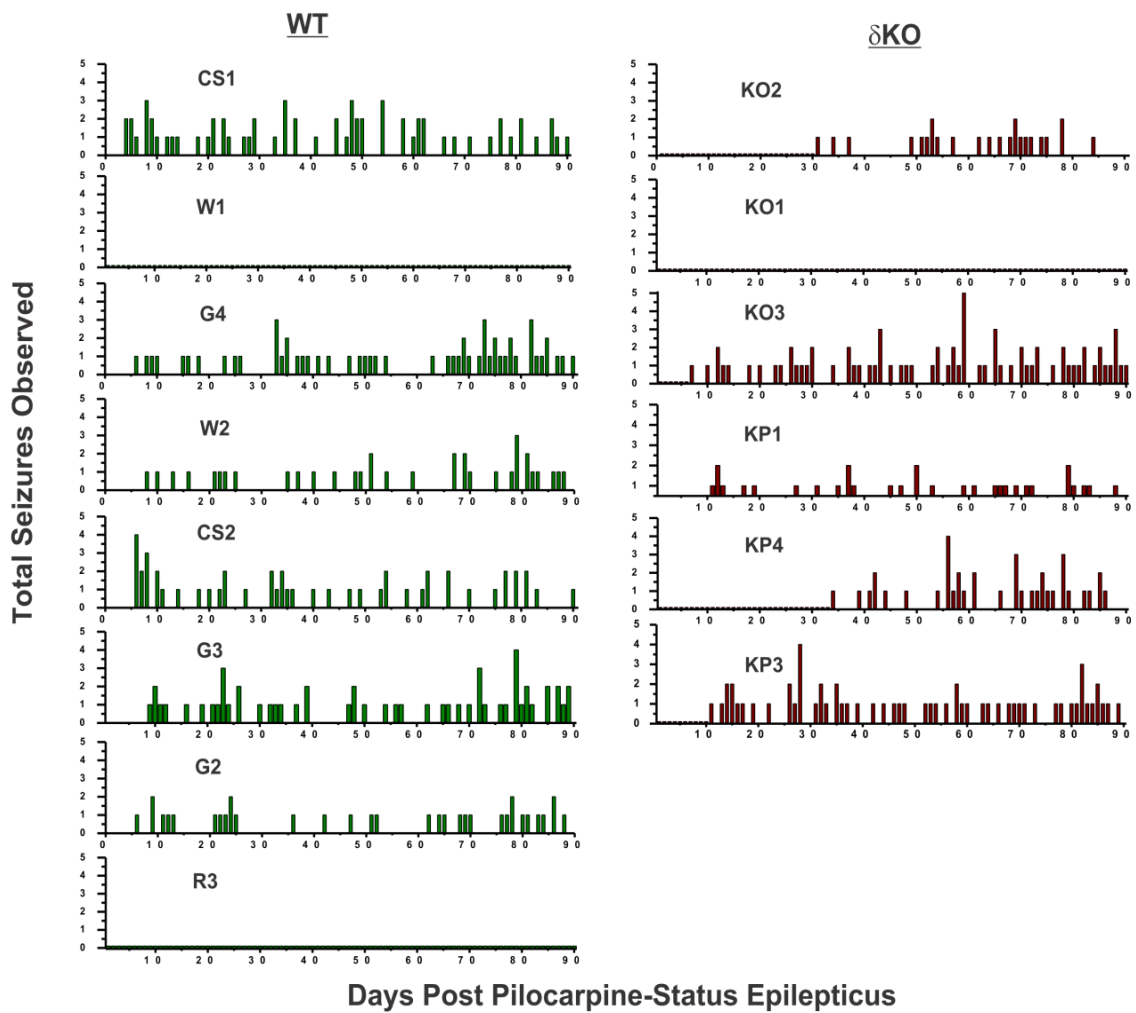
**Figure 15. Individual progression of SRS occurrence in WT and δKO mice following pilocarpine-SE.** Progression of SRS in WT and δKO mice in the 90 days following pilocarpine-SE as represented by total seizures per day for each subject in both cohorts.
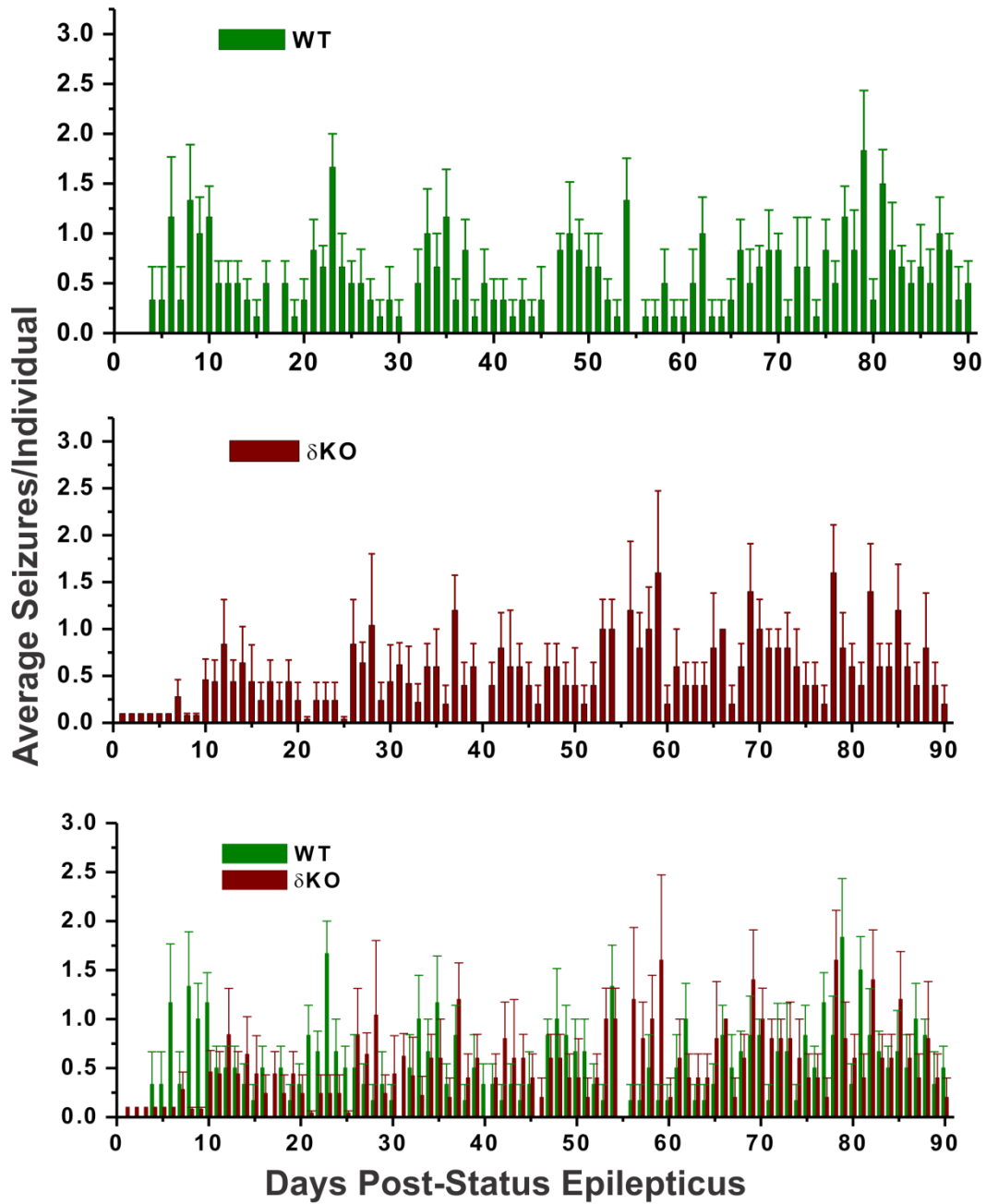
**Figure 16. Cohort progression of SRS occurrence in WT and δKO mice following pilocarpine-SE.** Progression of SRS in WT and δKO mice in the 90 days following pilocarpine-SE as represented by average seizures per day for an individual in each cohort. A direct visual comparison of SRS expression in WT and δKO mice following SE is provided by superimposing the graphs for both groups.
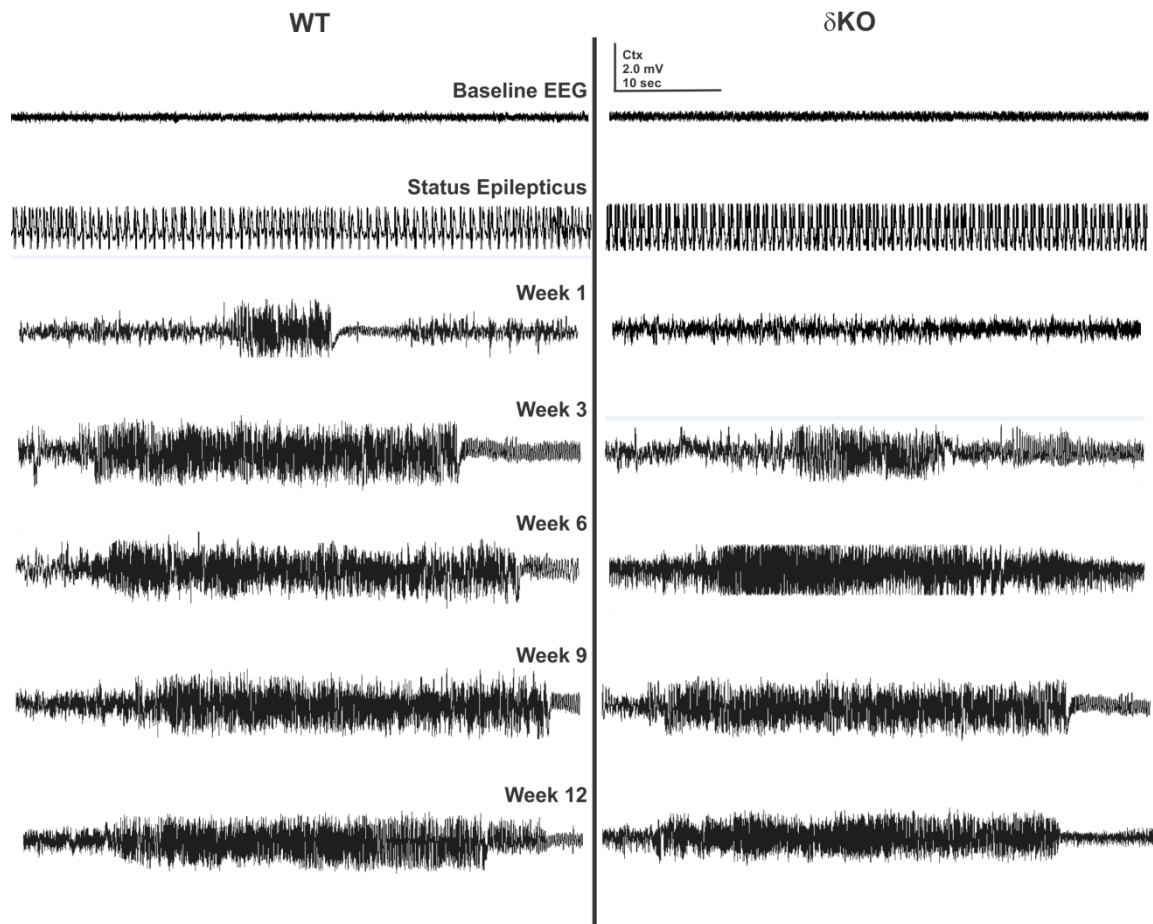
**Figure 17. Progression of electrographic seizures in WT and δKO pilo-epilepsy mice.** Comparison of representative EEG traces and electrographic seizures from WT and δKO mice throughout the 90 days of recording post-SE.

**IV.2.3 Morphological changes and exacerbated neurodegeneration in δKO mice in the pilocarpine model of temporal lobe epilepsy**

Although quantifying the development and progression of seizures is a critical component of examining epileptogenesis in animal models, it provides only one aspect of epileptic pathology. SE usually results in the degeneration of neurons within a number of areas of the hippocampus. In addition to electrographic and behavioral analyses of seizures, careful examination of brain tissue from patients and animal models provides investigators with the insight into the mechanistic cellular and circuit-level changes involved in the pathology of epileptogenesis. From these examinations, a number of structural and biochemical changes have been associated with epilepsy and epileptogenesis, including: principle cell death and neurodegeneration, alteration in BBB permeability and neurotransmitter imbalance, loss of inhibitory interneurons, localized and systemic inflammation, gliosis, and aberrant neurogenesis and neuronal sprouting. These changes are not limited to the epileptic foci, and the timeline and causal-nature of their appearance are an area of rigorous investigation. In the context of δ-GABA-AR role in epileptogenesis, we examined a number of these changes in the hippocampus (CA1, CA3, DG, and Hilus) and associated extra-hippocampal areas (thalamus, hypothalamus, amygdala, and piriform-, somatosensory-, entorhinal- cortices) that exhibit neurodegeneration in chronic epilepsy. Brain tissue was collected from WT and δKO mice subsequent to the pilocarpine-SE chronic SRS studies at 90 days following SE. Aged-matched naïve WT and δKO mice were used as controls. Overall cytoarchitecture and cell loss were visualized using Nissl stain (Figs. 18 & 19). Principle neuron loss was examined through NeuN staining of serial sections and subsequently quantified or characterized through stereological counts (hippocampus) or scoring (extra-hippocampal) (Figs. 20 & 21). Loss of a subset of inhibitory interneurons (parvalbumin-positive) was visualized and quantified via PV$^+$ (parvalbumin) staining of serial sections of the hippocampus (Figs. 22 & 23), and the extent of mossy fiber sprouting was visualized with Timm stain and quantified via densitometric analysis (Fig. 24).

Overall, the cytoarchitecture of the areas of focus was similar across control WT and δKO mice, with individuals of both genotypes displaying cell loss/decreased Nissl staining density at 90D post-SE in all areas of the hippocampus (Fig. 18) and extrahippocampal areas (Fig. 19). This loss was particularly notable in the hippocampal subfields, as well as in the thalamus and hypothalamus. Principal neuron loss in post-SE WT and δKO mice was similar striking in all areas of focus, and did not differ between genotypes. Absolute cell counts and normalized degeneration measures reflected the changes visualized in each section, with both WT and δKO mice experiencing similar overall principle cell loss in the HPC and HSF (WT 30.8% ± 7.7%; δKO 24.4% ± 4.7% Fig. 20). In both genotypes this effect was most pronounced in the DH, where cell loss averaged 60.6% ± 5.6% for δKO and 47.1% ± 3.8% for WT. Although changes were not significantly different between genotypes, δKO mice tended to average greater losses in the DG/DH (DG: δKO 29.7% ± 7.0%; WT 16.4% ± 1.5%), whereas WT mice averaged greater losses in the CA1 (δKO 21.8% ± 2.1%; WT 37.2% ± 10.3%) and CA3 (δKO 22.5% ± 5.1%; WT 38.8% ± 11.4%). This observation suggests the germline deletion of δ-GABAA receptors may affect the normally δ-rich dentate more than the rest of the HPC.

To investigate neurodegeneration in a different subset of neurons, parvalbumin (PV+) staining was utilized to examine the distribution and quantify the loss PV expressing inhibitory interneurons, which have been observed to be highly susceptible to loss in epileptic models (Kuruba et al., 2011). Parvalbumin is a calcium-binding protein expressed by several subsets of fast-spiking inhibitory interneurons which each target distinct populations of pyramidal cells in the hippocampus. Contrary to our observations in principal neuron loss, (Figs. 20 & 21), epileptic δKO mice displayed greater parvalbumin-positive interneuron loss than WT counterparts in all hippocampal regions except for the DG (Fig. 22). Total PV+ interneuron loss was greater in δKO mice (59.6% ± 8.47) than in WT mice (42.4% ± 8.33%). In δKO mice, CA1 loss of PV+ interneurons was the most pronounced, with δKO mice experiencing nearly double the

loss (62.4% ± 7.9%) of WT counterparts (31.5% ± 8.6%). Interestingly PV+ loss in CA3 was lowest but most variable among δKO mice (48.4% ± 12.5%), with WT animals displaying significantly lower levels of loss in CA3 (29.9% ± 3.4%), similar to that observed in CA1. PV+ interneuron loss in the DG was extensive and nearly identical in WT (65.7% ± 4.4%) and δKO mice (67.7% ± 5.0%). Observation of interneuron loss in extra-hippocampal areas showed similar effects in post-SE mice of both genotypes (Fig. 23). These observations suggest loss δ-GABAARs leads contributes to increased susceptibility of PV(+) inhibitory interneurons to damage and degeneration from epileptic seizures.

To investigate structural changes associated with chronic epileptogenesis, mossy fiber sprouting was analyzed in WT and δKO mice that experienced SRS following piocarpine-induced SE. Extensive sprouting of granule cell axons (mossy fibers), as detected by Timm staining, was observed in WT and δKO post-SE mice (Fig. 24). Timm staining is a technique of histochemical identification of the zinc-rich granules in the synaptic terminals of axons projecting from DGGCs (Cavazos et al., 1991), called mossy fibers due to moss-like appearance. A number of prior studies in convulsive models have observed mossy fiber sprouting into the granule layer as well as increased zinc accumulation in the dentate hilus (Gombos et al., 1999; Watanabe et al,. 1996). Serial sections were processed from control and epileptic WT and δKO mice and anaylzed with densitometric software and reported as normalized stain intensities from the DG granule layer and DH. Compared to WT mice, δKO mice displayed greater staining intensities under control conditions in both regions examined. Pilocarpine-SE and subsequent epileptogenesis resulted in significantly increased staining intensity in the the granule layer and hilus of the dentate in both genotypes. Following SE, the extent of MF sprouting in the hilus was higher in δKO mice than in WT, although post-SE animals of both genotypes exhibited similar stain densities within the DG granule layer. As observed in our other histopathologic analyses, the pattern of MF sprouting was similar but enhanced in δKO mice following pilocarpine-SE.

Overall, these results are in alignment with those presented in the SRS progression of WT and δKO mice: taken together our observations suggest a minor, but perhaps complex role of δ-GABAARs and tonic inhibition in modulating epileptogenesis and neuroprotection following SE in mice with germline loss of the δ-subunit containing GABA-ARs.
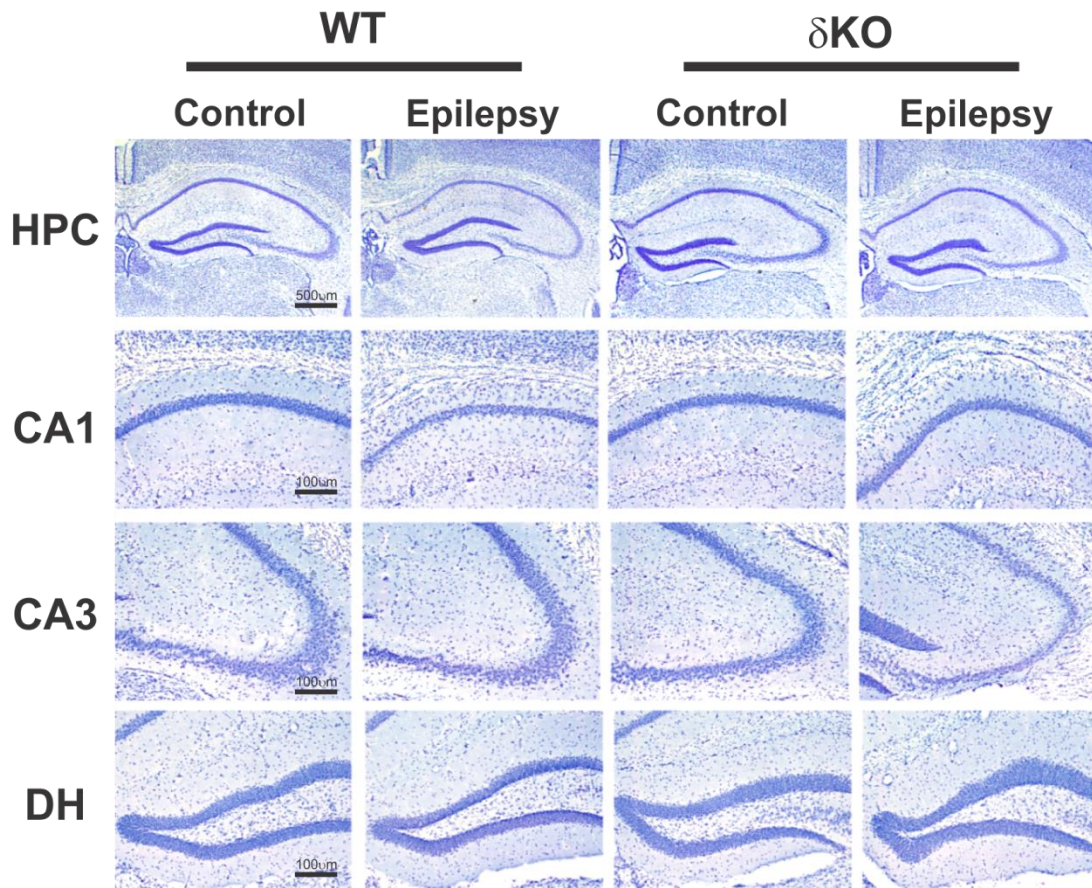


**Figure 18. Hippocampal cytoarchitecture of WT and δKO pilo-epilepsy mice.** Hippocampal cytoarchitecture visualized with Nissl stain. Cell loss following pilocarpine-SE was observed in WT and δKO mice across the hippocampus proper, with similar overall losses in the primary hippocampal sub-regions.
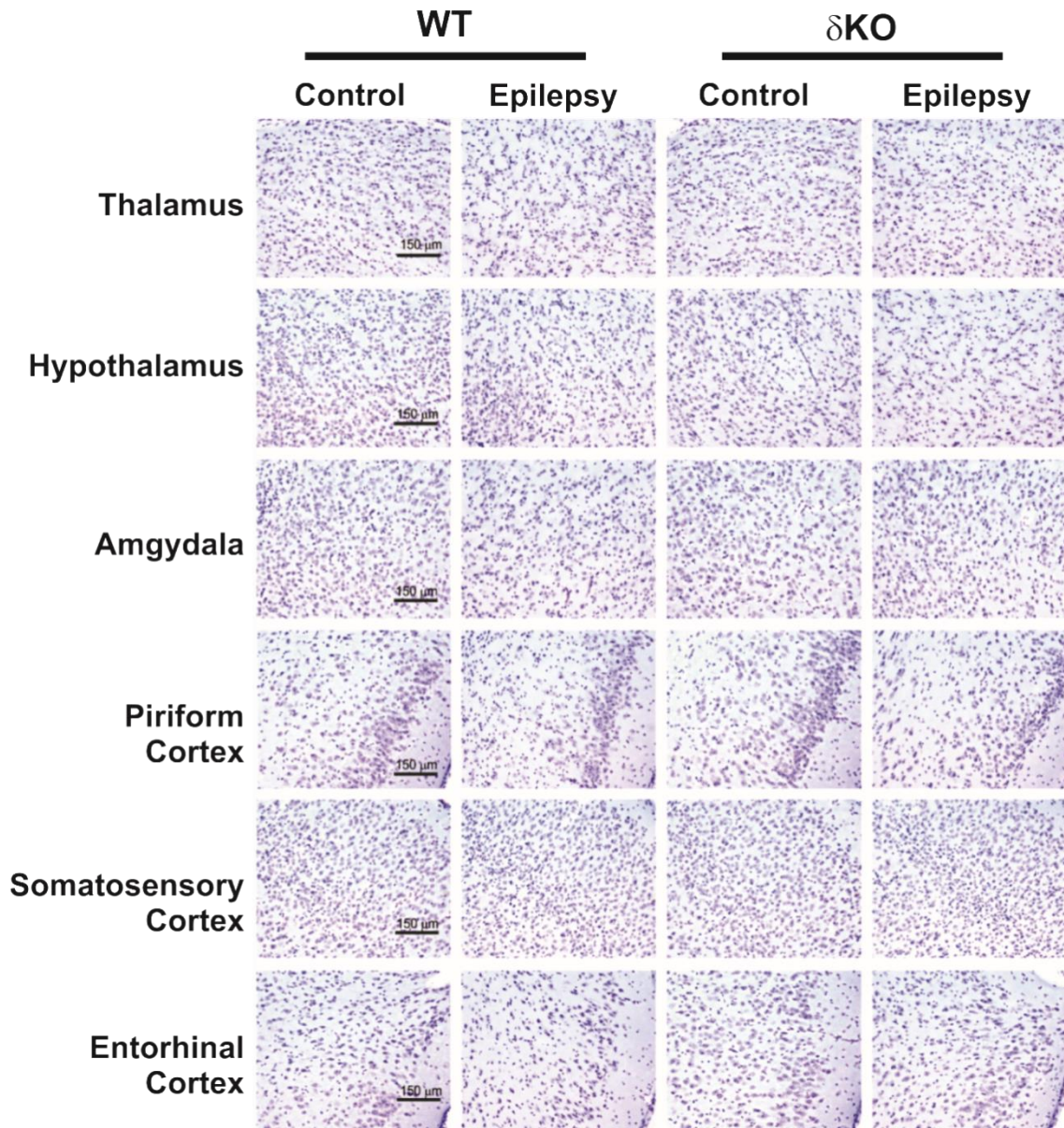
**Figure 19. Changes in the cytoarchitecture of extra-hippocampal regions δKO and WT mice following pilocarpine-SE.** Extra-Hippocampal cytoarchitecture visualized with Nissl stain. Cell loss at 90 days following pilocarpine-SE was observed in WT and δKO mice in a number of extra-hippocampal regions, highlighted above.
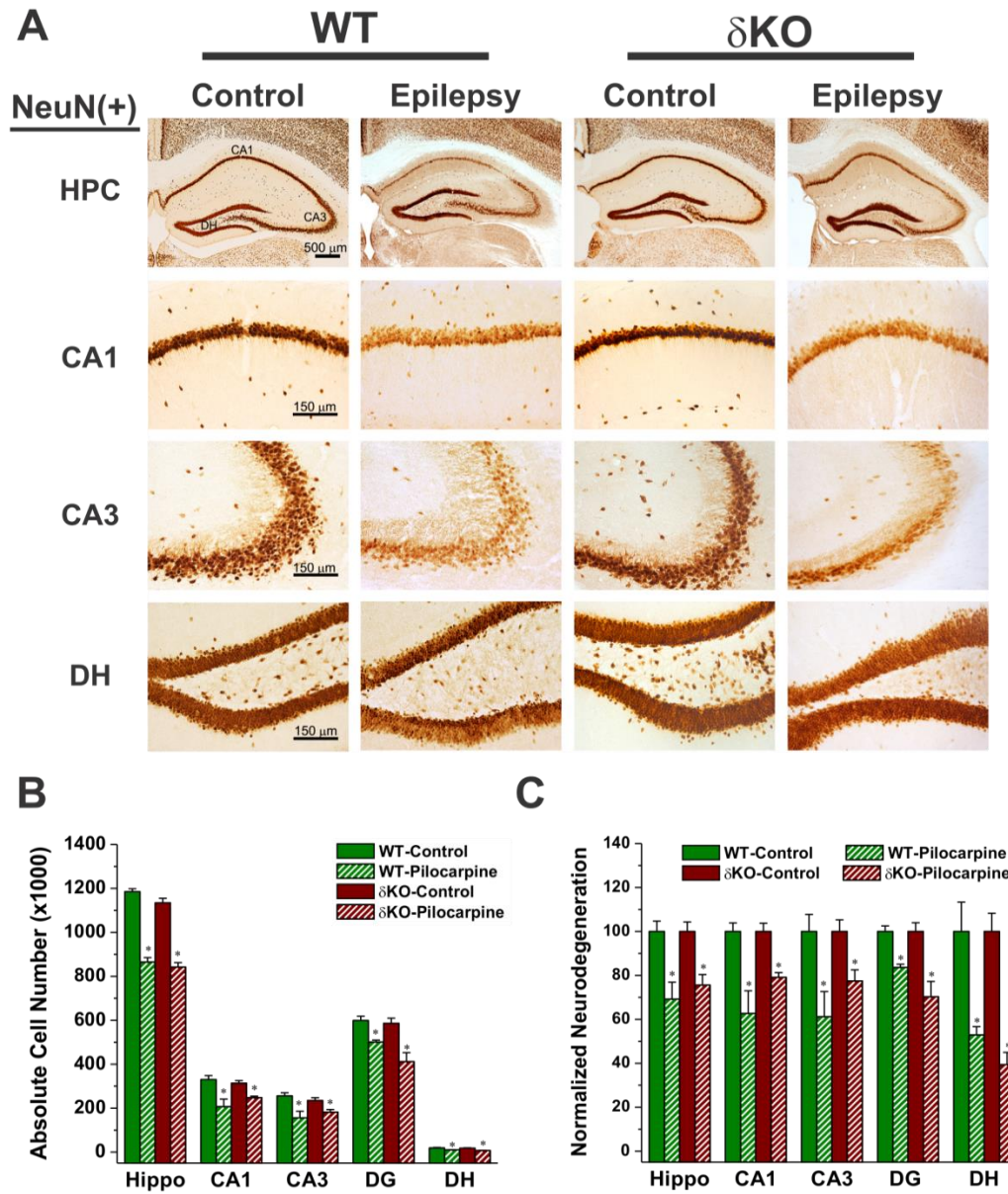
**Figure 20. NeuN(+) immunohistochemistry in WT and δKO mice. (A)** Distribution of NeuN(+) principal neurons in the hippocampus subfields at 3 months post-pilocarpine in WT and DKO mice. **(B)** Absolute cell counts for the hippocampus and hippocampal sub-regions in control and pilocarpine-epilepsy WT and δKO mice 90 days following SE. Similar trends in cell loss were noted between genotypes across the hippocampal areas analyzed. **(C)** Neurodegeneration results from (B), normalized to control counts. Scores compiled for 4 animals in each group (* $p < 0.05$ vs. WT of same condition, # $p < 0.05$ vs. control of same genotype, n = 12 sections per group, independent t-test).
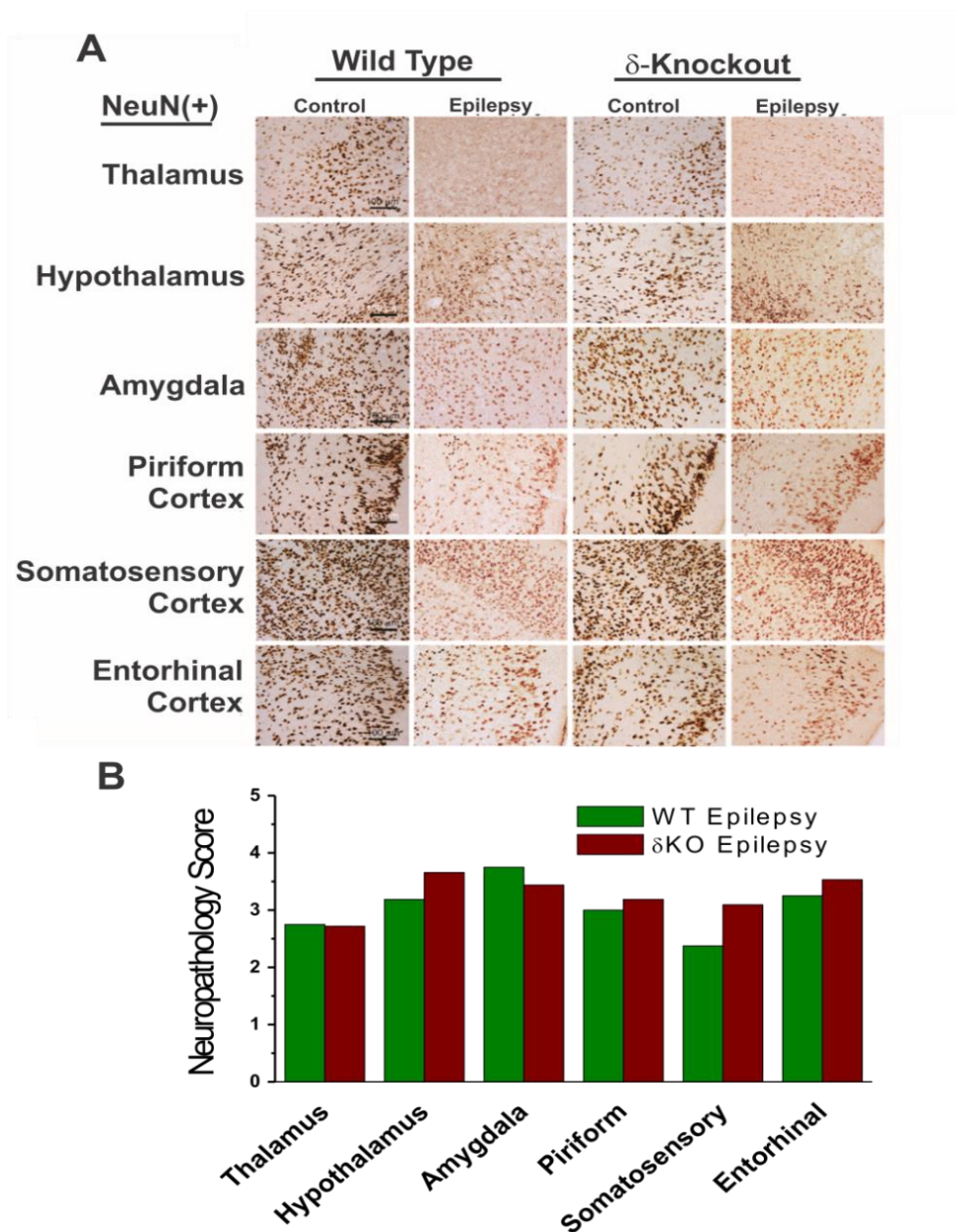
**Figure. 21. NeuN(+) immunohistochemistry in WT and δKO mice in extra-hippocampal regions. (A)** Distribution of NeuN(+) principal neurons in the extra-hippocampus regions at 3 months post-pilocarpine in WT and DKO mice. **(B)** Extra-Hippocampal Neurodegeneration Scores for WT and δKO Pilocarpine-Epilepsy Mice. Bar graph showing relatively more neurodegeneration in WT and δKO mice within some extra-hippocampus regions 90 days following SE. The extent of neurodegeneration in each structure was scored as following: 0 = no neuropathology; 1 = minimal neuropathology (1–10%); 2 = mild neuropathology (11–25%); 3 = moderate neuropathology (26–45%); and 4 = severe neuropathology (>45%). Scores compiled from 4 animals from each group, 12 sections per group.
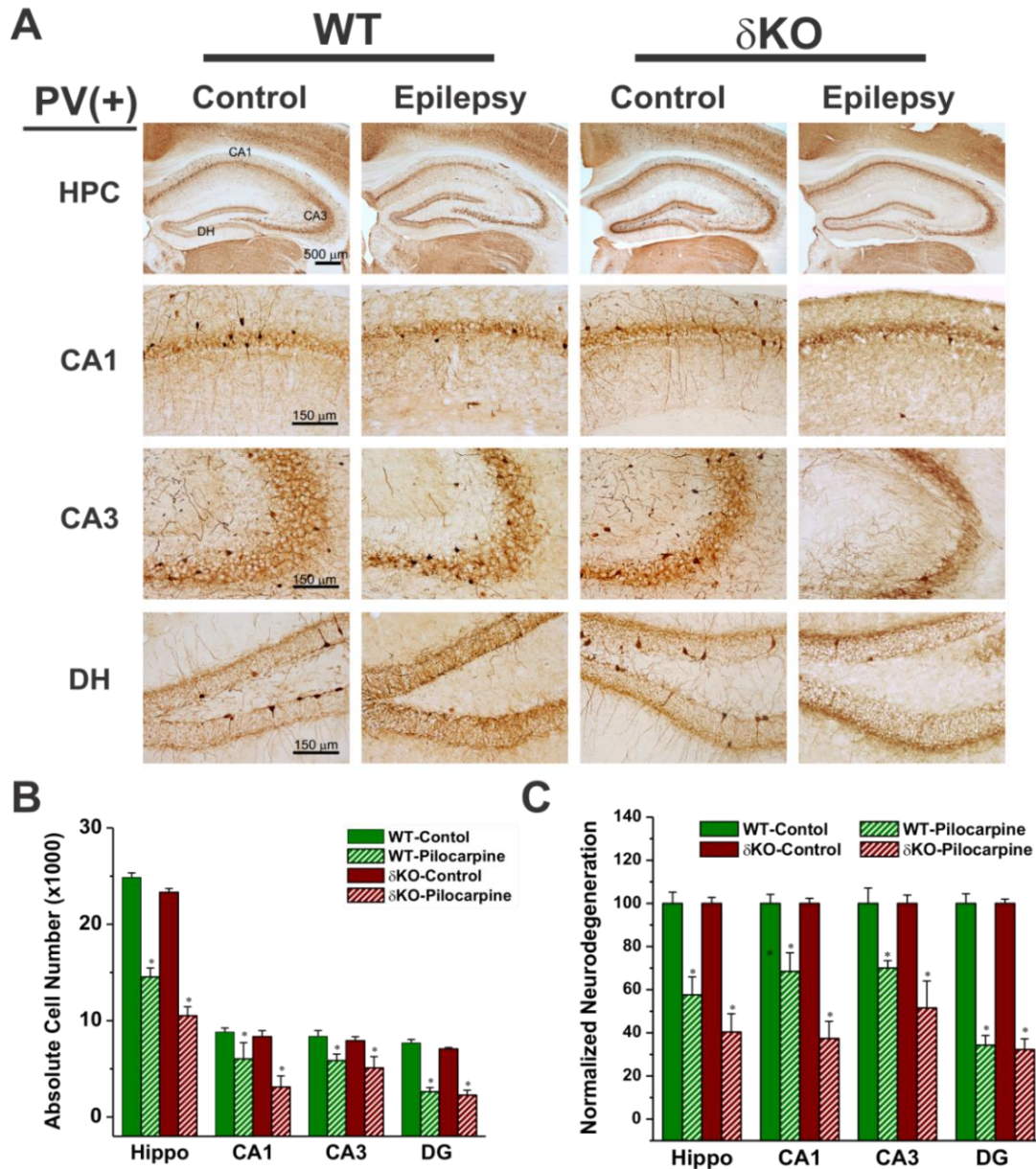
**Figure 22. PV(+) immunohistochemistry in WT and δKO mice.** (A) Distribution of PV(+) GABAergic interneurons in the hippocampus subfields at 3 months post-pilocarpine in WT and δKO mice (B) Absolute cell counts for the hippocampus and hippocampal sub-regions in control and pilocarpine-epilepsy WT and δKO mice 90 days following SE. (**C**) Neurodegeneration results from **(B)**, normalized to control counts δKO mice experienced greater overall loss of PV(+) interneurons and Scores compiled for 4 animals in each group (* $p < 0.05$ vs. WT of same condition, # $p < 0.05$ vs. control of same genotype, n = 12 sections per group, independent t-test). Sections were analyzed with the experimenter blinded to the genotype and condition.
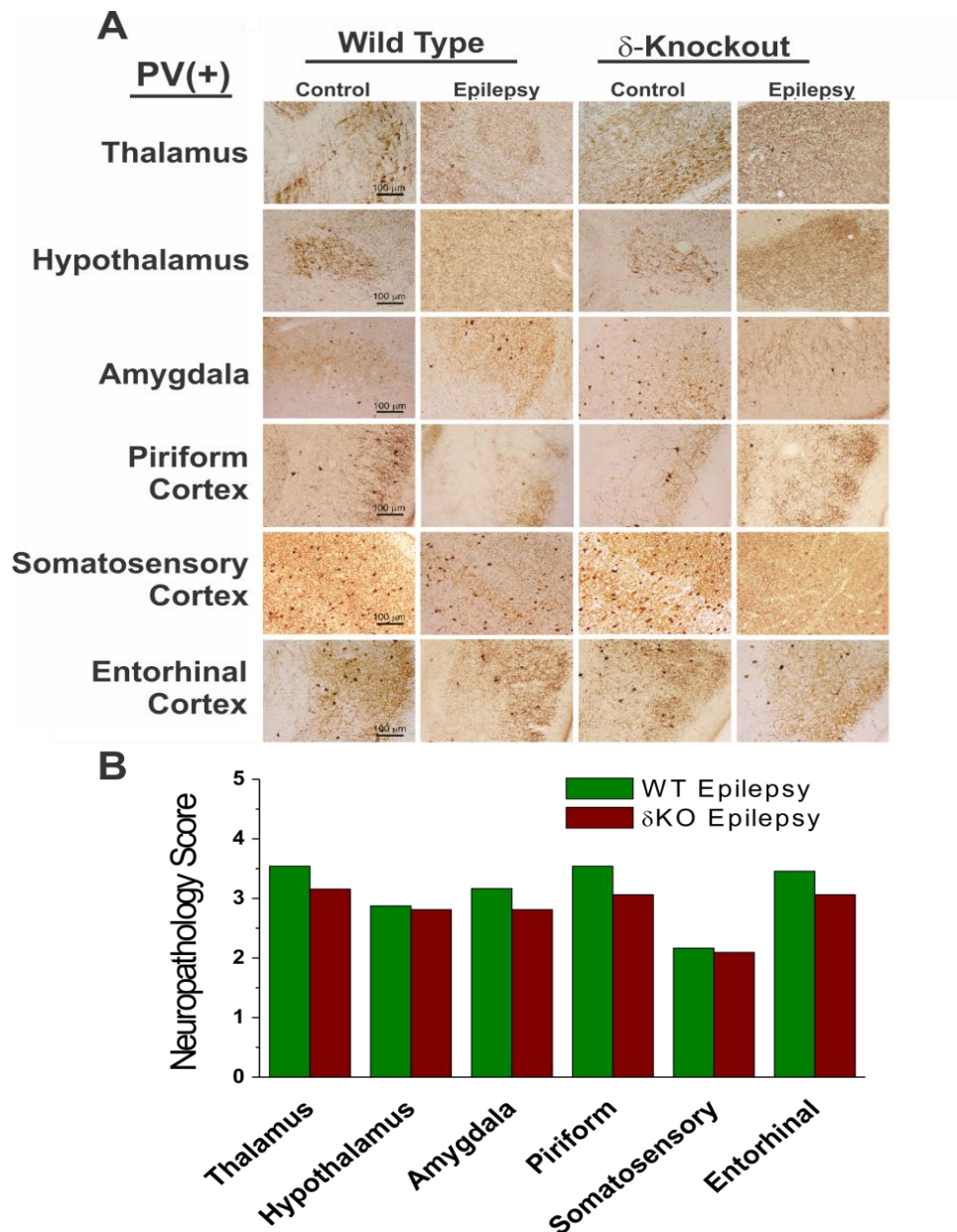
**Figure 23. PV(+) immunohistochemistry in WT and δKO mice in extra-hippocampal regions. (A)** Distribution of PV(+) GABAergic interneurons in the extra-hippocampus regions at 3 months post-pilocarpine in WT and DKO mice. **(B)** Extra-Hippocampal Neurodegeneration Scores for WT and δKO Pilocarpine-Epilepsy Mice. Bar graph showing more neurodegeneration in WT and δKO mice within extra-hippocampus regions 90 days following SE. The extent of neurodegeneration scored as following: 0 = no neuropathology; 1 = minimal neuropathology (1–10%); 2 = mild neuropathology (11–25%); 3 = moderate neuropathology (26–45%); and 4 = severe neuropathology (>45%). Scores compiled from 4 animals from each group, 12 sections per group.
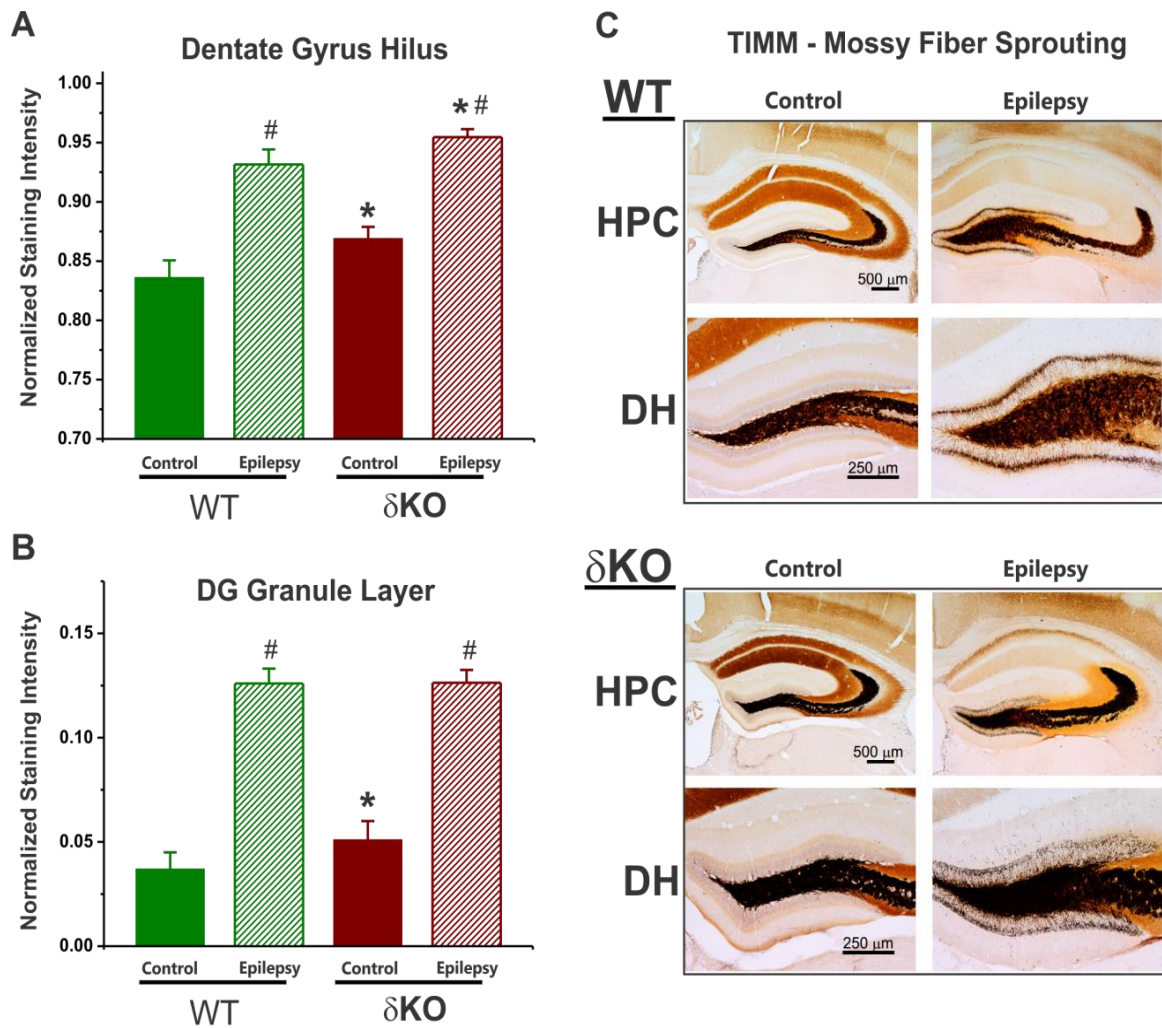
**Figure 24. Timm staining of mossy fiber sprouting. (A)** Stain intensity in DG hilus in control and pilocarpine-epilepsy WT and δKO mice. **(B)** Stain intensity in DG granule layer in control and pilocarpine-epilepsy WT and δKO mice. **(C)** Representative images from control and pilocarpine-epilepsy WT and δKO mice. Staining was significantly denser in the hilus than the granule layer in all conditions. Both genotypes displayed increased histochemical density in the hilus. Furthermore, the δKO mice subject to pilocarpine-SE had significantly greater staining intensity in the granule cell layer than seizure-naïve δKO control animals, indicative of seizure activity-induced mossy fiber sprouting. Scores compiled for 3 animals in each group (* $p < 0.05$ vs. WT of same condition, # $p < 0.05$ vs. control of same genotype, n = 9 sections per group, independent t-test). Sections were analyzed with the experimenter blinded to the genotype and condition.

**IV.3 The role of δ-subunit extrasynaptic GABA-A receptors in the antiepileptogenic actions of neurosteroids**

**IV.3.1 Modulation of endogenous neurosteroids in kindling epileptogenesis**

Our initial studies showed accelerated kindling epileptogenesis in mice lacking δ-GABA-ARs, which lack the sensitivity to neurosteorids and level of tonic inihibition found in WT mice. This provided evidence that neurosteroid activity or the tonic inhibition to which δ-GABA-ARs contribute may play a mediating role in kindling epileptogenesis. To investigate the potential role of endogenous neurosteorids in mediating kindling epileptogenesis, we utilized the same hippocampal kindling protocol with key adaptations providing for multiple distinct routes by which we could modulate neurosteorid levels in WT and δKO mice during the kindling process. We first sought to investigate the effects of increasing endogenous levels of neurosteroids immediately prior to kindling, and utilized the sequential gonadotropin treatment protocol to produce robust and lasting increases in circulating and brain levels of gondal homrones and their neuroactive deritaves, such as AP. Our second strategy for modulating neurosteroids during kindling epileptogenesis involved reducing circulating neurosteroid levels by inhibiting the conversion of progesterone and other hormones to their neurosteroid derivatives using finasteride, a 5α-reductase inhibitor. Using daily injections of finasteride (50 mg/kg, i.p.), we rapidly inhibit neurosteroid synthethis during the kindling process. This provides an opportunity to observe the impact of reduced endogenous neurosteroid levels on kindling epileptogenesis, as well as observe differences imparted by the loss of δ-GABA-ARs using the δKO mice in conjunction with WT cohorts. The third strategy for neurosteroid-based interventions in kindling epileptogensis was centered on the effect of exogenous neurosteroid administration on kindling development. We chose AP and GX, a synthetic analog of AP, for these tests. Daily injection with AP (0.5 mg/kg) reproduced circulating AP levels similar to that observed in gonadotropin treated mice (Fig.25). Furthermore, we also investigated potential dose-dependent effects and to better characterize the differences in

**IV.3 The role of δ-subunit extrasynaptic GABA-A receptors in the antiepileptogenic actions of neurosteroids**

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effectiveness of treatment in WT and δKO mice. Finally, we used combination treatments (gonadotropins or exogenous neurosteroids, and finasteride) to further characterize the nature and potential mechanisms underlying the changes observed in kindling epileptogenesis among our trial cohorts.
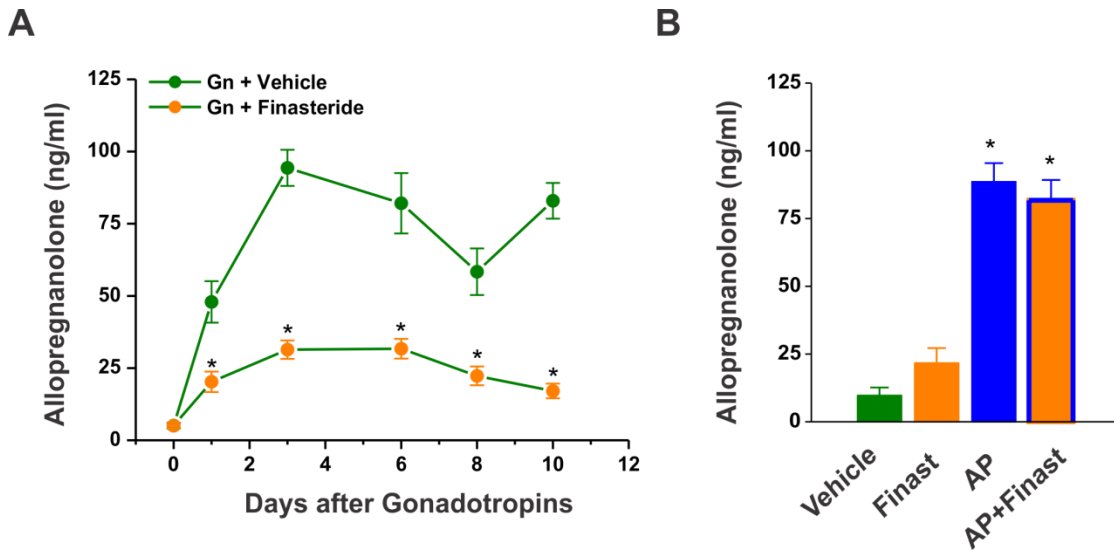


**Figure 25. Plasma levels of neurosteroids in control and drug-treated mice. (A)** Time-course of the AP levels after gonadotropin administration. **(B)** Plasma levels of AP after exogenous administration of AP (0.5 mg/kg, sc), finasteride (50 mg/kg, ip) or their combination. *p<0.05 vs. vehicle control group (N=6-8 mice per group, independent t-test).

### IV. 3.1.1 Enhancing endogenous neurosteroids delays kindling epileptogenesis

To study the role of δ-subunit extrasynaptic GABA-ARs in antiepileptogenic interventions in kindling epileptogenesis, we chose to observe the effect of enhanced endogenous neurosteroids in the hippocampal kindling model of temporal lobe epilepsy in WT and δKO mice. Using the sequential gonadotropin regimen (Reddy et al., 2012), female WT and δKO mice were kindled beginning on day 1, 4 hours following the HCG (5 IU s.c.) injection. We hypothesized that increased levels of circulating AP would increase tonic inhibition in the DG and serve to inhibit or delay the progression of kindling development, and that this effect would likely be observed primarily or most

strongly in WT animals, who express the highly neurosteroid-sensitive δ-GABA-ARs that the δKOs lack. Indeed, gonadotropin treatment conveyed a marked delay in kindling epileptogenesis in WT mice, with Gn treated groups requiring an average of 24.8 stimulations to reach kindled state (Fig.26). This effect was primarily defined by the elimination of the late-stage kindling rate acceleration observed in WT female mice undergoing standard hippocampal kindling. Seizure stage scores for vehicle and gonadotropin treated female mice were nearly identical until day 10 of kindling, when vehicle treated animals average rate of kindling increased. Interestingly, average AD duration did not differ between vehicle and Gn treated WT cohorts. To our surprise, we also observed a robust delay in kindling epileptogenesis in Gn treated δKO mice. Gn treated δKO mice required an average of 19.3 stimulations to reach kindled state, vs. 15.9 in vehicle treated counterparts. While the overall effect of Gn on kindling rate in δKOs was similar to that observed in the WT mice, the dynamics of the effects varied greatly. Gn treated δKOs displayed marked decreases in average seizure score and AD duration from very early in the kindling process. This effect was maintained throughout the kindling process. Evaluating kindling progression by average seizure stage/stimulation reflects the overall kindling rate changes in both genotypes, and reveals a significantly more powerful effect in WT animals. Examination of electrographic seizure traces reflected these findings, and representative traces of each group at key points in kindling are provided following the graphs (Fig 26). These results suggest a protective role for neurosteroids in kindling epileptogenesis.
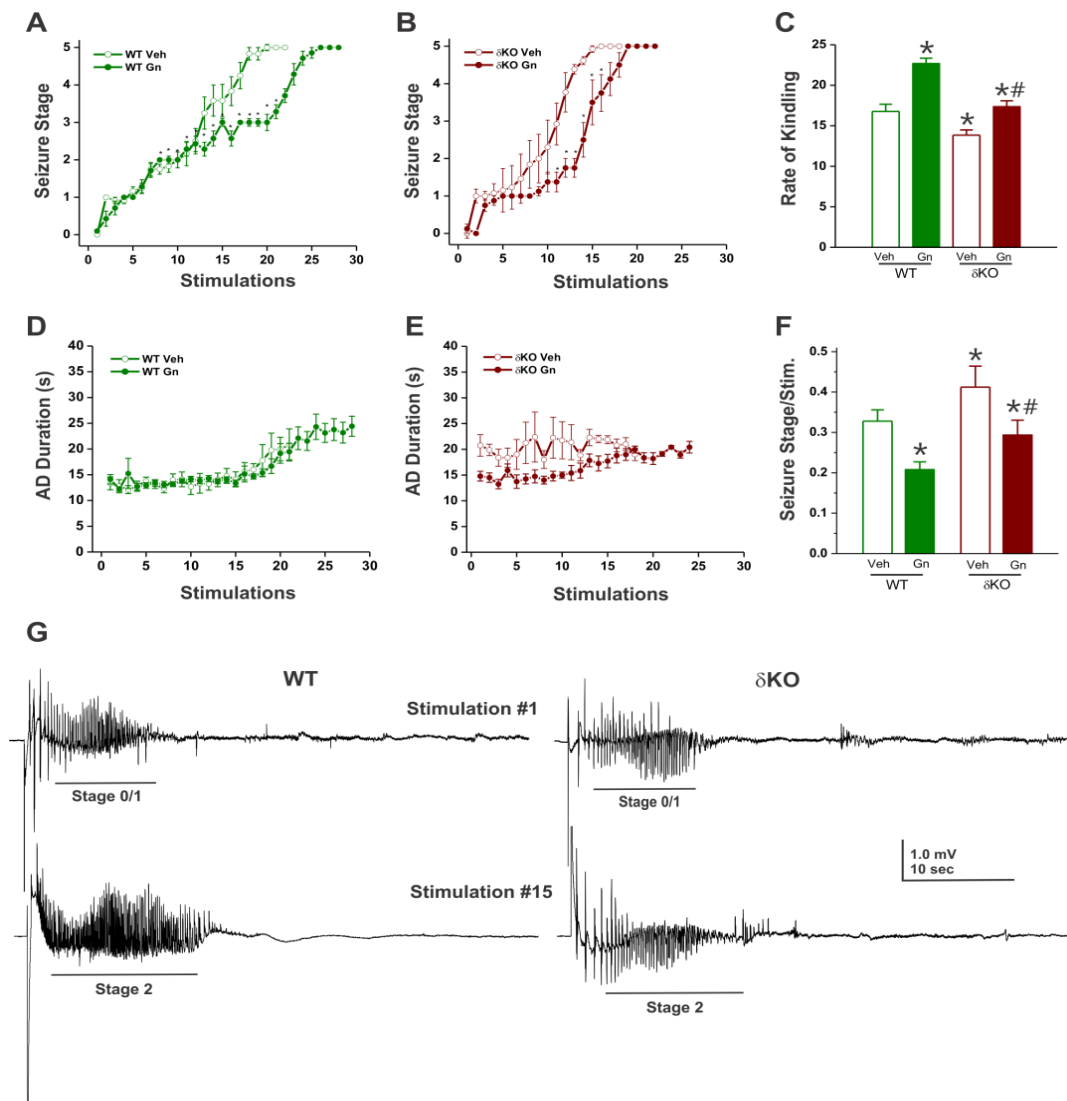
**Figure 26. Gonadotropin-treatment retards kindling epileptogenesis in female WT and δKO mice.**
**(A)** WT female mice treated with gonadotropins displayed delayed kindling epileptogenesis, as expressed by lower seizure stages at corresponding stimulation sessions. **(B)** Female δKO mice treated with gonadotropins also displayed delayed kindling epileptogenesis, as expressed by lower seizure stages at corresponding stimulation sessions. **(C)** AD duration in WT females was not significantly affected by gonadotropin treatment. **(D)** Gonadotropin-treated female δKO mice displayed significantly reduced AD durations than vehicle-treated mice at corresponding stimulations. **(E)** Comparison of gonadotropin effect on hippocampus kindling rates in female WT and δKO mice. **(F)** Analysis of kindling rates in control and Gn-treated female WT and δKO mice, as expressed by seizure stage advancement per stimulation session. (G) Representative traces comparing stimulation-induced electrographic seizures from WT and δKO mice. Values represent mean ± SEM (*$p < 0.05$ vs. control of same genotype, # $p < 0.05$ vs. WT of same condition, n = 8-10 mice/group, independent t-test).

**IV.3.1.2 Inhibiting neurosteroid synthesis accelerates kindling epileptogenesis**

Our second strategy for investigating the role of δ-GABA-ARs in antiepileptogenic neurosteroid-based interventions for kindling epileptogenesis focused on observing the effects of reducing or eliminating circulating neurosteroids during kindling epileptogenesis. We administered daily finasteride (5α-reductase inhibitor) to female WT and δKO mice beginning on day 1 of kindling, 4 hours prior to the first stimulation. AD thresholds were determined prior to initiating kindling. We hypothesized that the inhibition of neurosteroid synthesis and subsequent loss of tonic inhibition in the DG would lead to accelerated kindling and exacerbated seizures. This effect was expected to be more pronounced in WT mice, in which the sudden reduction in neurosteroid levels would result in a greater shift in inhibition than would be experienced by the less neurosteroid-sensitive δKO mice. However, finasteride treated mice of both genotypes experienced a significant acceleration of kindling epileptogenesis and acute seizure exacerbation starting from the first stimulation (Fig.27). Treated WT mice reached fully kindled state in 9.2 stimulations, more than twice as fast as vehicle treated counterparts. Fn-treated δKO mice also reached fully-kindled state significantly faster than δKO controls, reaching kindled-state in 8.6 stimulations, compared to 15.9 in controls. As predicted, the left-shift of the kindling curve was more significant in WT mice, though clearly powerful in both genotypes. Initial AD durations (Day 0) were similar between treatment and vehicle groups in both genotypes, though the AD durations in Fn treated WT and δKO mice increased significantly and consistently through each seizure stage and continued to increase until the 10th stimulation, where they were 150% (WT) and 52% (δKO) higher than those observed in respective vehicle groups. Examination of electrographic seizure traces reflected these findings, and representative traces of each group at key points in kindling illustrate the rapid epileptogenesis described above (Fig. 27).
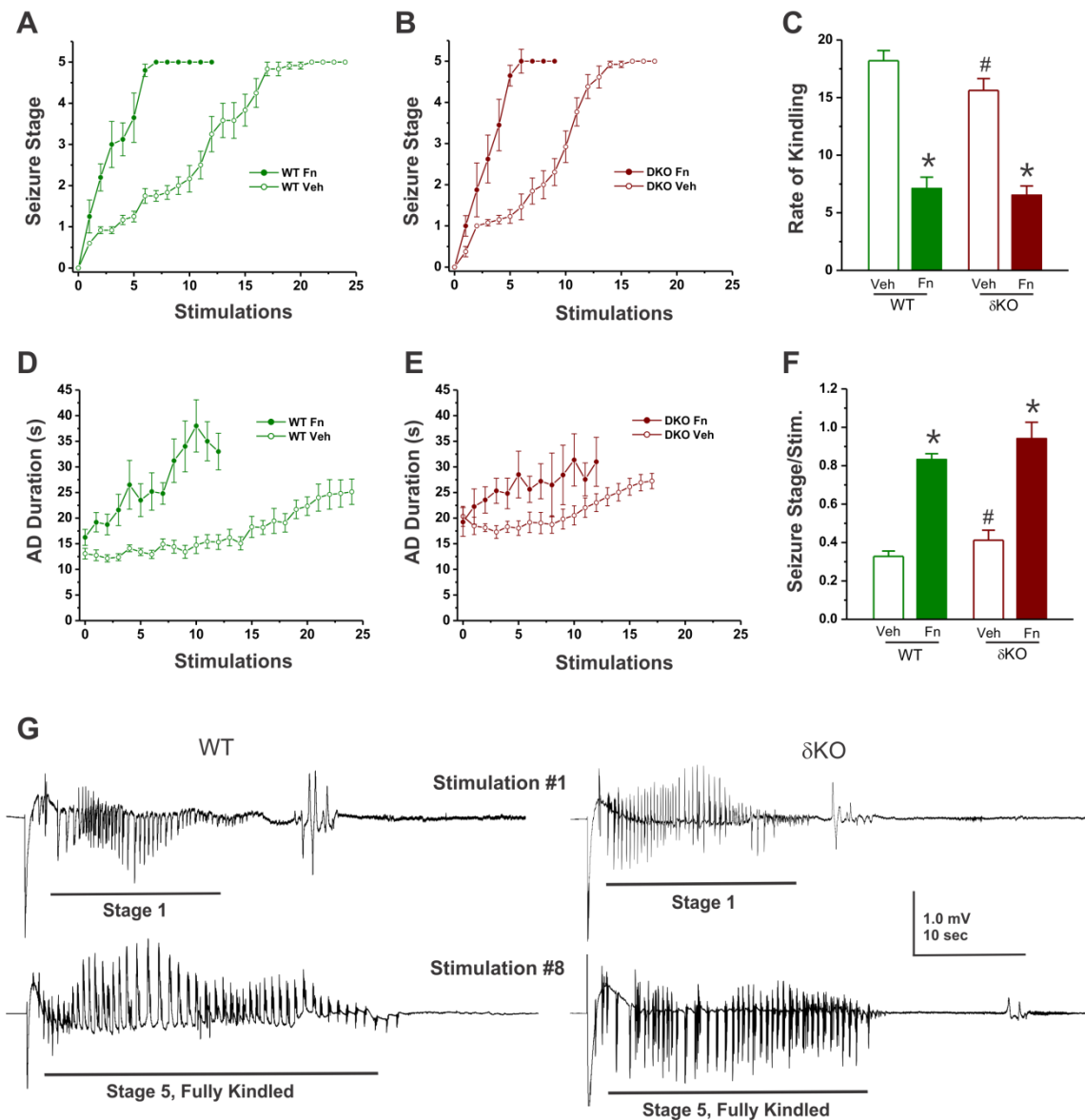
**Figure 27. Effect of finasteride-inhibition of NS synthesis on kindling epileptogenesis in WT and δKO mice. (A) & (B)** Female WT (A) and δKO (B) mice treated with finasteride prior to stimulations displayed marked acceleration in kindling epileptogenesis, as expressed by higher seizure stage scores than controls at corresponding stimulations. **(C)** Comparison of finasteride's effect on hippocampus kindling rates in female WT and δKO mice. **(D) & (E)** Finasteride-treated WT (D) and δKO (E) mice displayed significantly increased AD durations across all stimulations sessions. **(F)** Analysis of kindling rates in control and Fn-treated female WT and δKO mice, as expressed by seizure stage advancement per stimulation session. **(G)** Representative traces comparing stimulation-induced electrographic seizures from Fn-treated WT and δKO cohorts. Values represent mean ± SEM (*p<0.05 vs. control of same genotype, # p < 0.05 vs. WT of same condition, n = 8-10 mice/group, independent t-test).

**IV.3.1.3 Finasteride inhibits the disease-modifying effects of gonadotropins on kindling epileptogenesis**

We next sought to confirm the pivotal role of neurosteroids in this effect through a combination treatment consisting of both sequential gonadotropin pretreatment and daily finasteride administration. If hormonal changes or other effects of gonadotropin treatment were responsible for the inhibition of kindling epileptogenesis observed in our prior study, we would expect to observe some degree of antiepileptogenic effect persisting after the loss of neurosteroid synthesis due to finasteride administration. In WT mice, finasteride completely abolished the antiepileptogenic effects of gonadotropin treatment and resulted in accelerated epileptogenesis, particularly beyond stimulation 5 (Fig.28). WT animals treated with Gn+Fn required an average of 16.7 stimulations to reach fully kindled state, ~15% faster than vehicle counterparts. In δKO mice, finasteride eliminated the antiepileptogenic effects of gonadotropin treatment, but did not result in accelerated epileptogenesis compared to vehicle treated kindling rates. AD duration was increased in treated WT mice, with particularly prominent differences during late stage kindling. Interestingly, inhibition of neurosteroids following gonadotropin treatment in δKO mice resulted not in an increase, but in a decrease in AD duration. Compared to vehicle δKO mice, treated animals expressed shorter ADs for the duration of the kindling process, supporting the notion that modulation of endogenous neurosteroids have a less powerful impact on the inhibitory state of mice lacking δ-GABA-ARs. Indeed, the observation of unchanged kindling rate and decreased AD durations in δKOs points to persisting, neurosteroid- or δGABA-AR-independent antiepileptogenic effect of gonadotropin treatment in these mice. Examination of electrographic seizure traces reflected these findings, and representative traces of each group at key points in kindling illustrate the effect described above (Fig 28).
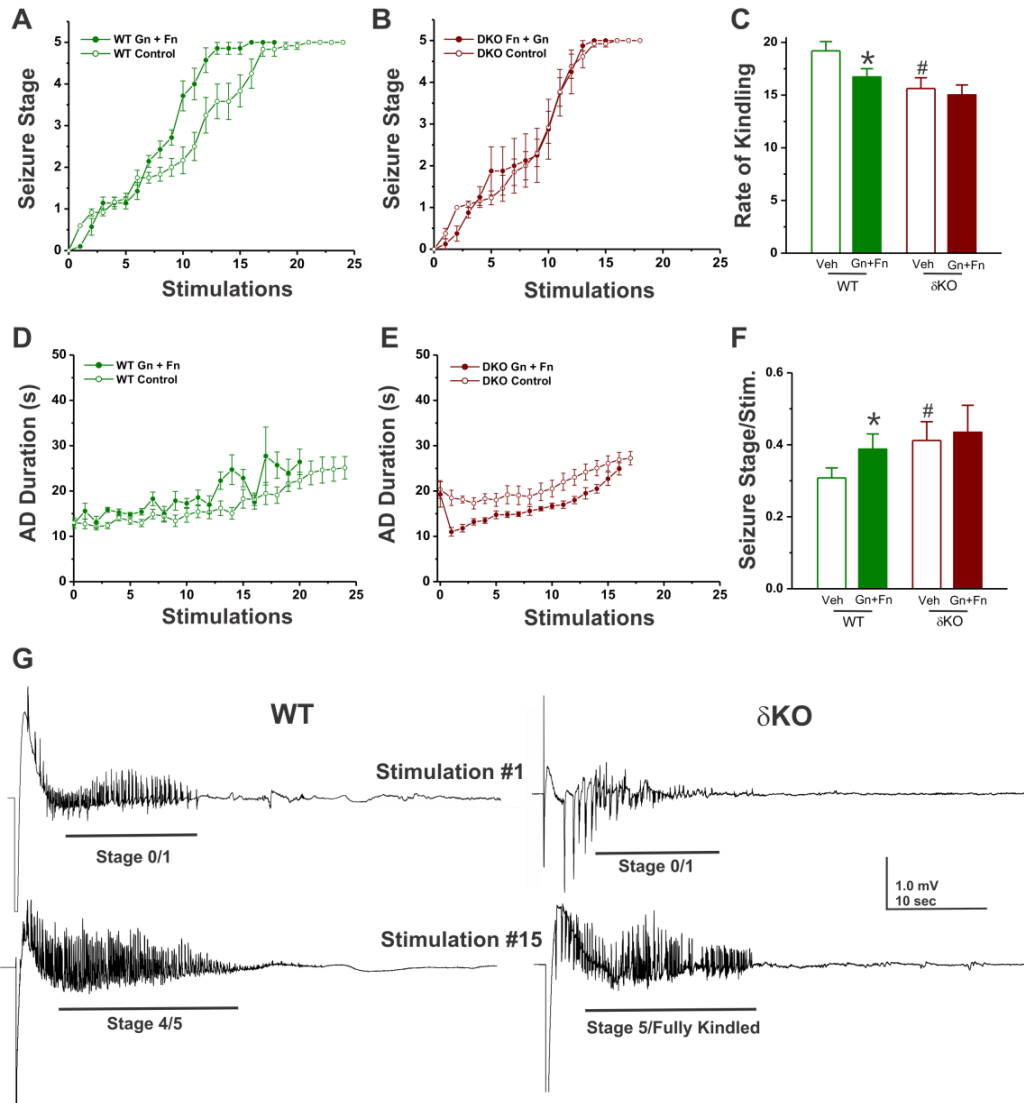
**Figure 28. Finasteride inhibition of disease-modifying effects of gonadotropins on kindling epileptogenesis in female WT and δKO mice. (A) & (B):** Finasteride-treatment inhibited the gonadotropin-derived retardation of kindling epileptogenesis in female WT (A) and δKO (B) mice, as expressed by increased average seizure stage scores at corresponding stimulation sessions. **(C)** Comparison of finasteride's effect on hippocampus kindling rates in gonadotropin-treated female WT and δKO mice. **(D) & (E):** Finasteride treatment also inhibited the AD-reducing effect of gonadotropin treatment in female WT mice (D), and resulted in a small reduction in AD duration in δKO (E) mice. **(F)** Analysis of kindling rates in control and treated WT and δKO mice, as expressed by seizure stage advancement per stimulation session.   **(G)** Representative traces comparing stimulation-induced electrographic seizures from Fn and Gn-treated WT and δKO mice. Values represent mean ± SEM (*p<0.05 vs. control of same genotype, #p<0.05 vs. WT of same condition, n = 8-10 mice/group, independent t-test).

**IV.3.2 Exogenous administration of natural and synthetic neurosteroids in hippocampal kindling epileptogenesis**

Our investigations into the role of δ-GABA-ARs in the disease-modifying effects of neurosteroid modulation during kindling epileptogenesis revealed enticing data supporting a critical mediating role for endogenous neurosteroid signaling. To further investigate the antiepileptic potential of neurosteroid-based interventions in kindling epileptogenesis, we sought to examine the effects of administration of exogenous neuosteroids. For these experiments we selected AP and its synthetic analog Ganaxolone. Hippocampal kindling was performed as in previous experiments, with daily injections of the selected neurosteroid 15 minutes prior to kindling. We also sought to expand our investigation by using female and male cohorts of both genotypes for our studies using AP and GX, respectively.

**IV.3.2.1 Exogenous allopregnanolone inhibits kindling epileptogenesis**

Our first investigation of δ-GABA-ARs role in the disease-modifying effects of exogenous neurosteroid administration consisted of observing kindling epileptogenesis in female WT and δKO mice given daily AP (0.5 mg/kg, s.c.) 15 minutes prior to kindling stimulations, starting with the first kindling stimulation. Considering the evidence provided by our gonadotropin experiments, we hypothesized that chronic AP treatment would result in a similar inhibition of kindling epileptogenesis in WT and δKO mice (Fig.29). Indeed, AP treatment resulted in a striking inhibition of kindling epileptogenesis in WT mice. This effect was most prominent in late stage kindling, in a manner similar to that observed in gonadotropin treated WT mice. To our surprise this effect was equally strong in δKO mice. This effect was observed throughout the kindling process, in a stronger but similar manner to that observed in gonadotropin treated δKO mice. AD durations in WT mice treated with AP did not significantly differ from that of controls until late stage kindling, when control mice neared fully kindled state. In δKO mice, AP treatment significantly reduced AD duration across all stimulations. Examination of electrographic seizure traces reflected these findings, and representative

traces of each group at key points in kindling illustrate the powerful antiepileptogenic effect conveyed by AP treatment (Fig. 29).
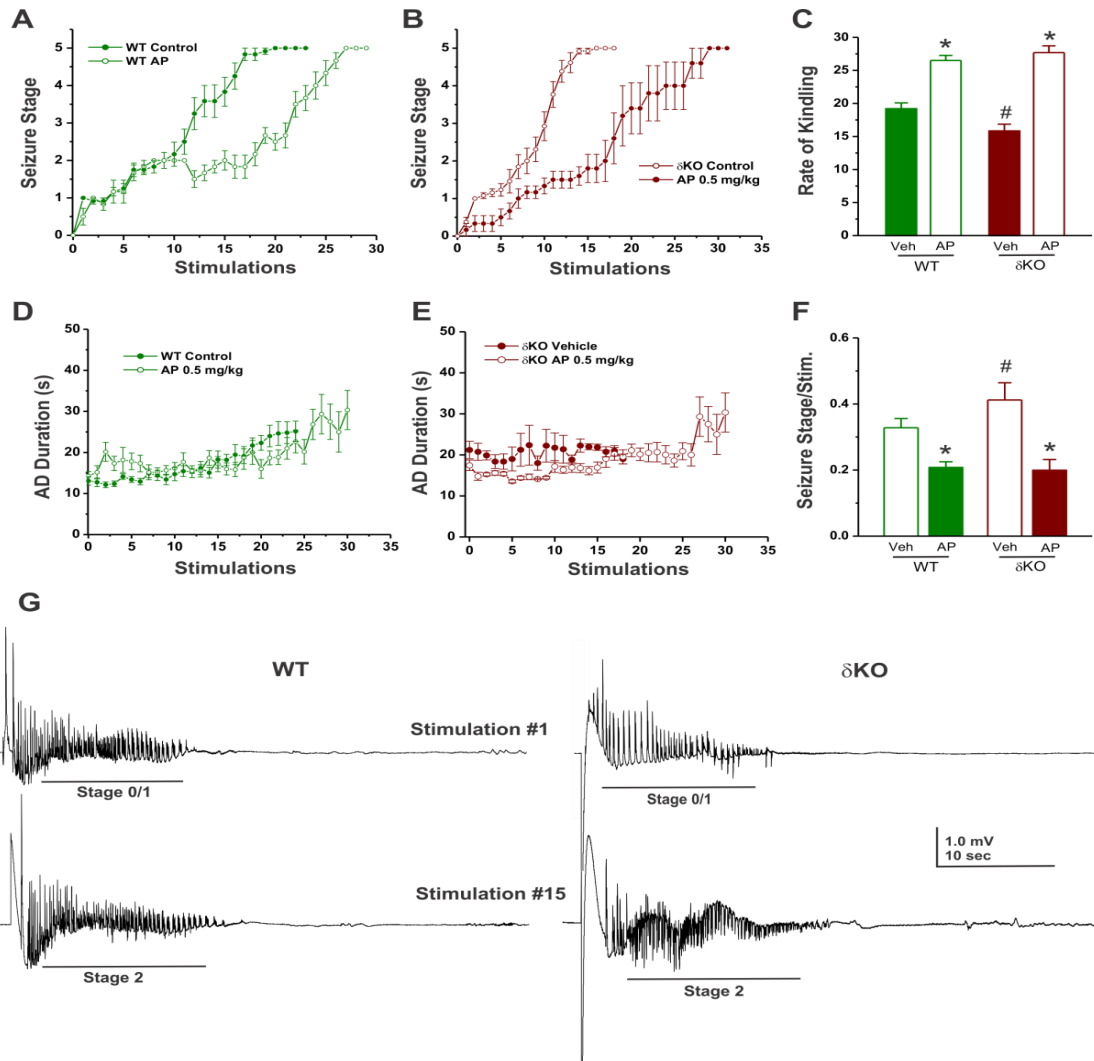


**Figure 29. Effect of allopregnanolone on kindling epileptogenesis in female WT and δKO mice. (A & B)** Female WT (A) and δKO (B) mice treated with AP displayed significantly retarded kindling epileptogenesis, as expressed by lower seizure stages at corresponding stimulation sessions. **(C)** Comparison of AP's effect on hippocampus kindling rates in female WT and δKO mice. (**D**) AD duration in WT females was not significantly affected by AP-treatment until late stage kindling. (**E**) AP-treated female δKO mice displayed reduced AD durations. (**F**) Analysis of kindling rates in control and AP-treated female WT and δKO mice, **(G)** Representative traces of electrographic kindling seizures from AP-treated WT and δKO mice. Values represent mean ± SEM (*p<0.05 vs. control of same genotype, #p<0.05 vs. WT of same condition, n = 8-10 mice/group).

**IV.3.2.2 Finasteride does not inhibit the disease-modifying effects of allopregnanolone on kindling Epileptogenesis in WT female mice**

Having observed modulation of kindling epileptogenesis with gonadotropin treatment as well as daily AP treatment, we sought to confirm the role of neurosteroids in this effect through a combination treatment consisting of daily AP and finasteride administration. If hormonal changes or other neurosteroid-independent effects of gonadotropin treatment were partially responsible for the inhibition of kindling epileptogenesis observed in our prior studies, we would expect to observe a diminished antiepileptogenic effect of AP treatment in animals concurrently receiving daily finasteride as well. Compared to AP-only cohorts, concurrent finasteride treatment had no effect on the course of kindling progression in AP-treated WT female mice (Fig.30). Seizure stage scores at corresponding stimulations were not significantly different between AP and AP + Fn groups for the duration of kindling, with both groups experiencing a significant delay in kindling epileptogenesis compared to vehicle-treated WT controls. AD duration in the AP + Fn cohort was more variable than that observed in either control or AP treated cohorts, and tended to be slightly higher than vehicle cohorts during early stage kindling. AD durations were not significantly different between AP + Fn and control cohorts beyond the 9th kindling stimulation (Fig.30). Rate of kindling for AP + Fn-treated WT female mice were significantly higher than controls, reflecting a delay in kindling epileptogenesis similar to that observed in AP-only cohorts (Fig.30). These observations serve as strong evidence for the neurosteroid-specificity of the disease-modifying effect of gonadotropin treatment on kindling in our previous studies, and further demonstrate a protective role for neurosteroids in kindling epileptogenesis.
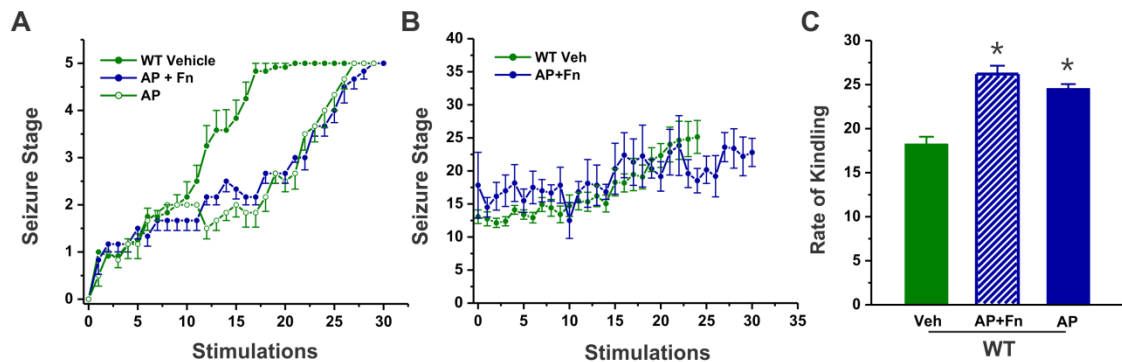
**Figure 30. Finasteride does not inhibit the disease-modifying effects of allopregnanolone on kindling epileptogenesis in female WT mice. A:** Daily finasteride-treatment failed to alter the observed retardation of kindling epileptogenesis in female WT treated with AP (0.5 mg/kg) (A), expressed no change in average seizure stage scores between AP-only and AP + Fn treated mice at corresponding stimulation sessions. **(B)** AP + Fn treated animals displayed AD durations similar to those of control animals across all stimulation sessions. **(C)** Comparison of finasteride's effect on hippocampus kindling rates in control, AP-treated, and AP + Fn-treated female WT mice. Data represents the mean ± SEM (N=8-10 mice/group, *p<0.05 vs. WT vehicle treated mice, independent t-test).

## IV.3.2.3 Effect of high-dose allopregnanolone and 3β-allopregnanolone on kindling epileptogenesis in WT female mice

To further verify our findings of kindling modulation using low-dose AP, we sought to investigate the effect of daily high-dose AP during kindling epileptogenesis. Furthermore, we replicated this study using high-dose 3β-AP, which is inactive at δ-GABA-ARs, to investigate potential mechanisms of disease-modification outside of our hypothesis. Significant inhibition of kindling epileptogenesis was observed in female WT mice treated with 3.0 mg/kg AP, as expressed by significantly reduced seizure stage scores at corresponding stimulation sessions (Fig.31). Indeed, we observed a lack of kindling completion in these animals even after a greatly extended number of hippocampal kindling stimulations, with treated animals never exceeding level 2 seizures after 35 stimulations. High-dose AP (3.0 mg/kg) treatment reduced AD duration in WT female mice in later stages where control groups exhibit increased AD durations compared to consistent ADs in the high-dose AP cohort. Conversely, treatment with high-dose 3B-AP (3.0 mg/kg) had no effect on hippocampal kindling rates in WT female

mice, as expressed by similar seizure stage scores at corresponding stimulation sessions. 3B-AP had no effect on AD duration in WT female mice (Fig.31). Comparison of high-dose 3B-AP and AP effect on hippocampus kindling rates in female WT mice supports the neurosteroid specificity of the observed antiepileptogenic actions of AP. Analysis of kindling rates in control, high-dose 3B-AP, and high-dose AP treated female WT mice confirm the lack of effect and near-complete inhibition of kindling epileptogenesis.
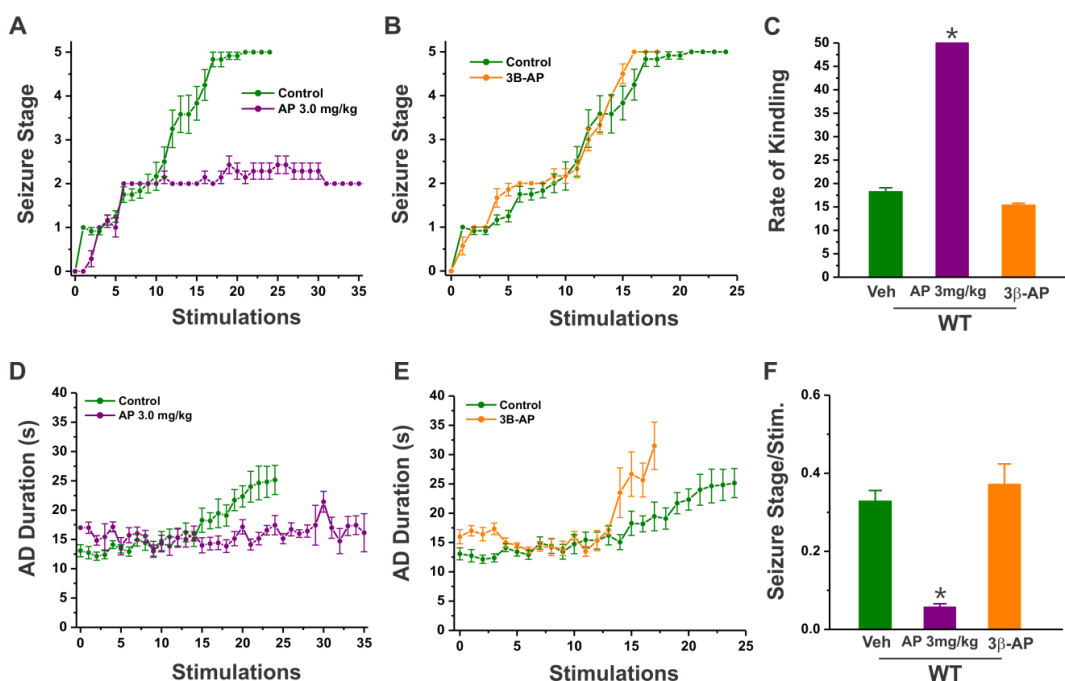


**Figure 31. Effect of high-dose allopregnanolone and 3β-allopregnanolone on kindling epileptogenesis in WT female mice. (A)** Significant inhibition of kindling epileptogenesis was observed in female WT mice treated with 3.0 mg/kg AP, as observed by significantly reduced seizure stage scores at corresponding stimulation sessions, reflecting a lack of kindling completion in these animals even after greatly extended hippocampal kindling stimulations. **(B)** Treatment with high-dose 3B-AP (3.0 mg/kg) had no effect on hippocampal kindling rates in WT female mice, as expressed by similar seizure stage scores at corresponding stimulation sessions. **(C)** High-dose AP (3.0 mg/kg) treatment reduced AD duration in WT female mice, as expressed by reduced average AD durations at corresponding stimulation sessions. **(D)** 3B-AP had no effect on AD duration in WT female mice. **(E)** Comparison of high-dose 3B-AP and AP effect on hippocampus kindling rates in WT mice. **(F)** Analysis of kindling rates in control, high-dose 3B-AP and high-dose AP treated female WT mice. Data represents the mean ± SEM (N=8 mice/group, *$p < 0.05$ vs. WT vehicle treated mice, independent t-test).

**IV.3.3 Exogenous administration of synthetic neurosteroid ganaxolone in kindling epileptogenesis**

We sought to evaluate the antiepileptogenic effectiveness of the synthetic neurosteroid ganaxolone in kindling epileptogenesis. We selected three doses for this test: 0.5 mg/kg, 1.0 mg/kg, and 3.0 mg/kg. The lowest dose corresponds to the circulating NS levels observed following gonadotropin treatment. The 1.0 mg/kg and 3.0 mg/kg doses were selected as incremental increases from our "baseline" 0.5 mg/kg dose such that we might investigate for the potential of dose-dependent nature of antiepileptogenic effects of synthetic neurosteroid treatment during kindling epileptogenesis. GX treatment inhibited kindling epileptogenesis in a dose-dependent manner in both WT and δKO mice. 0.5 mg/kg GX caused a significant delay in kindling in WT and δKO mice, and this effect was observed much more strongly in WT mice than in δKO mice (Fig.32), reflecting the lower sensitivity of these animals to neurosteroids. AD durations in 0.5 mg/kg groups followed a pattern similar to that observed in seizure stage progression, with treated WT and δKO mice displaying significantly lower AD durations than their respective controls after the 5th kindling stimulation. In WT mice the effect 1.0 mg/kg GX on seizure stage and AD progression was similar to that of 0.5 mg/kg, with nearly identical kindling rates and AD durations observed in both groups. In δKO mice 1.0 mg/kg provided greater protection than 0.5 mg/kg, with δKO mice displaying reduced AD durations similar to that of WT mice undergoing the same treatment. Interestingly, δKO mice tended to exhibit low level seizure stages (1-2) longer than WT mice, but experienced more rapid progression in late stage kindling, leading to similar kindling rates in both genotypes at this dose. 3.0 mg/kg GX had a profound effect on kindling epileptogenesis that was similar in both WT and δKO mice (Fig.32). Neither WT nor δKO mice progressed beyond level 2 Racine seizures, even after a greatly extended kindling process. AD durations were greatly reduced across all stimulations in both genotypes, and beyond an initial drop and increase observed in WT, remained constant throughout the kindling process. Data from each dose is provided in Fig. 33, and a summary of these findings is

presented in Fig. 33, along with an assessment of kindling rates and seizure progression. The dose-dependent nature of the effect can be clearly seen in the sequential right-shift of kindling curves, the reduction in AD durations, and the delayed seizure stage progression. Representative seizure traces of each group at key points in kindling illustrate the powerful antiepileptogenic effect conveyed by GX treatment (Fig. 34).
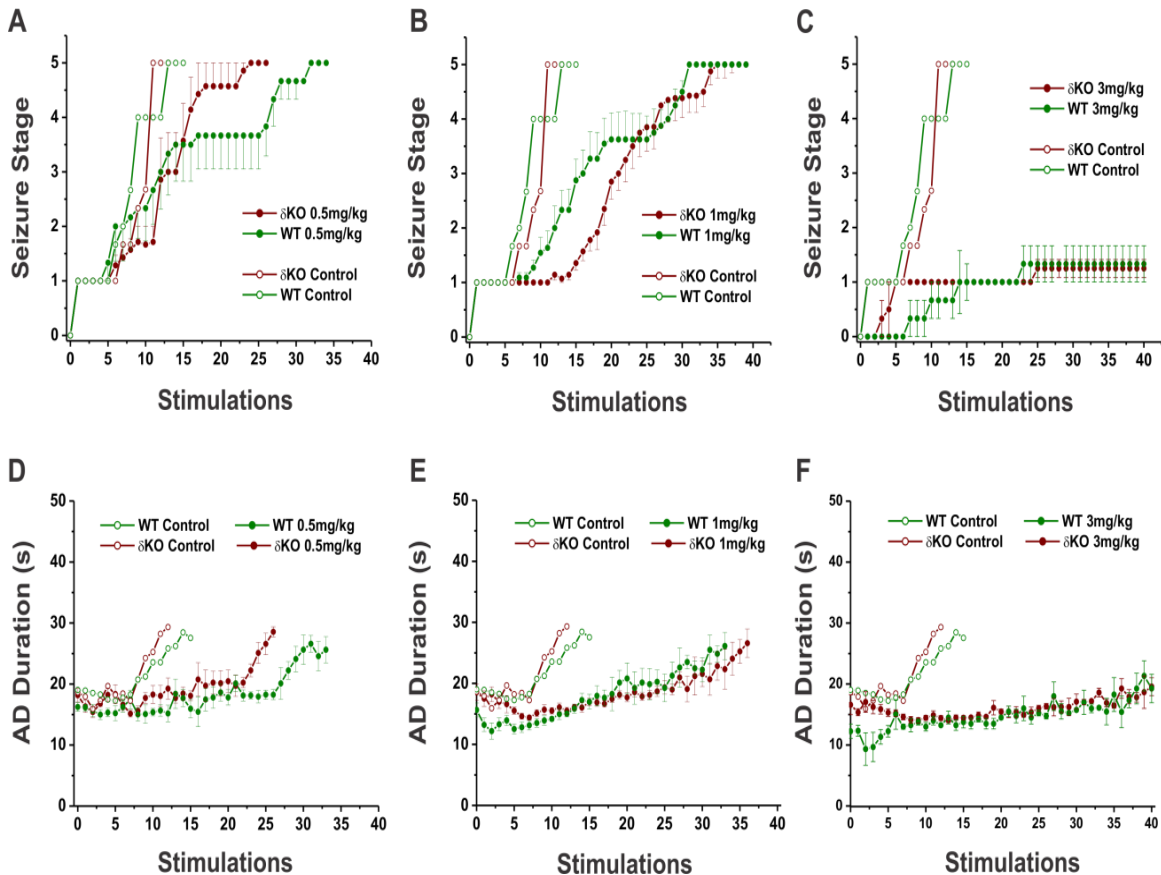


**Figure 32. Effect of synthetic neurosteroid ganaxolone on Kindling epileptogenesis in WT and δKO male mice. (A, B, C):** Comparison of kindling progression between control and GX treated (A - 0.5 mg/kg, B – 1.0 mg/kg, C – 3.0 mg/kg) male WT and δKO mice. GX-treatment retarded progression of kindling epileptogenesis in a dose-dependent manner in both WT and DKO cohorts. (**D, E, F):** Comparison of AD duration across all GX-treated cohorts (D – 0.5 mg/kg, E – 1.0 mg.kg, F – 3.0 mg/kg). GX treatment reduced AD durations in a similar manner, regardless of dose, in both WT and δKO mice. Values represent mean ± SEM (n = 8-10 per group).
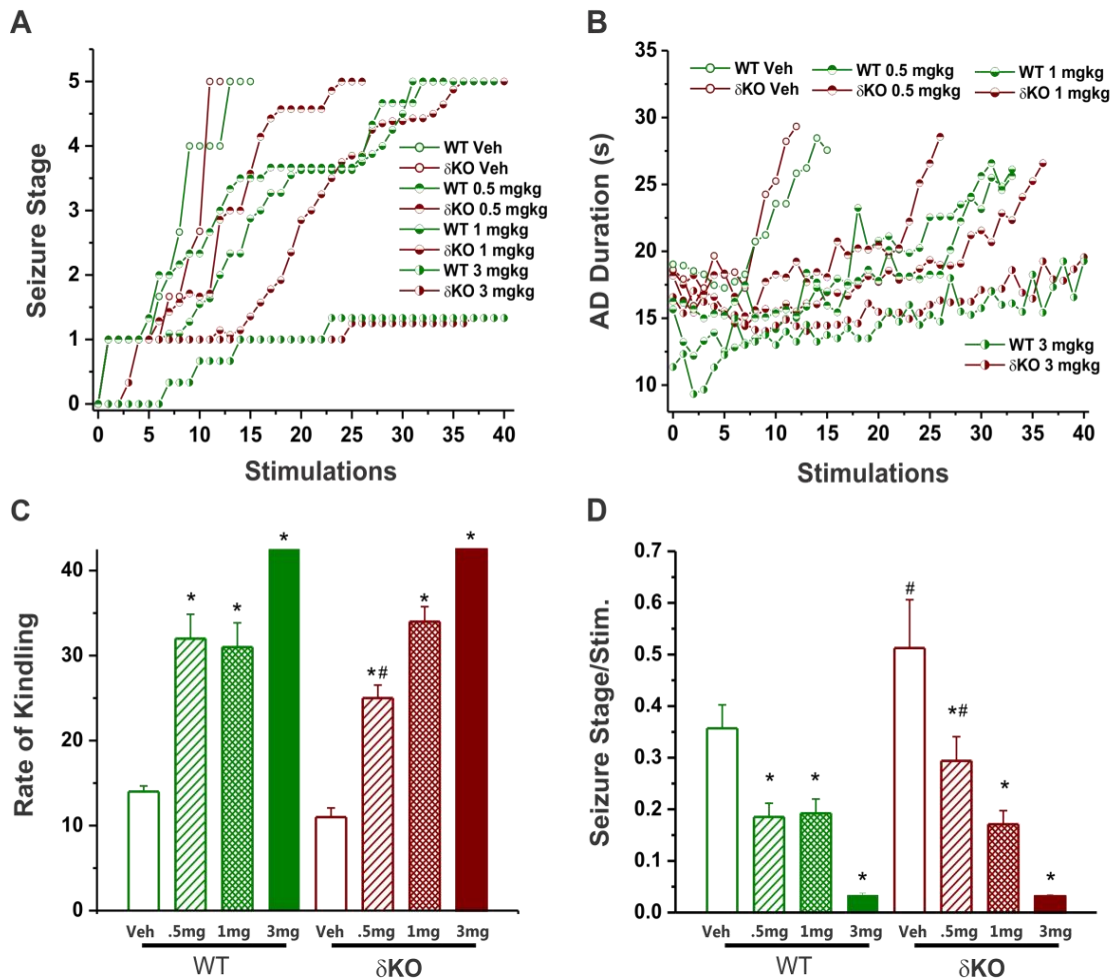
**Figure 33. Summary of ganaxolone effect on hippocampal kindling in WT and δKO mice. (A)** Comparison of kindling progression across all GX-treated WT and δKO male cohorts, as expressed by seizure scores at corresponding stimulation sessions. **(B)** Comparison of AD duration progression across all GX-treated WT and δKO male cohorts. **(C)** Comparison of hippocampus kindling rates across all GX-treated WT and δKO male cohorts, as expressed by number of stimulations required to reach fully kindled state. **(D)** Analysis of Kindling Progression across all GX-treated WT and δKO male cohorts, as expressed as seizure stage advancement per stimulation. **(E)** Representative traces comparing stimulation-induced electrographic seizures from WT and δKO mice during GX-treated kindling epileptogenesis, at 0.5, 1.0, and 3.0 mg/kg doses. Values represent mean ± SEM (*$p<0.05$ vs. control of same genotype, # $p < 0.05$ vs. WT of same condition, n = 8-10 per group).
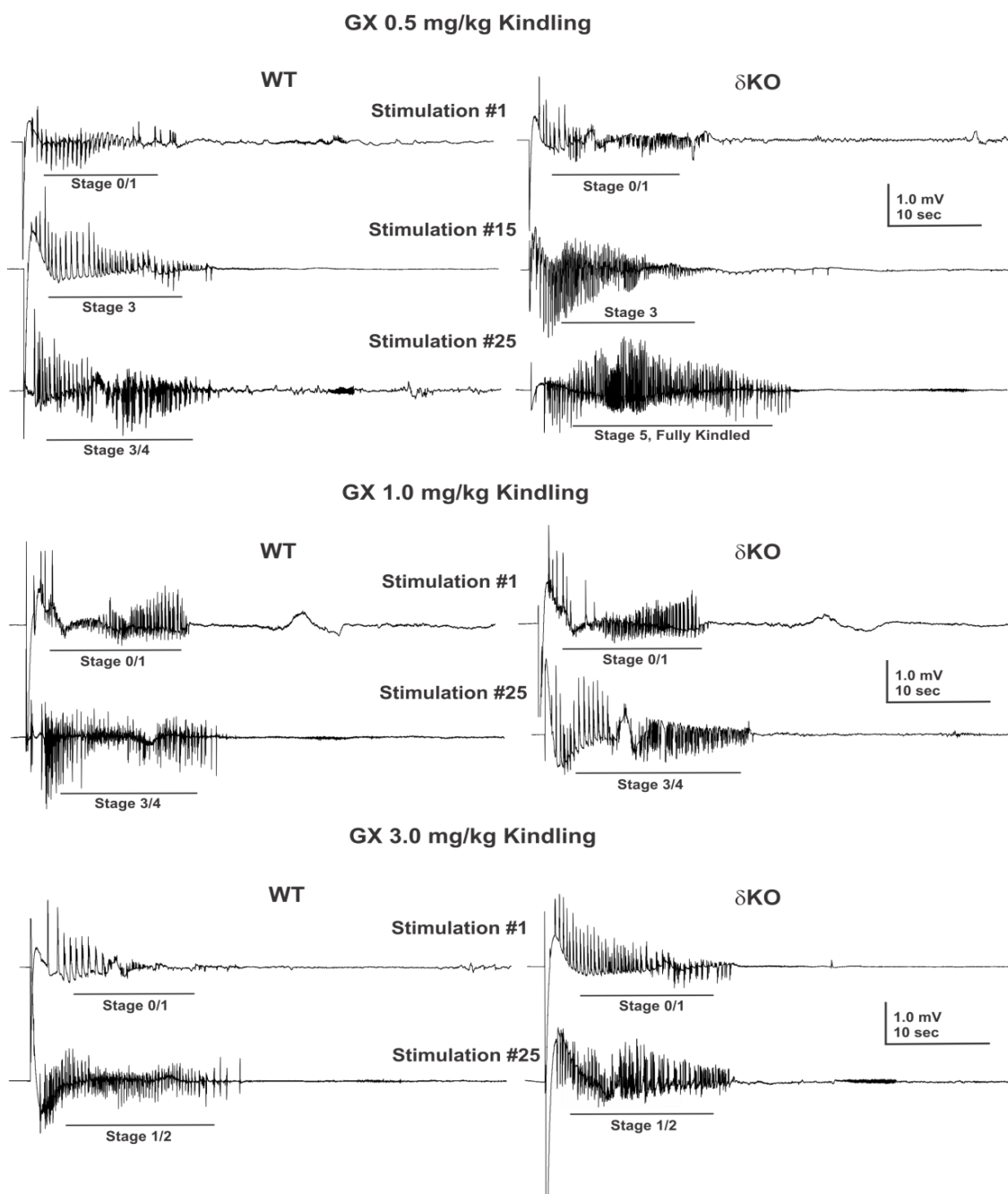
**Figure 34. Representative kindling traces from ganaxolone treated WT and δKO mice.** GX treatment was associated with a marked inhibition of kindling epileptogenesis. At 0.5 mg/kg dose, WT animals displayed a greater delay in epileptogenesis than δKO mice. At 1.0 and 3.0 mg/kg, WT and δKO mice did not significantly differ in kindling rate. Representative traces from WT and δKO mice at critical points in kindling are presented above.

### IV.3.3.1 Mossy fiber sprouting in kindled animals

Mossy fiber sprouting is one of the hallmark characteristics of chronic epilepsy. We investigated the extent of mossy fiber sprouting in naïve WT and δKO mice, as well as in fully kindled animals of each genotype. In addition to these groups, we also examined the potential effect of ganaxolone treatment during kindling, including fully-kindled animals of both genotypes from 0.5 mg/kg and 1.0 mg/kg groups. 3.0 mg/kg groups never reach the fully kindled state and were excluded from this assessment. Staining was significantly denser in the hilus than the granule layer in all conditions. Control δKO mice displayed greater stain density than their WT counterparts, and kindled animals of both genotypes displayed increased histochemical stain density in the hilus and DG granule layer than their respective controls (Fig.35). Furthermore, the δKO mice subject to kindling had significantly greater staining intensity in the granule cell layer than seizure-naïve δKO control animals, indicative of seizure activity-induced mossy fiber sprouting. GX treatment conveyed a dose-dependent reduction in stain intensity in the granule layer of WT and δKO mice when compared to respective kindled control animals. However, this effect was not as clear in the hilus. Kindling induced increased stain density in the hilus of both genotypes, and WT mice treated with 0.5 mg/kg had reduced stain intensity when compared to kindled controls. A similar effect was observed in δKO mice treated with 1.0 mg/kg GX. Interestingly, in WT mice treated with 1.0 mg.kg GX, and δKO mice treated with 0.5 mg/kg, GX did not have a significant effect on stain density in the granule layer (Fig.35). These results provide evidence for increased mossy fiber sprouting in δKO mice, and for the potential role of neurosteroids in ameliorating seizure induced mossy fiber sprouting in kindled animals.
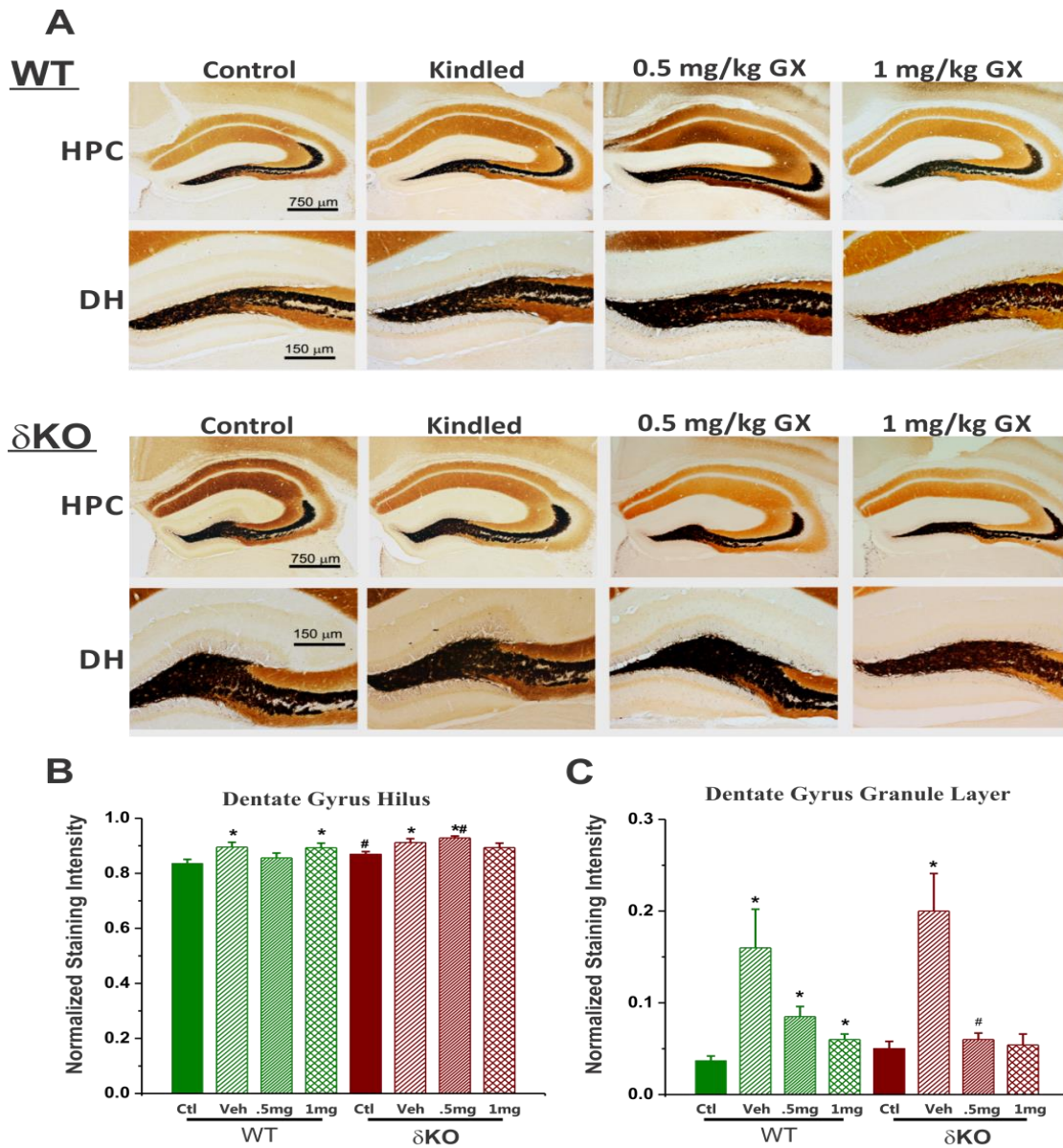
**Figure 35. TIMM staining of mossy fiber sprouting in kindled WT and δKO mice. (A)** Representative images from control, kindled mice, and kindled mice treated with GX. **(B)** Stain intensity in DG hilus in control and kindled cohorts. **(C)** Stain intensity in the DG granule layer in control and kindled cohorts. Staining was significantly denser in the hilus than the granule layer in all conditions. Kindling caused significant increases in stain density in both genotypes. In both genotypes GX-treatment reduced kindling-induced increases in stain intensity in the DG granule layer in a dose-wise manner. Scores compiled for 3 animals in each group (*p<0.05 vs. control of same genotype, #p < 0.05 vs. WT of same condition, n = 9 sections per group, independent t-test). Sections were analyzed with the experimenter blinded to the genotype and condition.

**Table 5. Rates of hippocampal kindling in WT and δKO mice.** Table shows average number of stimulations required to reach each stage 1-5 of the Racine Seizure Scale. Values represent mean ± SEM (n=8-12 per group).

| WT Female | Control | Gn | AP 0.5 mg/kg | Fn | δKO |
|---|---|---|---|---|---|
| Stage 1 | 1.0 ± 0.0 | 2.80 ± 0.33 | 1.5 ± 0.19 | 1.10 ± 0.21 | 1.0 ± 0.0 |
| Stage 2 | 8.10 ± 0.24 | 7.20 ± 0.23 | 6.1 ± 0.46 | 2.1 ± 0.1 | 7.20 ± 0.39 |
| Stage 3 | 10.2 ± 0.30 | 13.3 ± 0.60 | 19.3 ± 0.86 | 3.00 ± 0.37 | 10.1 ± 0.27 |
| Stage 4 | 13.8 ± 0.32 | 21.7 ± 0.29 | 22.0 ± 0.49 | 6.10 ± 0.17 | 11.7 ± 0.35 |
| Stage 5 | 17.2 ± 0.89 | 22.8 ± 0.64 | 24.5 ± 0.56 | 7.21 ± 0.77 | 13.9 ± 0.64 |

| WT Female | Control | Gn + Fn | AP 0.5 + Fn | AP 3.0 mg/kg | 3B-AP |
|---|---|---|---|---|---|
| Stage 1 | 1.0 ± 0.0 | 1.20 ± 0.11 | 1.17 ± 0.17 | 3.70 ± 0.18 | 1.4 ± 0.16 |
| Stage 2 | 8.10 ± 0.24 | 7.59 ± 0.21 | 6.00 ± 1.37 | 6.00 ± 0.44 | 7.2 ± 0.21 |
| Stage 3 | 10.2 ± 0.30 | 9.40 ± 0.43 | 16.7 ± 1.58 | 29.1 ± 4.65 | 9.3 ± 0.27 |
| Stage 4 | 13.8 ± 0.32 | 11.3 ± 0.38 | 23.7 ± 0.42 | N/A | 12.1 ± 0.25 |
| Stage 5 | 17.2 ± 0.89 | 14.7 ± 0.77 | 26.2 ± 2.22 | N/A | 15.3 ± 0.46 |

| δKO Female | Control | Gn | AP 0.5 mg/kg | Fn | Gn + Fn |
|---|---|---|---|---|---|
| Stage 1 | 1.0 ± 0.0 | 3.10 ± 0.35 | 4.67 ± 1.05 | 1.0 ± 0.0 | 1.0 ± 0.0 |
| Stage 2 | 7.20 ± 0.39 | 10.3 ± 0.44 | 12.3 ± 1.50 | 2.5 ± 0.19 | 5.8 ± 0.64 |
| Stage 3 | 10.1 ± 0.27 | 13.8 ± 0.22 | 17.7 ± 0.42 | 3.4 ± 0.24 | 9.2 ± 0.41 |
| Stage 4 | 11.7 ± 0.35 | 15.2 ± 0.32 | 20.7 ± 0.80 | 4.8 ± 0.14 | 10.1 ± 0.53 |
| Stage 5 | 13.9 ± 0.64 | 17.3 ± 0.71 | 25.7 ± 1.01 | 6.6 ± 0.78 | 12.4 ± 0.93 |

| WT Male | Control | GX 0.5 | GX 1.0 | GX 3.0 | δKO |
|---|---|---|---|---|---|
| Stage 1 | 1.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 | 10.3 ± 1.04 | 1.0 ± 0.0 |
| Stage 2 | 3.78 ± 0.40 | 5.67 ± 0.21 | 10.4 ± 0.49 | >25 | 3.67 ± 0.39 |
| Stage 3 | 7.45 ± 0.45 | 10.5 ± 0.67 | 14.6 ± 0.34 | N/A | 6.75 ± 0.21 |
| Stage 4 | 10.6 ± 0.43 | 22.0 ± 3.07 | 23.4 ± 1.93 | N/A | 8.50 ± 0.40 |
| Stage 5 | 13.8 ± 0.76 | 31.2 ± 2.93 | 29.8 ± 3.17 | N/A | 10.5 ± 0.89 |

| δKO Male | Control | GX 0.5 | GX 1.0 | GX 3.0 | WT |
|---|---|---|---|---|---|
| Stage 1 | 1.0 ± 0.0 | 1.0 ± 0.0 | 1.0 ± 0.0 | 4.18 ± 0.26 | 1.0 ± 0.0 |
| Stage 2 | 3.67 ± 0.39 | 8.00 ± 0.57 | 15.8 ± 0.65 | >25 | 3.78 ± 0.40 |
| Stage 3 | 6.75 ± 0.21 | 10.9 ± 0.50 | 19.7 ± 0.27 | N/A | 7.45 ± 0.45 |
| Stage 4 | 8.50 ± 0.39 | 14.7 ± 0.28 | 23.7 ± 0.59 | N/A | 10.6 ± 0.43 |
| Stage 5 | 10.5 ± 0.89 | 25.2 ± 1.09 | 33.7 ± 1.54 | N/A | 13.8 ± 0.76 |

**IV.3.4 Investigating the role of neurosteroids in modulating epileptogenesis in the pilocarpine-SE model of epilepsy**

**IV.3.4.1 Ganaxolone ameliorates epileptogenesis following pilocarpine-SE**

After observing the disease-modifying effects of neurosteroid-based therapies in kindling epileptogenesis, we sought to investigate the potential of endogenous neurosteroids in modulating epileptogenesis following status epilepticus. For this we utilized the pilocarpine-SE model, and treated WT male mice with ganaxolone immediately following SE, and then twice daily for 20 days. As in previous studies, mice were implanted with wireless telemetry devices and allowed to recover prior to the experiment. SE was induced scopolamine (1 mg/kg, s.c.) 30 minutes prior to administration of pilocarpine (300 mg.kg, i.p.). SE was allowed to continue uninterrupted for 90 minutes before being terminated with diazepam (10 mg/kg, i.p.), followed by ganaxolone (5 mg/kg, s.c.). Injections with GX (5 mg/kg, s.c.) continued twice daily until D20 post-SE. GX treatment significantly ameliorated epileptogenesis following pilocarpine-SE (Fig. 36). Development of SRS occurred in 25% of ganaxolone treated mice, compared to 75% in the control group. Onset of SRS was also significantly delayed by GX treatment, with first detected SRS in GX groups developing 30 days after the majority of vehicle animals displayed first SRS. Of note, SRS expression did not vary much between the two responding members of the GX cohort, although larger studies are needed to further characterize the nature of SRS emergence following GX treatment. Compared to vehicle controls, GX-treated mice exhibited significantly reduced seizure expression and lower average number of SRS in the 90 days following SE. Seizure duration in GX-treated mice tended to be lower than in controls, although this difference was not of statistical significance. Average total time seizing was significantly lower in GX-treated mice, reflecting the lower number of seizures and shorter period of seizure expression within the 90-day study window. This effect is visualized with individual snapshots of seizure expression in GX and control cohorts

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over 90 days following SE (Fig. 37), as well as in the overall average seizure expression of each cohort (Fig. 38).
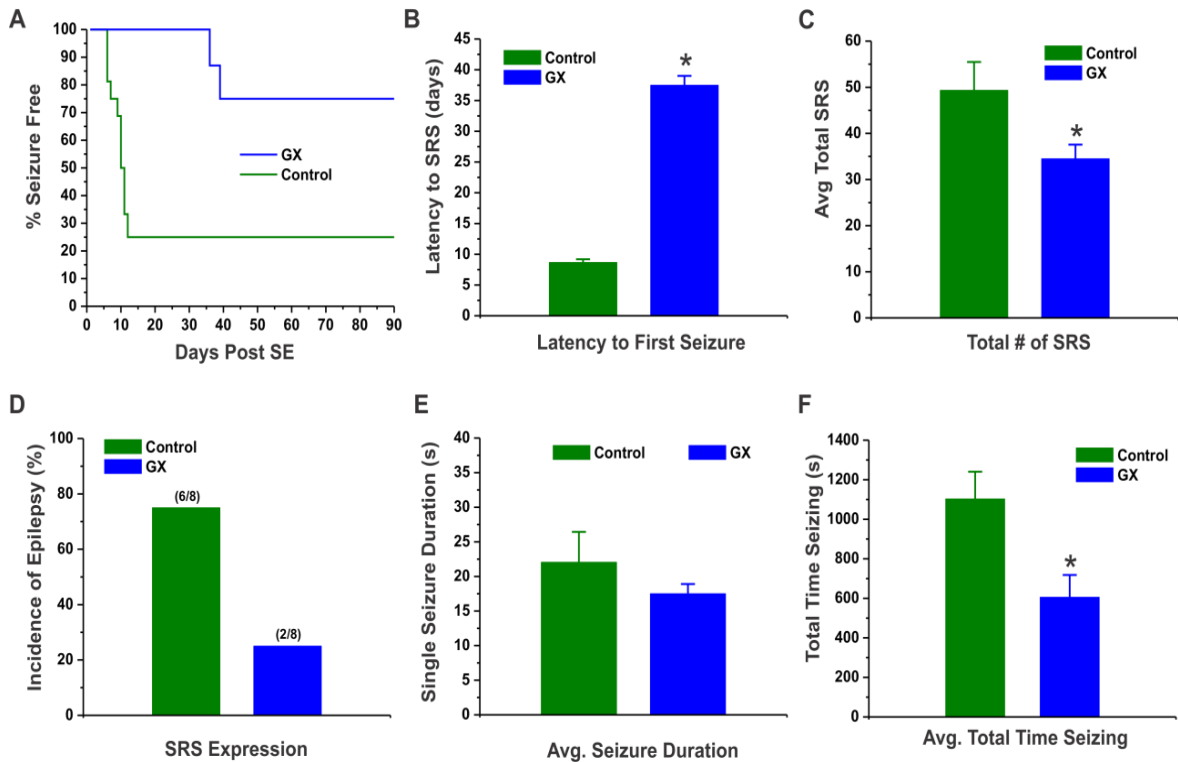


**Figure 36. Ganaxolone treatment delays epileptogenesis following pilocarpine SE. (A)** Seizure expression as % of cohort in control and ganaxolone treated mice. (**B**) Average latency to first SRS. GX treated animals had a significantly greater latency to SRS than controls. (**C**) Average number of seizures per responding individual in each cohort. GX treatment increased overall expression of SRS compared to controls. (**D)** Bar graph showing total incidence of epilepsy in each cohort. (**E)** Average duration of individual SRS was not significantly different between control and GX treated animals. (**F)** Average combined duration of SRS for seizure-expressing mice in GX and control cohorts. GX treated animals spent significantly less time seizing than control mice. Values represent mean ± SEM (*p<0.05 vs. control).
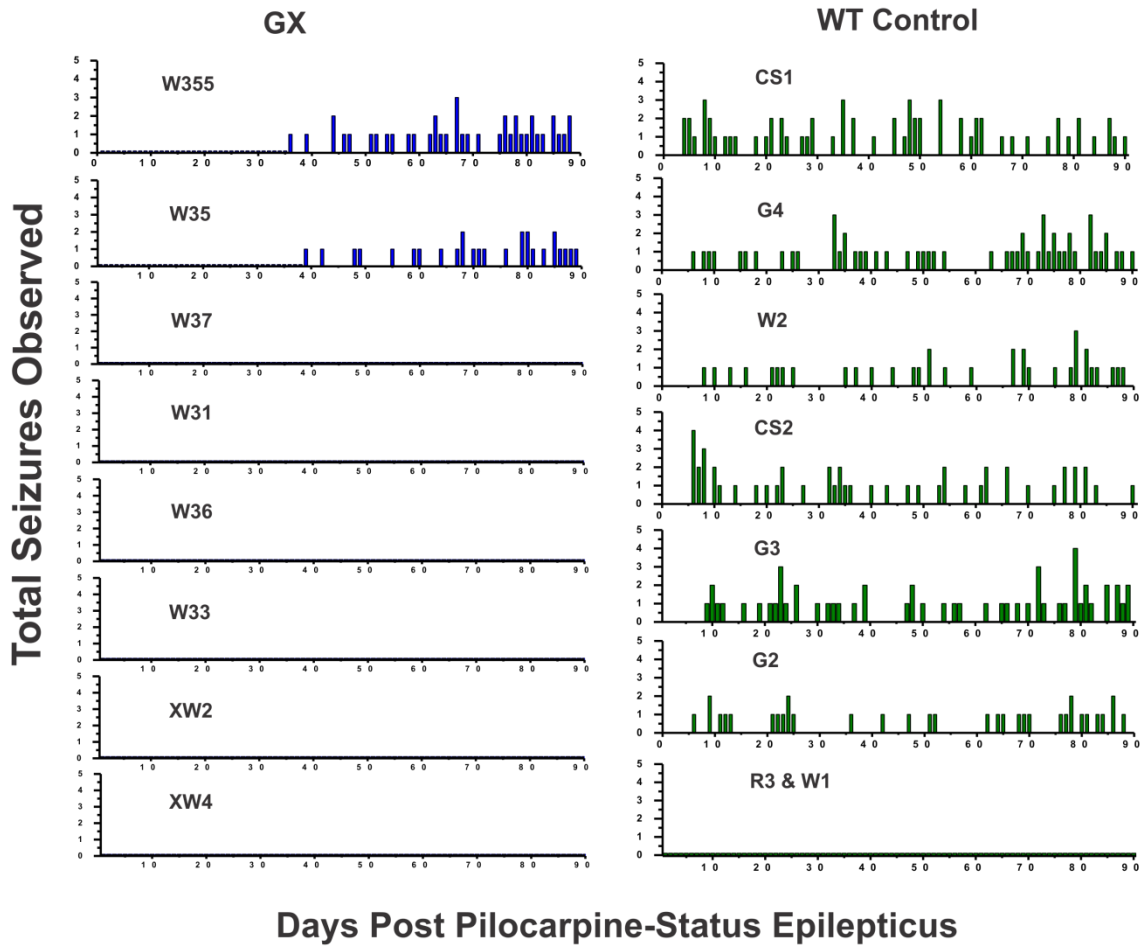
**Figure 37. Individual progression of SRS expression in GX pilocarpine-epilepsy mice.** Progression of SRS in GX-treated WT mice and vehicle-treated controls in the 90 days following pilocarpine-SE as represented by total seizures per day for each subject in both cohorts.

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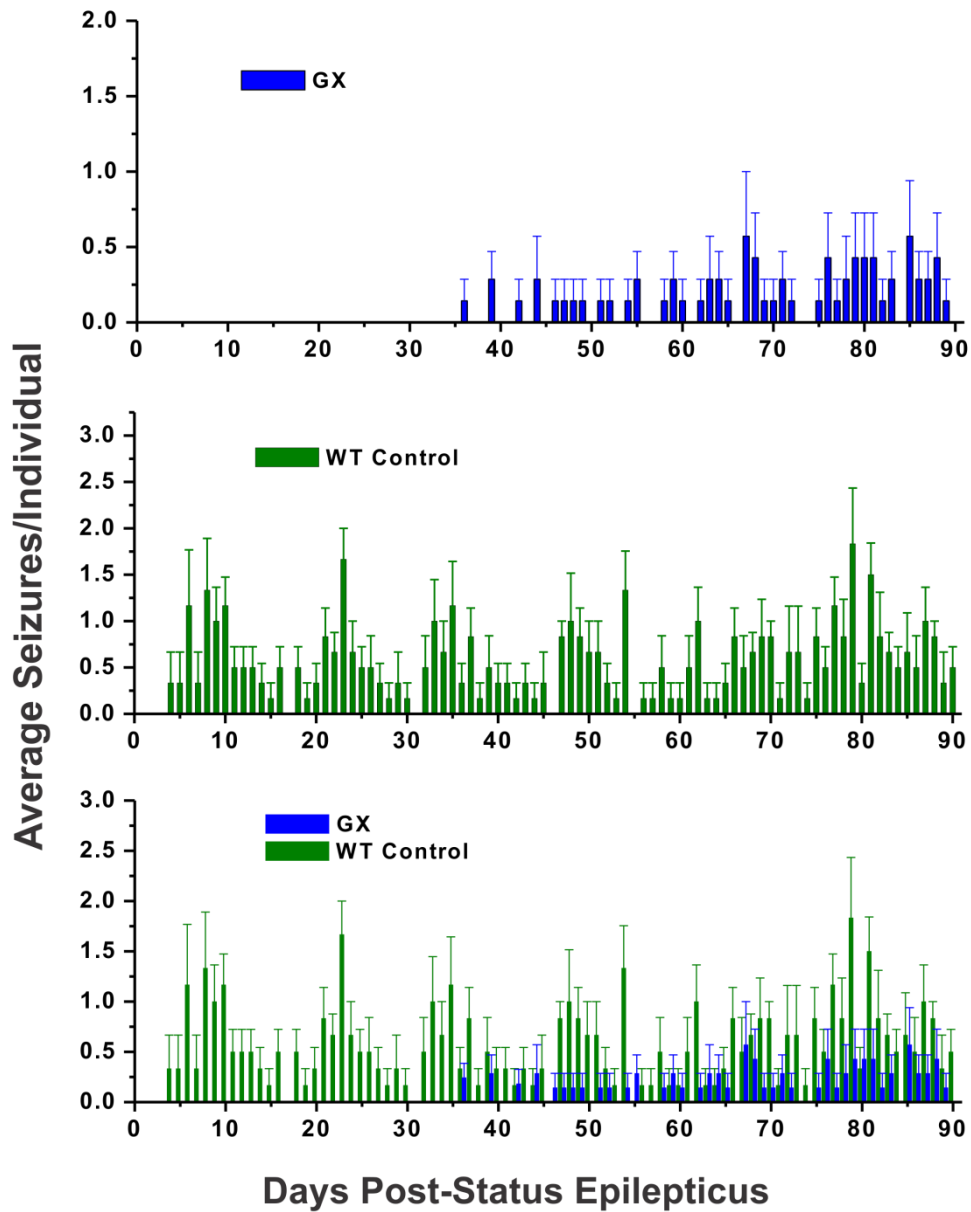
**Figure 38. SRS progression in GX treated and control WT pilocarpine-SE mice.** Progression of SRS in GX-treated WT mice and vehicle-treated controls in the 90 days following pilocarpine-SE as represented by average seizures per day for an individual in each cohort. A direct visual comparison of SRS expression following SE is provided by superimposing the graphs for both groups. Values represent mean ± SEM (n = 8 per group).

**IV.3.4.2 Inhibition of neurosteroid synthesis accelerates and augments epileptogenesis following pilocarpine-SE**

To conduct a further investigation into the potential mediating role of neurosteroids in epileptogenesis following SE, we used daily finasteride treatment to inhibit neurosteroid synthesis starting immediately following SE, and continued for 14 days. Finasteride treated animals displayed accelerated and augmented epileptogenesis, expressing seizures an average of 3 days earlier than vehicle-control mice. Neurosteroid inhibition with finasteride also led to increased overall incidence of epilepsy, with 100% of finasteride treated animals developing SRS, compared to 75% of untreated controls (Fig.39). Although the duration of individual seizures did not significantly differ between finasteride and control groups, the number of seizures expressed in finasteride treated mice was much higher than in controls, particularly during the first week following pilocarpine-SE. Accordingly, finasteride treatment led to a significant increase in the average total time spent seizing per animal. The nature of this effect is further characterized when examining the individual seizure expression figures (Fig. 40), where large clusters of seizures were observed in all animals during the treatment phase in the finasteride cohort. Following the treatment phase, clusters of SRS occur in finasteride mice in a manner similar to that of vehicle treated animals, visualized in the cohort SRS expression overlay below (Fig.41).
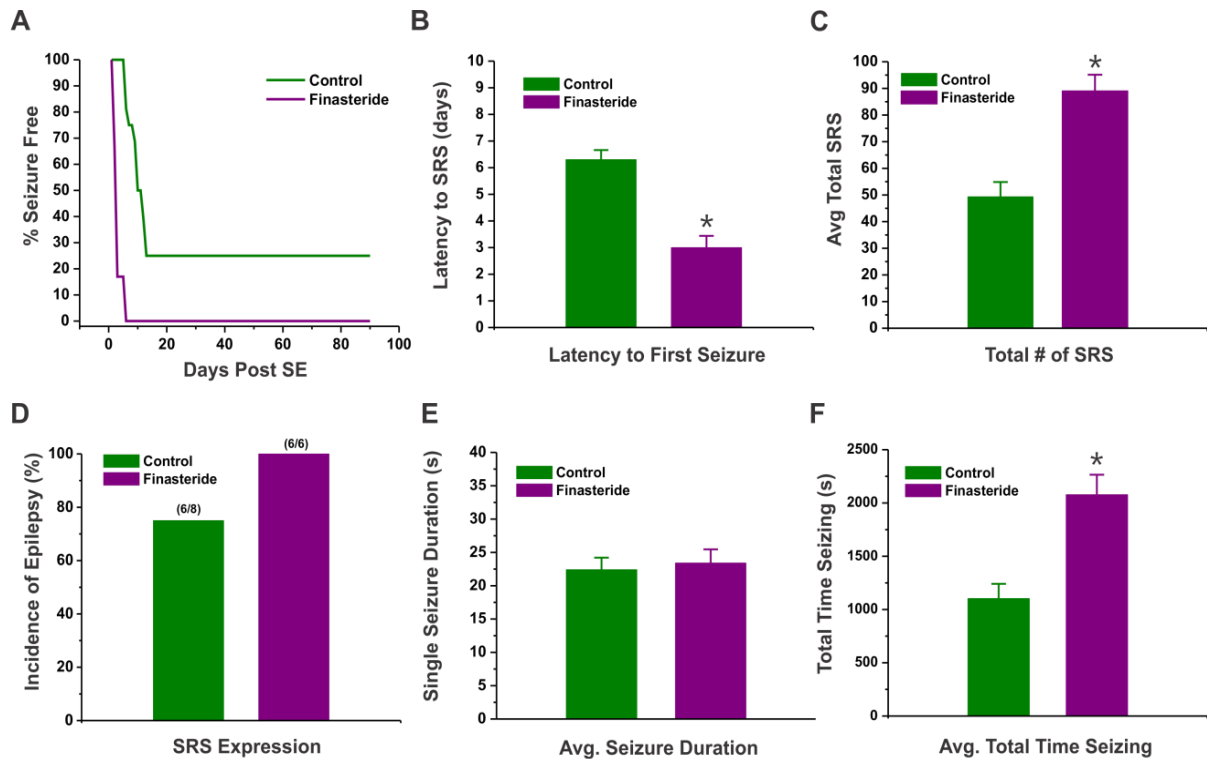
**Figure 39. Accelerated and augmented epileptogenesis in finasteride-treated pilocarpine-epilepsy mice. (A)** Seizure expression as % of cohort in control and finasteride-treated mice. (**B**) Average latency to first SRS. Finasteride treated animals displayed SRS an average of 3 days prior to control animals. (**C**) Average number of seizures per individual in each cohort. Finasteride treatment increased overall expression of SRS compared to controls. (**D**) Bar graph showing total incidence of epilepsy in each cohort. (**E**) Average duration of individual SRS was not significantly different between control and finasteride-treated animals. (**F**) Average combined duration of SRS for individuals in finasteride and control cohorts. Finasteride treated animals spent significantly more time seizing than control mice. Values represent ± SEM (N=6-8 animals per group,*p<0.05 vs. control).

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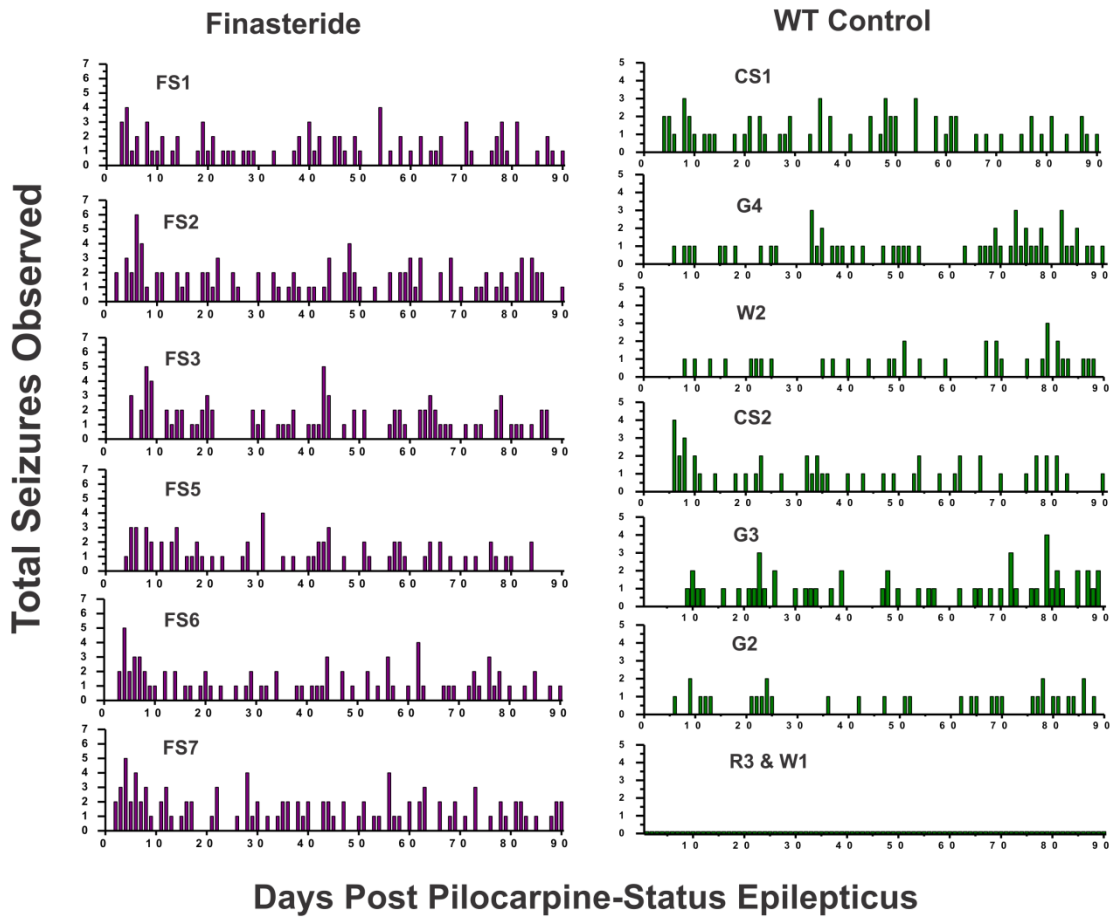
**Figure 40. Individual progression of SRS expression in finasteride pilocarpine-epilepsy mice.** Progression of SRS in Fn-treated WT mice and vehicle-treated controls in the 90 days following pilocarpine-SE as represented by total seizures per day for each subject in both cohorts.

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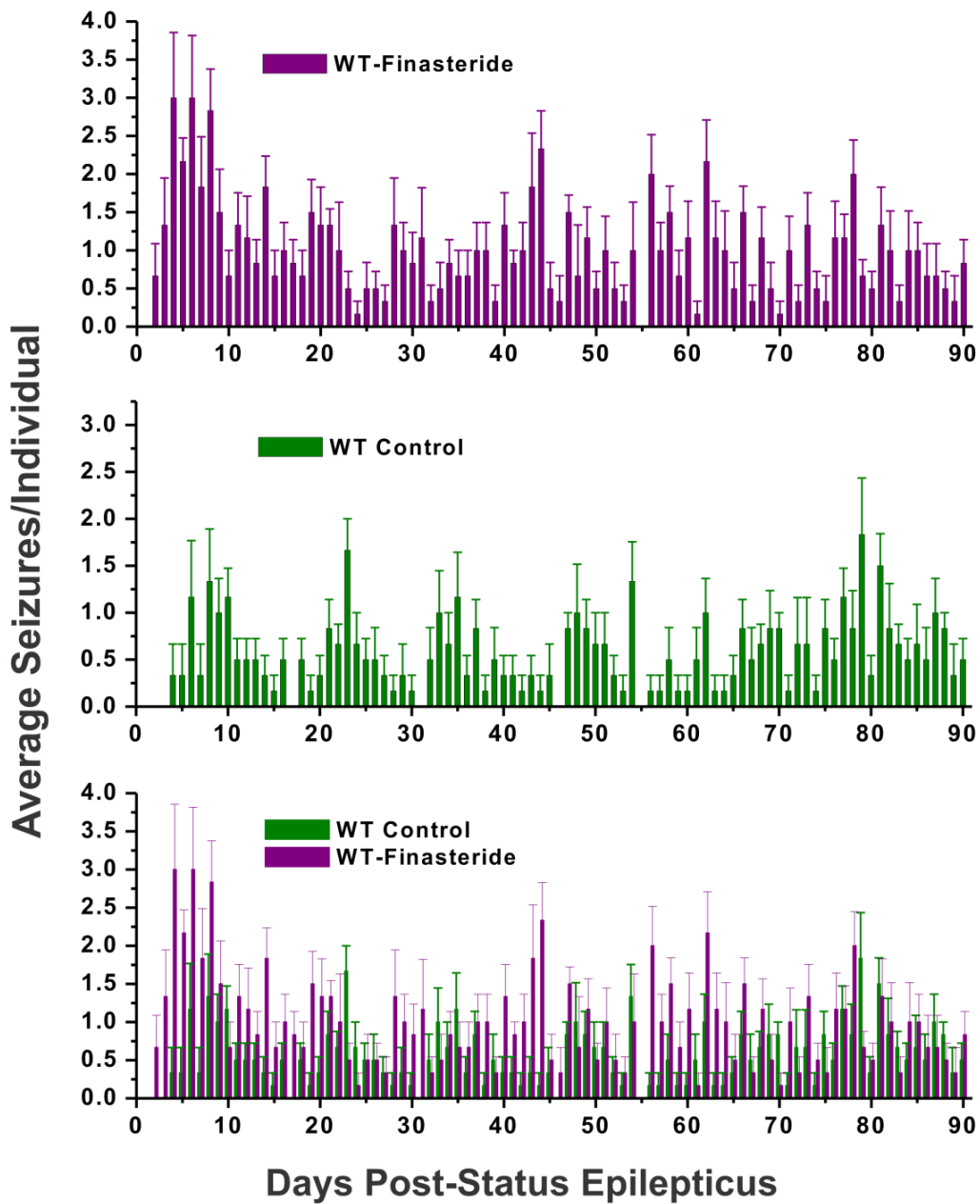
**Figure 41. Cohort progression of SRS expression in finasteride and control pilocarpine-epilepsy Mice.** Progression of SRS in finasteride-treated WT mice and vehicle-treated controls in the 90 days following pilocarpine-SE, as represented by average seizures per day for an individual in each cohort. A direct visual comparison of SRS expression following SE is provided by superimposing the graphs for both groups. Values represent mean ± SEM (N=6-8 animals per group).

**IV.3.4.3 Cognitive Assessment in Ganaxolone and Finasteride Treated Pilocarpine-Epilepsy Mice**

As cognitive deficits such as anxiety and learning and memory issues are common comorbidities in epilepsy, we sought to assess these attributes in the context of our neurosteroid augmentation or inhibition in pilocarpine-epilepsy mice. We hypothesized that animals that experienced fewer seizures would display less signs of anxiety and perform memory-related tasks better than their seizure laden counterparts. Conversely, we sought to investigate the impact of increased seizure expression on cognitive performance. Object memory was assessed using a 24-hour novel object recognition test (NORT), in which animals are repeatedly exposed to two identical 'familiar' objects, and subsequently introduced to a new object in place of one of the familiar ones. Scoring is based on the ratio of novel object explorations vs. familiar object explorations. Anxiety-like behavior was assessed in the Open-Field Test and Elevated Plus-Maze (in finasteride, control, and naive cohorts only), which measure anxiety-like behavior by assessing time in the center of the open field (OFT) or open arm sections (EPM). In the NORT, control epilepsy mice scored significantly lower than naïve mice, and both GX- and Fn- treated cohorts also scored well below naïve mice, demonstrating the blanket impact of neuronal loss and seizures on object memory (Fig. 42). However, within our post-pilocarpine cohorts, GX-treated mice performed significantly better than control mice, although still not at the level of naïve animals. FN-treated animals' scores were more variable than either control or GX-treated, and were not significantly different from either. Compared to naïve mice, all three post-pilocarpine groups showed higher scores of anxiety-like behavior in the OFT, although GX-treated mice tended to spend more time in the center compared to epilepsy controls, and FN-treated mice tended to spend less. Results from the EPM reflected those of the OFT, with both epilepsy controls and Fn-treated mice spending significantly less time in the open arms than mice in the naïve group (Fig.42). Here again the scores from the Fn group were more variable and tended to be lower than that of the epilepsy controls, however there was no significant difference between those groups.
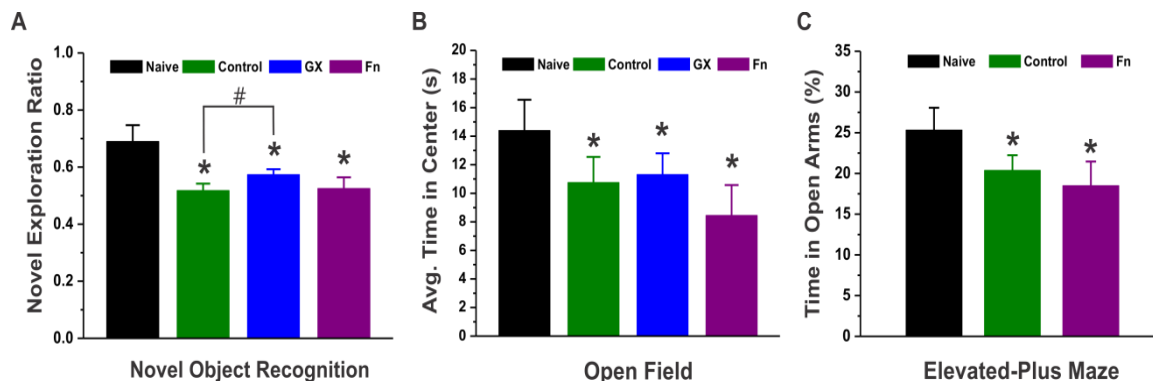
**Figure 42. Cognitive assessment in ganaxolone and finasteride treated epilepsy mice. (A)** NORT - Average individual Novel Exploration Ratio for naïve, control, GX-, and Fn-treated mice. All pilocarpine epilepsy groups scored lower than naïve mice, although GX treated mice displayed a greater propensity for exploring the novel object than control or FN-treated epilepsy mice. **(B)** OFT – Average time spent in the center of the open field apparatus. All 3 epileptic groups spent less time in the center than did naïve mice, with no significant difference between those cohorts. **(C)** EPM - % time in open arms. There was no significant difference between Fn treated and epilepsy control mice, which both spent significantly less time in the open arms than did naïve mice. Values represent mean ± SEM (N=6-10 animals per group, *p<0.05 vs. WT of same condition, #p<0.05 vs. indicated group).

## IV.3.4.4 Morphology of ganaxolone- and finasteride-treated epilepsy mice

We next characterized the morphological changes occurring in the brains of mice treated with GX and Fn following pilocarpine-SE. Our observation of disease-modifying effects on seizure development led us to hypothesize that morphological examination of these mice might reveal differences in neurodegeneration and inflammation in the brains of GX or FN-treated cohorts. Accordingly, we also examined glial fibrillary acidic protein (GFAP+) and ionized calcium-binding adapter molecule 1 (IBA1) stains to examine extent of inflammation in astrocytes and activated microglia in the brains of naïve, post-pilocarpine epileptic mice, and GX-treated post-pilocarpine mice. In the brain, GFAP is expressed by astrocytes and is upregulated in response to inflammation and neuronal injury. GFAP is often upregulated in the brains of epileptic animals and is commonly used as a marker for inflammation following experimental procedures that incite neuroinflammation, such as pilocarpine-SE. Within the brain, IBA1 is specifically expressed by microglia and is upregulated upon their activation in response to

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inflammation, allowing for discrimination of activated microglia from those that are surveying. Brains were taken from naïve mice as well as control/vehicle treated, and GX- and FN-treated mice 90 days following pilocarpine-SE. Brains were fixed and sliced (see methodology), and sections were stained for each marker of interest.

Nissl staining revealed that chronic GX-treatment preserved cytoarchitecture and reduced cell death following pilocarpine-SE, as indicated by increased Nissl staining density (Fig.43). Compared to naïve controls, vehicle and FN treatment groups both displayed significant cell loss across the hippocampal subfields, while we observed only slight loss in GX-treated. The presence of NeuN(+) principal neurons across the hippocampus and amygdala is visualized in Fig. 44A. We observed a significant decrease in NeuN(+) staining following SE. However, the extent of neurodegeneration observed in GX-treated mice was significantly reduced compared with vehicle or Fn-treated cohorts. Compared to vehicle-treated mice, GX-treated mice showed significantly greater NeuN(+) staining, and analysis of cell counts (Fig.44B) revealed that GX-treatment reduced principal neuron losses to across all hippocampal subfields. A qualitative loss was observed in the amygdala, although cell counts were limited to CA1-3, DG, and DH. Losses were greatest in the DH, with GX-treated mice averaging 31.7% fewer neurons than naïve mice (Fig.44C). However, the observed losses were still significantly reduced compared to those in post-pilocarpine controls (74%).

Principal neuron loss across the CA subfields and DG showed high intergroup variation in GX-treated mice, with reduction of 15-20% in the CA sub-regions, and ~8% in the DG. It is important to note that these observations represent neurodegeneration of only a fraction of the extent observed in control post-pilocarpine mice. On the contrary, principal neuron loss was not significantly affected by finasteride treatment, we observed significantly decreased NeuN(+) staining compared to naïve and GX-treated mice. Overall cell counts for the hippocampus revealed that vehicle and finasteride mice had 60% fewer surviving NeuN(+) neurons than were observed in naïve controls. Regional neurodegeneration was similar in control and finasteride-treated groups in all

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areas examined, with the lowest extent of NeuN(+) principal neuron loss in the DG (50%). Immunohistochemical staining of brain sections with PV(+) antibody revealed that GX-treatment also significantly reduced the loss of PV(+) inhibitory interneurons in the hippocampus and dentate gyrus observed in post-pilocarpine epileptic mice. The distribution of PV(+) interneurons across the hippocampus and amygdala of naïve and post-pilocarpine control and treatment groups is visualized in Fig. 45A. A qualitative reduction of PV(+) interneurons was observed in the amygdala, although cell counts were limited to CA1-3, DG, and DH. Cell counts in GX-treated animals show a decrease of 12% of PV(+) interneurons across the hippocampus as a whole, compared to a substantial 55%  loss observed in vehicle post-pilocarpine mice (Fig.45C). This protective effect was greatest in the DG and CA2, where we observed decreases of 14% and 6% (respectively), representing degeneration to a quarter of the extent observed in control post-pilocarpine epileptic mice. The difference was least-pronounced (while still significant) in the CA1, where PV(+) losses were 15% in GX-treated mice, compared to 42% in control post-pilocarpine mice. Interestingly, finasteride inhibition of neurosteroid synthesis did not significantly alter the reduction in PV(+) interneurons in post-pilocarpine epileptic mice, as we observed similar reduction of staining intensity in finasteride and control post-pilocarpine cohorts across all regions examined.

We observed a significant increase in astrocyte activation, as measured by GFAP(+) staining, in the brains of post-pilocarpine epileptic mice (Fig. 46). Epileptic mice displayed a 159% increase (compared to controls) in astrocyte activation in the hippocampus as whole. Subfield-specific increases in the CA1 and DG were 157% and 92% above controls, with the CA3 and amygdala displaying the highest increases, at 295% and 214% over controls, accordingly (46B). GX treatment ameliorated this effect, with GX mice displaying significantly reduced levels of GFAP(+) staining increases across all regions examined except for the DG. GX had the most significant effect on increases in astrocyte activation in the CA3 and amygdala, the regions of highest increase in control post-pilocarpine mice. Here we observed an 84% and 65% increase compared to naïve animals. This is in stark contrast to the observations in vehicle-treated

post-pilocarpine mice, where GFAP(+) staining was most elevated in these regions. Compared to epileptic controls, GX-treatment resulted in significantly lower increases in astrocyte activation, suggesting a powerful neuroprotective effect following pilocarpine-SE. The observed increases in GFAP(+) staining intensity in GX mice likely reflects an unavoidable consequence of prolonged SE, which produces robust inflammation and neurodegeneration.

Immunohistochemical examination using IBA1 antibody revealed significant and easily visualized increases in levels of activated microglia following pilocarpine-SE (Fig. 47A). Furthermore, GX-treatment had no effect on the levels of activated microglia observed in post-pilocarpine mice. Area fractionation analysis produced similar results for both vehicle-treated and GX post-pilocarpine mice across all hippocampal regions and the amygdala (Fig.47B). When normalized to control values, the levels of microglia activation in vehicle and GX pilocarpine group were nearly identical, at 88% and 78% over control levels, with the most activation observed in CA3 (Fig.47C). Considering the powerful neuroprotective effect of GX indicated by our analysis of neurodegeneration and astrocyte activation in post-pilocarpine mice, the lack of effect observed in our characterization of microglia activation came somewhat as a surprise. However, SE induces neuroinflammation and promotes macrophage infiltration of the brain, and the current observation likely reflects microglia infiltration and activation resultant from the initial exposure to SE in both pilocarpine cohorts. Overall GX-treatment resulted in a powerful neuroprotective effect, ameliorating neuronal loss and neuroinflammation in post-pilocarpine mice. These observations reflect the beneficial effect on epileptogenesis and cognitive function in GX –treated mice. On the contrary, finasteride inhibition of neurosteroid synthesis following SE did not affect levels of neurodegeneration or inflammation as measured in our study, despite resulting in an increase in seizure activity. These data demonstrate that SE and the resultant epileptogenesis and SRS occurrence lead to significant neuronal loss and inflammation, and that chronic treatment with GX may provide a disease-modifying intervention following SE.
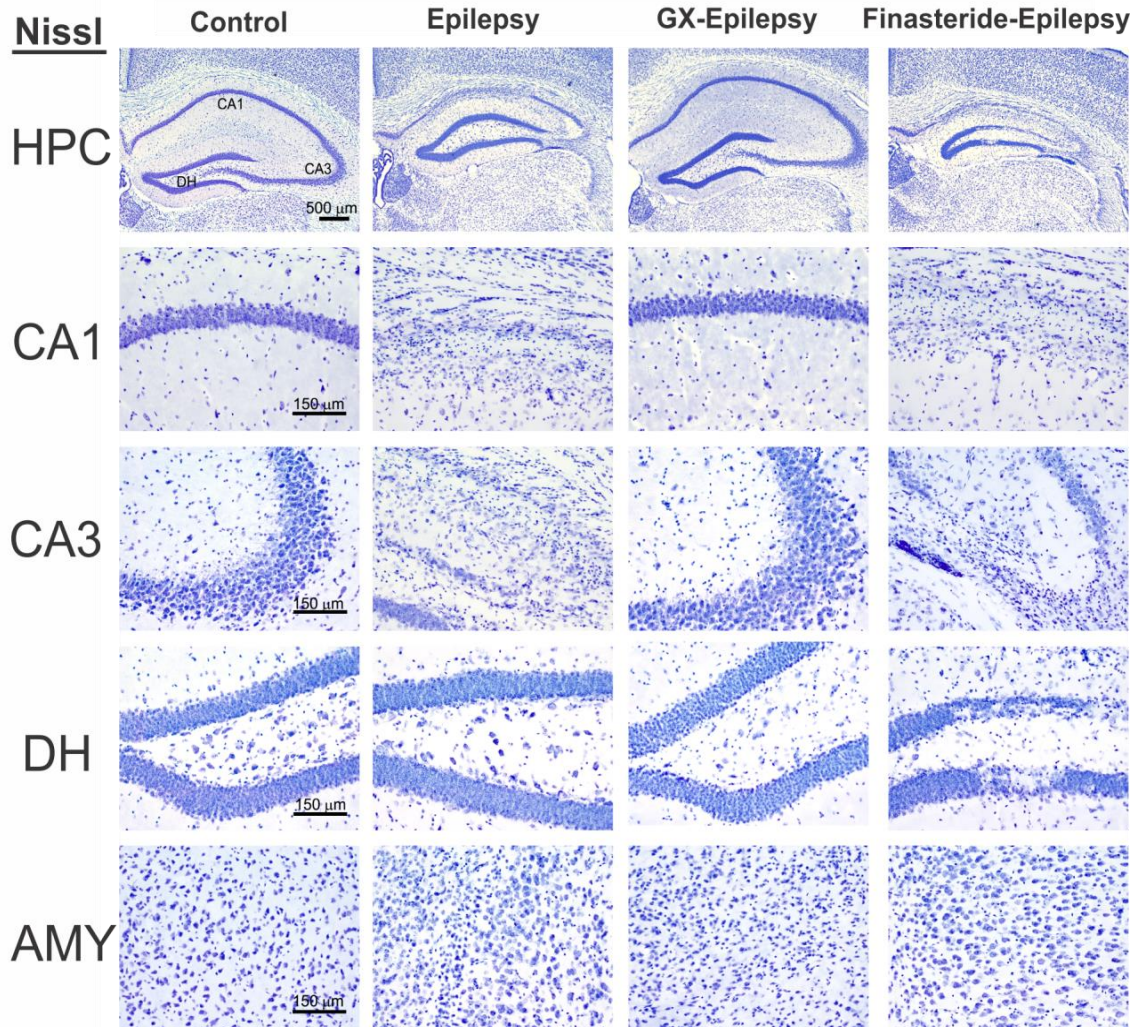
**Figure 43. Changes in hippocampal cytoarchitecture of pilo-epilepsy WT mice treated with ganaxolone or finasteride.** Hippocampal cytoarchitecture visualized with Nissl stain. Significant cell loss was observed in epileptic-control and finasteride-treated mice across the hippocampus, while GX-treated animals displayed cytoarchitecture that more closely resembled that of naïve controls.
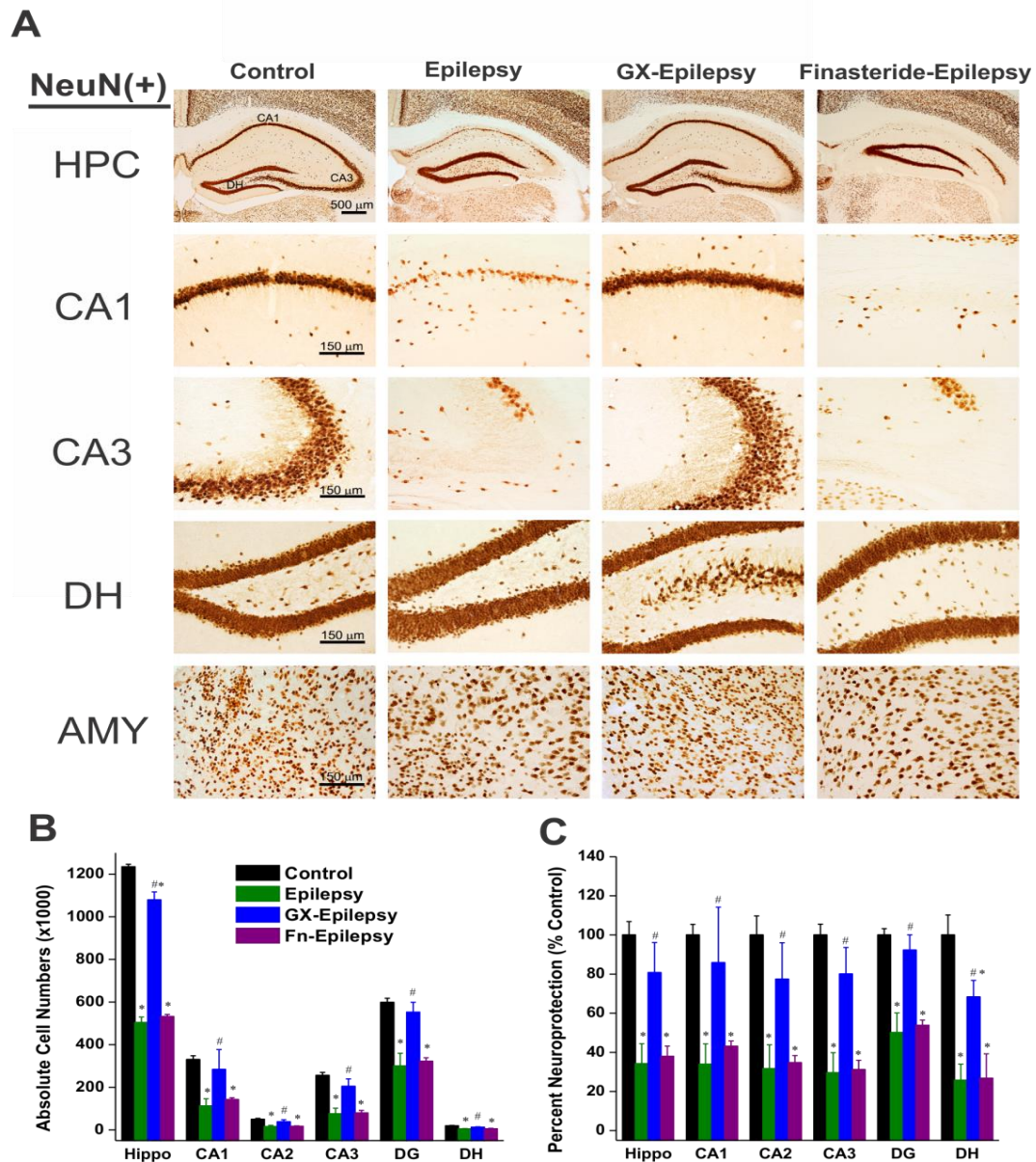
**Figure 44. NeuN(+) immunohistochemistry in pilo-epilepsy WT mice treated with ganaxolone or finasteride. (A)** Distribution of NeuN(+) principal neurons in the hippocampus subfields at 3 months post-pilocarpine in WT mice post-treated with vehicle, GX, or Fn. **(B)** Absolute cell counts for the hippocampus and hippocampal sub-regions in control and WT mice post-treated with GX or Fn, 90 days following SE. **(C)** Neurodegeneration results from **(B)**, normalized to control counts. Scores compiled for 4 animals in each group (* $p < 0.05$ vs. naïve control, # $p < 0.05$ vs. epilepsy and Fn-epilepsy, n = 12 sections per group, independent t-test). Sections were analyzed with the experimenter blinded to the genotype and condition.
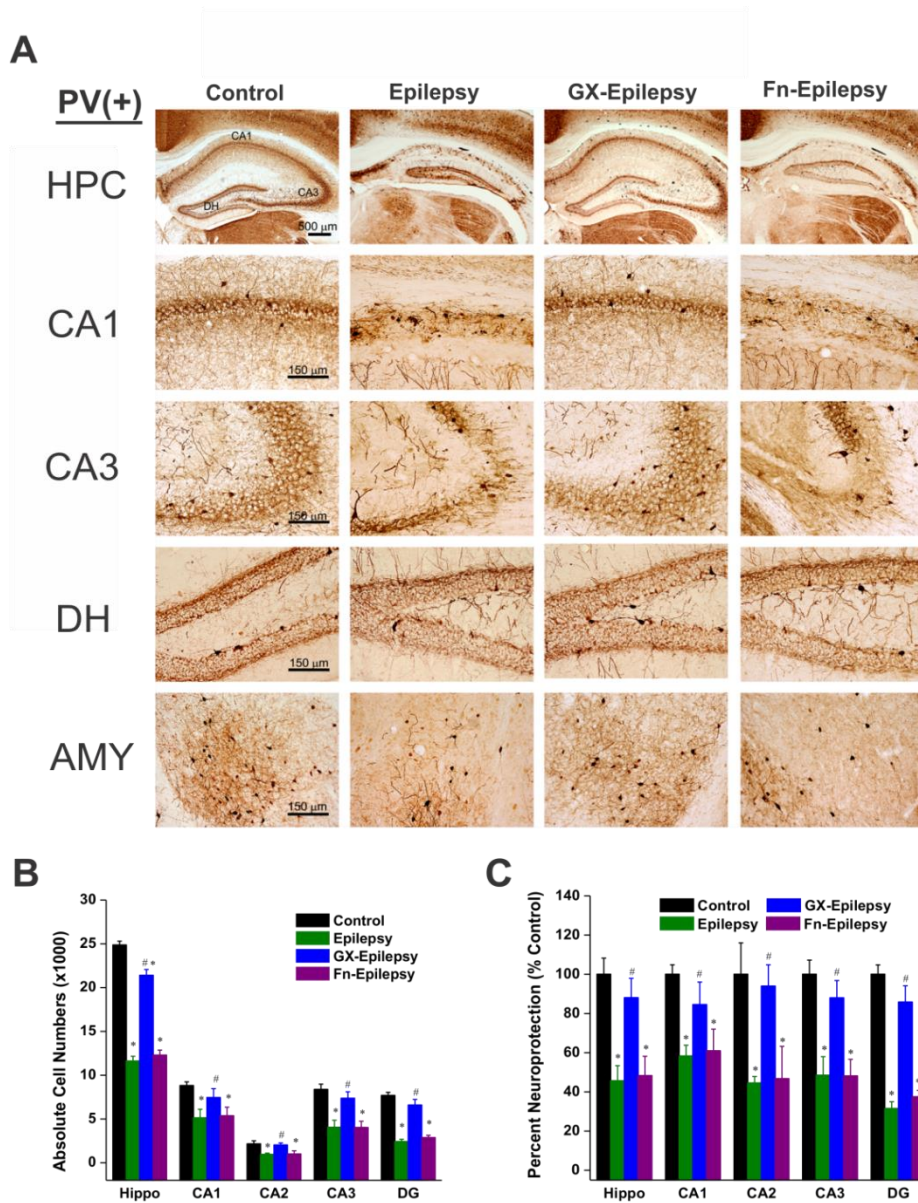
**Figure 45. PV(+) immunohistochemistry in pilo-epilepsy WT mice treated with ganaxolone or finasteride.** (A) Distribution of PV(+) GABAergic interneurons in the hippocampus subfields at 3 months post-pilocarpine in WT mice post-treated with vehicle, GX, or Fn. **(B)** Absolute cell counts for the hippocampus and hippocampal sub-regions in control and pilocarpine-epilepsy WT post-treated with GX or Fn, 90 days following SE. **(C)** Neurodegeneration results from (B), normalized to control counts. Values represent mean ± SEM. Scores compiled for 4 animals in each group (* $p < 0.05$ vs. naïve control, # $p < 0.05$ vs. epilepsy and Fn-epilepsy, n = 12 sections per group, independent t-test).
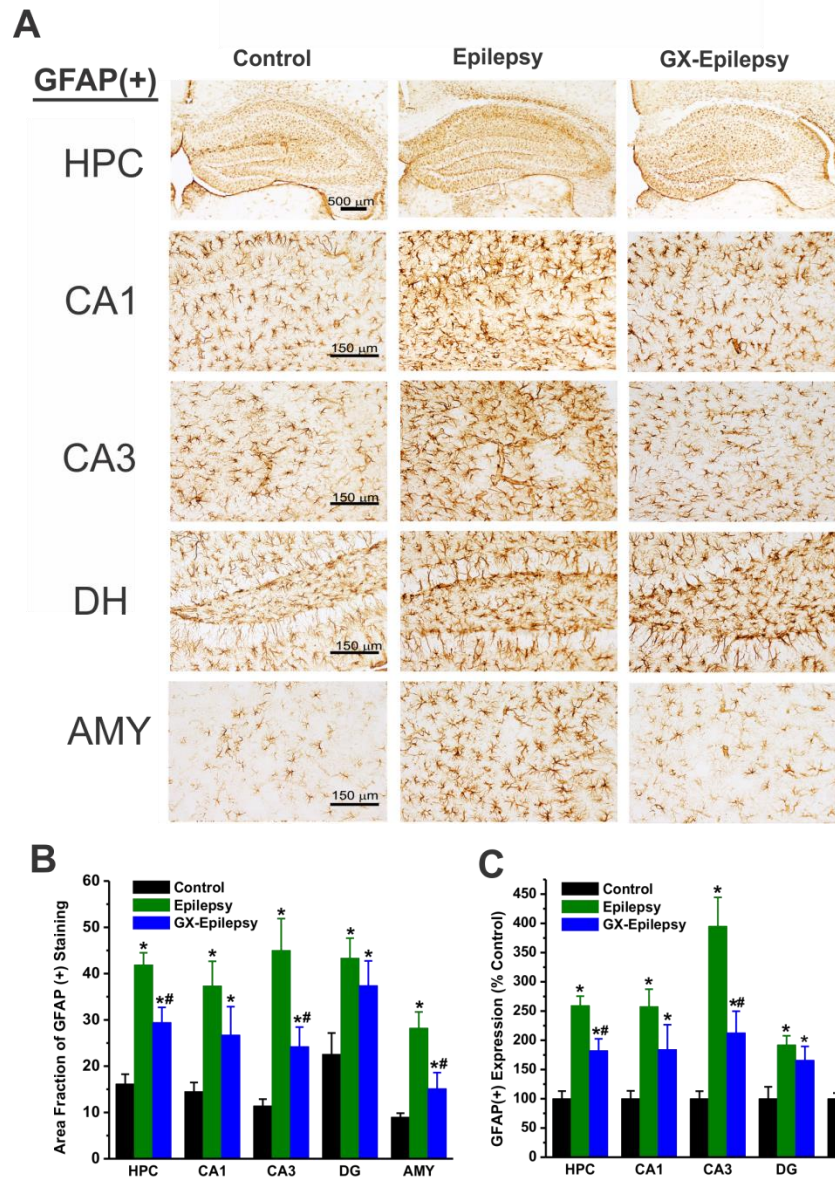
**Figure 46. GFAP(+) immunohistochemistry in pilo-epilepsy WT mice treated with ganaxolone. (A)** GFAP(+) immunostaining of astrocytes in the hippocampus and amygdala regions in naïve, epilepsy, and GX-epilepsy mice. **(B)** Quantification of GFAP(+) staining in the hippocampus and sub-regions by area fractionation. **(C)** Area data normalized to levels of naïve control mice. Values represent mean ± SEM. Scores compiled for 4 animals in each group (* $p < 0.05$ vs. naïve control, # $p < 0.05$ vs. epilepsy, n = 12 sections per group, independent t-test). Sections were analyzed with the experimenter blinded to the genotype and condition.
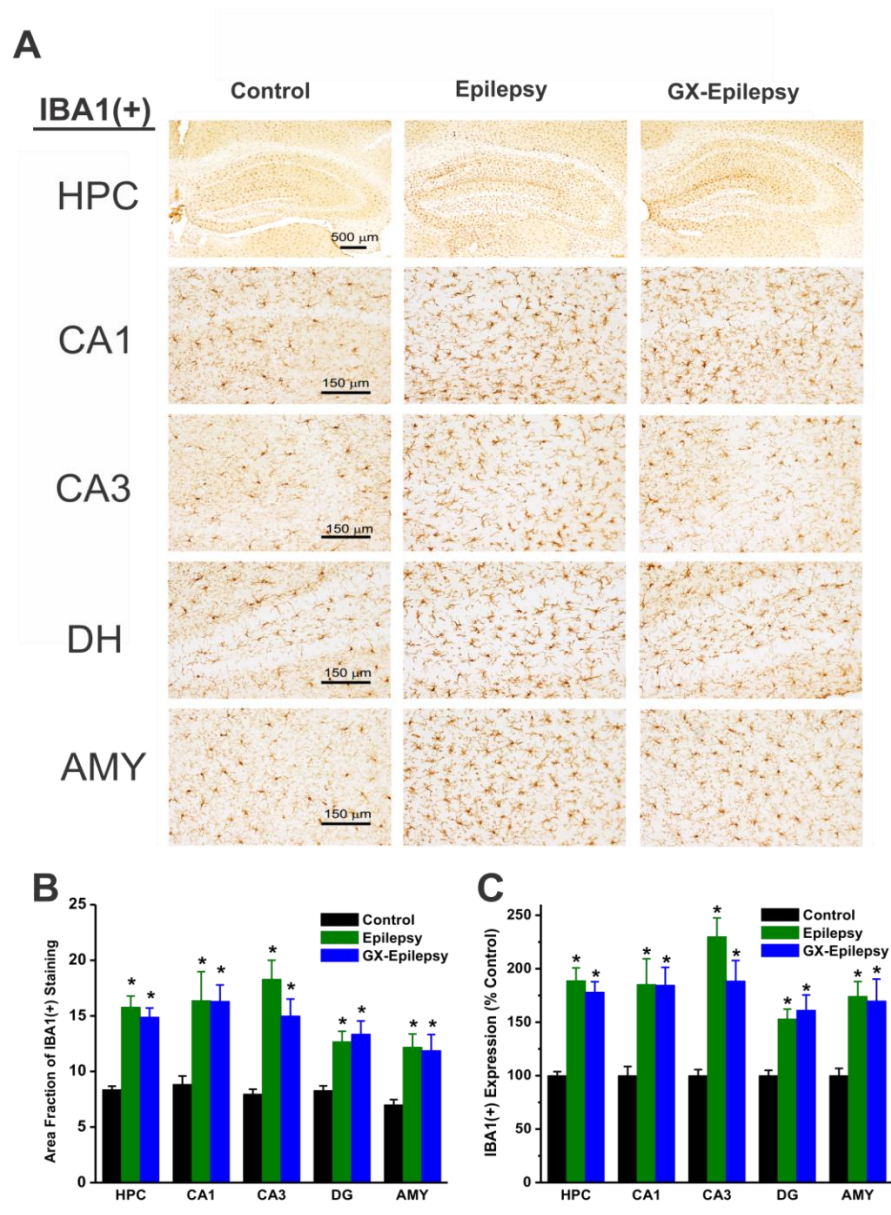
**Figure 47. IBA1(+) immunohistochemistry in pilo-epilepsy WT mice treated with ganaxolone.** **(A)** IBA1(+) immunostaining of microglia in the hippocampus and amygdala regions in naïve, epilepsy, and GX-epilepsy mice. **(B)** Quantification of IBA1(+) staining in the hippocampus and sub-regions by area fractionation. **(C)** Area data normalized to levels of naïve control mice. Values represent mean ± SEM (N=4-6 animals per group). Scores compiled for 4 animals in each group (* $p < 0.05$ vs. naïve control, n = 12-18 sections per group, independent t-test). Sections were analyzed with the experimenter blinded to the genotype and condition.

## CHAPTER V
## DISCUSSION

### V.1 The role of δ-subunit extrasynaptic GABA-A receptors in a perimenstrual model of catamenial epilepsy

The principal findings of the study are that loss of extrasynaptic δGABA-A receptors results in striking amplification of NSW-induced hyperexcitability and seizure exacerbation in the mouse model of catamenial epilepsy. In fully-kindled female mice, NSW has been shown previously to trigger a heighted exacerbation of seizure activity as evident by an increase in seizure severity, AD duration, generalized seizure duration (Reddy et al., 2012). In δKO mice, the seizure threshold for generalized seizures was markedly decreased 12–24 h after NSW resulting in manifestation of spontaneous generalized seizures, suggesting enhanced vulnerability of δKO mice to catamenial-like seizures. NSW is known to be associated with increased GABA-A receptor α4- and δ-subunit expression, reduced antiseizure sensitivity to diazepam, and enhanced antiseizure sensitivity to AP (Reddy et al., 2012). This potential pathophysiological profile is consistent with clinical catamenial seizure features. When exposed to the NSW paradigm, mice lacking the δGABA-A receptor-mediated tonic inhibition displayed conspicuous measures of increased hyperexcitability to a greater degree than WT counterparts. Moreover, δKO mice in NSW displayed marked differences in NSW-associated changes in pharmacological response to diazepam and AP, including reduced diazepam insensitivity and lack of enhanced neurosteroid-sensitivity. Overall, these results reinforce the notion that extrasynaptic δGABA-A receptors and their endogenous neurosteroid ligands play a critical role in protecting against perimenstrual catamenial seizures in women, and that loss of this signaling through natural fluctuations in neurosteroids, such as during menstruation, may exacerbate seizure activity (**Fig. 48**).
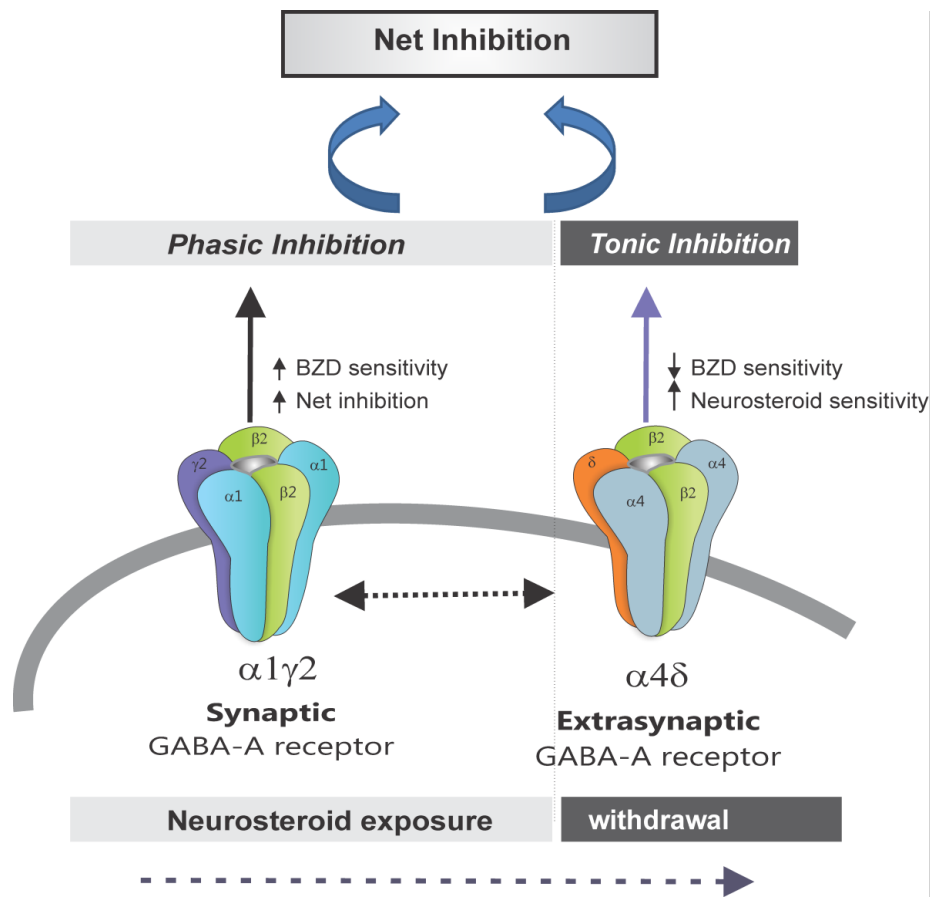
**Fig. 48. Overview of potential molecular changes in synaptic and extrasynaptic GABA-A receptor subunit plasticity in the NSW model of perimenstrual catamenial epilepsy.** A working model is devised to illustrate the alterations in pharmacology due to NSW-induced changes in extrasynaptic GABA-A receptor subunit composition within the hippocampus. NSW-induced upregulation of extrasynaptic δ-subunit-containing receptors may regulate tonic inhibition and neurosteroid sensitivity. Loss of such tonic inhibition in δKO mice leads to catamenial-like seizure exacerbation following NSW paradigm.

Previously we reported the development of a novel mouse model of catamenial epilepsy for use in mechanistic and therapeutic studies (Reddy et al., 2012). Our data on δ- and α4-subunit expression guided us towards a potential molecular mechanism for the enhanced seizure susceptibility and drug sensitivity in this epilepsy model. Alterations in ovarian hormones and modification of GABAergic inhibition are intensely investigated as potential pathophysiological mechanisms underlying catamenial epilepsy (Scharfman

and MacLusky, 2006; Tuveri et al., 2008; Reddy, 2009, Carver et al, 2014). Until recently, an overarching theme in the investigation into catamenial epilepsy revolved around the lack of a suitable mouse model to investigate the pathophysiological mechanisms by which seizure activity is exacerbated. Using the NSW paradigm, we developed first a rat model, followed by a mouse model, both of which were successfully used for pharmacological testing of agents to reduce catamenial seizures (Reddy et al., 2001; Reddy and Rogawski, 2001, Reddy et al, 2012). A series of follow up studies have led to the identification of an extrasynaptic molecular mechanism of catamenial epilepsy (Reddy, 2016b). The importance of δGABA-A receptors in protecting against hyperexcitability in catamenial epilepsy is highlighted by neurosteroid's increased activity at δGABA-A receptors, and the lack of change observed in pharmacological efficacy of neurosteroids between NSW and control δKO mice. Therefore, the findings from the present study demonstrate an important step in the investigation of mechanisms driving hyperexcitability and seizure activity in catamenial epilepsy.

Seizure exacerbation observed in the NSW mouse model is caused by reduced neurosteroid levels in the brain. Measurements of plasma AP levels are consistent with the NSW induction effect of finasteride (Reddy et al., 2012). Finasteride blocks the synthesis of neurosteroids such as AP and related 5α-reduced pregnane analogs that modulate GABA-A receptor function (Mukai et al., 2008), and accordingly, we observed a latent period of 12-24h after finasteride treatment before the start of NSW-related hyperexcitability. Exposing mice lacking δGABA-A receptors to NSW revealed seizure exacerbation beyond the catamenial-mimicking effects of NSW observed in fully-kindled WT mice. Compared to WT mice, δKO mice undergoing NSW displayed a striking increase in excitability, with electrographic and behavioral seizure exacerbation, including increased incidence of spontaneous generalized seizures. Such spontaneous catamenial-like seizures were not widespread in WT mice subjected to similar NSW. We have previously published similar results obtained after NSW paradigm in rats (Reddy et al., 2001) and fully-kindled mice (Reddy et al, 2012). Patients treated with finasteride

had significantly reduced levels of neurosteroids (Duskova et al., 2009) and finasteride may result in seizure exacerbation in women with epilepsy (Herzog and Frye, 2003). Therefore, these reports suggest that endogenous neurosteroids and δGABA-A receptors are regulated within the ovarian cycle and serve as compelling mediators of extrasynaptic inhibition in the hippocampus and overall seizure propensity (Reddy et al., 2012; Wu et al., 2013).

The potential molecular basis for catamenial-like seizures in δKO is illustrated in Fig.8. The enhanced seizure susceptibility following NSW is likely due, at least in part, to increased hippocampal expression of synaptic α4GABA-A receptors (Smith et al., 1998a, 2007; Reddy, 2009; Gulinello et al., 2001). The receptors exhibit faster decay kinetics than α1GABA-A receptors and confer transient benzodiazepine insensitivity. Prior studies have confirmed increases in the α4-subunit and its pharmacological properties after withdrawal from progesterone or neurosteroids (Smith et al., 1998a,b, Reddy et al, 2012, Carver et al, 2014). In the hippocampus dentate neurons, the δ-subunit preferentially coassembles with the α4 subunit to form perisynaptic/extrasynaptic GABA-A receptors (Sur et al., 1999). Tonic inhibition from DG neurons is critically mediated by δGABA-A receptors (Stell et al., 2003). Interestingly, both δ and α4-subunits are increased in the hippocampus of NSW animals (Gangisetty and Reddy, 2010; Reddy et al., 2012; Carver et al., 2014). Therefore, when neurosteroids are withdrawn at the time of menstruation, the α4, and in-turn, δ subunit, is up-regulated, diminishing synaptic inhibition, resulting in enhanced excitability and a predisposition to catamenial seizures. However, compensatory changes such as increased expression of α4δ-subunit-containing receptors appear to preserve GABAergic inhibition in the dentate gyrus, particularly around the perimenstrual period which is associated with heightened seizure symptoms in patients with catamenial epilepsy.

In the present study, the differential changes in antiseizure activity of AP and of diazepam observed between WT and δKO mice are consistent with the premise that up-regulation of α4/δ-subunit-containing GABA-A receptors following NSW is the major

contributing factor to these pharmacological changes (Gangisetty and Reddy, 2010; Reddy et al., 2012). Moreover, the increased NSW-associated seizure exacerbation observed in δKO mice supports previous studies which have pointed to δGABA-A receptors as important mediators of tonic inhibition and protection against catamenial seizures. A more defined picture of the molecular mechanisms underlying enhanced neurosteroid sensitivity in the catamenial model is now coming into view, and recent work has provided meaningful insights into the role of GABA-A receptors in the catamenial epilepsy model (Carver et al., 2014; Reddy, 2016a). As such, enhanced neurosteroid sensitivity in catamenial epilepsy has powerful therapeutic implications. Synthetic neurosteroids may provide a practical approach for suppressing catamenial seizures at low doses with little GABAergic side effects, and provide a unique opportunity for conjunctive therapies that work through related but divergent mechanisms. Such therapy could significantly reduce or prevent seizure occurrence in women with epilepsy (Reddy and Rogawski, 2009).

There are certain caveats of using constitutional knockout mice for epilepsy research. While receptor expression data from control knockout mice show signs of compensatory adaptation in many regions of the brain, the dynamics of NSW on the regulatory processes involved in subunit assembly and incorporation of receptors into the membrane remain unclear. Furthermore, genetic ablation of δ-GABA-A receptors signaling resulted in accelerated kindling epileptogenesis when compared to WT mice. Subunit expression analysis from δKO control mice indicates expression of γ2-subunit levels increase while α4-subunit expression levels decrease in the forebrain, implicating functional compensation in response to the loss of δ-subunit (Korpi et al., 2002; Peng et al., 200; Spigelman et al., 2003). The compensational changes to other subunits have been a challenge in understanding the putative role of δ-subunit receptors in the net inhibition of the brain.

In the present study, a significant increase in seizure exacerbation in δKO mice was evident in the NSW model of perimenstrual catamenial epilepsy, indicating the role of

extrasynaptic δGABA-A receptor-mediated tonic inhibition in catamenial epilepsy. As expected, δKO mice exhibited reduced antiseizure activity to benzodiazepines but not to neurosteroids. The heightened seizure susceptibility and altered antiseizure drug responses are consistent with the relative deficiency of δGABA-A receptors in the brain.

## V.2 The role of δ-subunit extrasynaptic GABA-A receptors in susceptibility to limbic epileptogenesis

Germline deletion of the δ-subunit and δ-containing extrasynpatic GABA-ARs in the brain resulted in significantly accelerated epileptogenesis in the hippocampus kindling model of temporal lobe epilepsy in mice. From the first stimulation, δKO mice exhibited enhanced seizure susceptibility (lower average afterdischarge threshold) and exacerbated seizure intensity (longer avergae afterdischarge duration), and required fewer stimulations to reach fully-kindled state. Together, these observations indicate that δ-containing receptors play a crucial mediating role in acute seizure susceptibility and kindling epileptogenesis. Our investigation also revealed sex-specific differences in kindling epileptogenesis: WT and δKO male mice displayed accelerated kindling epileptogenesis when compared to females of respective genotype. These observations align with previous reports of sex-based differences in seizure susceptibility and overall prevalence of epilpesy (Galanopolou, 2008ab; Pack et al., 2011; Reddy, 2009b; 2011; Reddy and Kulkarni 1999; Wu et al., 2013). A number of physiological mechanisms may underlie these observations, including differences in GABA-AR expression and variability of hormone and neuroendorince dyanimcs. These findings support the notion that alteration or loss of δ-containing extrasynaptic receptors results in a shift or imbalance of excitatory and inihbitory inputs in the hippocampus, specifially derived from reduction of tonic inhibtion in the dentate gyrus, and thus may play a mediating or protective role in the pathophysiology of epileptogenesis.

The hippocampus and related limbic structures are key components of temporal lobe epilepsy and associated pathophysiology. Within the limbic system, the dentate gyrus serves as an inhibtory "gate-keeper" of the hippocampus. It's unique connectiivty and rich expression of inhibitory receptors provide it with the ability to mediate excitatory inputs such as the perforant pathway efferents originating in the entorhinal cortex and other snesory association areas (Coulter and Carlson, 2007; Heinemann et al., 1992). The dentate gyrus normally is home to a large concentration of δ-GABA-ARs, which provide a major contribution to tonic inhibition and overall inhibitory tone in the limbic system. This is achieved through the shunting of exctiatory current, which leads to direct changes in the relationship between inputs and outputs, as well as through the regualting the baseline hyperpolarizing membrane current via subtractive inhibtion (Mitchell and Silver, 2003; Wlodarczyk et al., 2013). Despite a number of experiments aimed at charcaterizing the function and dynamics of extrasynaptic δ-GABA-ARs, our understanding of their impact and role within multi-structure networks and overarching systems in still not well understood. However, the results from hippocampus kindling in δKO mice provide some insight into the functional role of these extrasynpatic receptors in mediating epileptgenic processes.

It is important to note several caveats exist when interpreting the results of these experiments as they apply to the epileptogneic process as a whole. Primarily, these results are relevant only within the context of the hippocampus kindling model of temporal lobe epilepsy. As will be discussed in the following section, the effect of germline deletion of the δ-subunit and loss of extrasynaptic GABA-ARs was considerbaly less clear in the pilocarpine model of temporal lobe epilepsy. Although the kindling model is widely utilized to screen antiepileptogenic drugs, its translational relevancy regarding epileptogenesis is a subject of debate and continued inquiry. For instance, kindled-seizures replicate many characterisitcs of human complex seizures, but their stimulation-induced nature is contrary to the spontaneous nature of seizures in clinical cases of epilepsy. While human cases of temporal lobe epilepsy are often assocaited with (and perhaps partially born from) significant neurodegenration and

alterations in brain morphology and biochemistry, the hippocampus kindling model results in only mild neuropathology. Among the most prominent of the epileptic hallmarks that is typically lacking in the kindling model is expansive mossy fiber sprouting from granule cells of the dentate gyrus. Within this context, there is still much to be taken from these results, and provided here is substantial evidence for a mediating role of δ-continaing receptors in modulating seizure activity and epileptogenesis in the kindling model. Findings from studies utilizing a conditional knockout or other translational epileptogenic models will be key in extrapolating the greater role of δ-GABA-ARs in system excitability and susceptibility to epileptogenesis.

Loss of δ-subunit GABA-ARs resulted in lower afterdischarge threshold, and longer afterdischarge duration, both signs of increased excitability. While this observation alone stands intriguing, in the context of the kindling model of epileptogenesis, it begs the consideration of indirect mechanisms which may drive the accelrated epileptogensis observed in these mice. As kindling requires a focal electrographic discharge for the induction of epileptogenesis (Goddard et al., 1969), it is reasonable to consider that increased intensity of individual kindling stimlations may be responsible. Interestingly, during early stimulations when the difference between the afterdischarge durations of δKO and WT mice are the greatest, no difference in seizure stage or progression is observed. However, the effect of the more intense early stimulations may contribute to the accelerated progression that begins in δKOs between the 5th and 8th stimulation. Although a correlation was observed in kindling progression and afterdischarge duration, the afterdischarge duration to began normalize among genotypes during mid- or late-phase kindling, suggesting that increased afterdischarge duration may be more representative of excitability than reflective of the extent of epileptogenesis. What is clear is that targeted germline deletion of δ-subunit containing GABAARs results in hyperexcitability, decreased seizure threshold, and accelerated epileptogenesis within the hippocampal kindling model.

Germline deletion of the δ-subunit and δ-containing extrasynpatic GABAAR's in the brain resulted in a number of alterations to the course of epileptogenesis following pilocarpine SE. Considering the numerous differences in which the pilocarpine-SE and hippocampal kindling models generate epilepsy, this is not wholly unexpected. Pilocarpine-SE induces extensive neuronal damage and inflammation, and elicits spontaneous seizures, whereas seizures in the kindling modle are stimulation-induced, and brains from kindled animals display significant conservation of neuronal poplulations and far less inflammation. The pilocarpine-SE model is also associated with high mortality, with overall cohort mortality rates of 25-50% and rates of animals reaching SE and surviving ~33% commonly reported (Buckmaster and Haney, 2012). This aspect is strkingly different than the low mortality involved in hippocampus kindling, and represents a potential confound when attempting to draw conclusions from the data at hand. These primary differences, as well as a number of other factors that have been identified in influencing the outcomes of pilocarpine-SE, will be disucssed in the following sections.

In contrast ot the accelerated rate of epileptogenesis observed in δKO mice in the kindling model, rate of epileptognenesis (as represented by latency to seizure) observed in post-SE δKO mice was extended and considerably more varied than in WT mice. Unlike WT mice, in which latency to first seizure was observed within a fairly strict time window between 4 and 10 days post-SE, δKO mice were seemingly biphasic in exhibiting their first spontaenous seizure: between days 7 and 11, or between days 31 and 34. This observation suggests that a number of factors mediating the generation of spotaneous seizures after SE may be altered within the germline δKO mice. Within our implanted electrode-mice, overall incidence of epilepsy was higher within δKO mice, with 83% developing SRS vs. 75% of WT mice. Single seizure duration was not significantly different between WT and δKO mice, echoing the observations form our studies in the kindling model, in which differences in electrographic seizure duration

were diminshed as both gentypes approached the fully kindled (epileptic) state. SE was associated with increased mortality in δKO mice, with 45% mortality rate on day 1 for δKO cohort vs 34% in WT mice. Overall mortality rates for the 90-day study were also higher in δKO (55%) than in WT mice (44%), reflecting the higher initial mortality during SE.

The basic underlying pathophysiology of SE centers around a number of changes that alter endogenous mechanisms for controlling hyperexcitability, such as diminshed GABAergic inhibition (Feng et al., 2008). SE can induce alterations in GABA-ARs expression, with prominent changes in membrane-present GABA-ARs due to internalization of membrane receptors (Goodkin et al., 2008), although the nature and extent of these changes has been shown to be age-dependent (Zhang et al., 2004). Interestingly, the dynamics of GABA-AR internalization during SE vary drastically- in a subunit-dependent manner, with rapid internalization of γ2–containing (synaptic/benzodiazepine-sensitive) beginning shortly after SE, while δ-contaning receptors are largely spared and undergo a much slower rate of intracellular accumulation (Joshi and Kapur, 2009). Evidence from these studies suggests this internalization and reduction in available GABA-ARs at the plasma membrane underlies reduction in GABAergic transmission and subsequent benzodiazepine-resistance observed in prolonged SE (Goodkin et al., 2008; Terunuma et al., 2008). In δKO mice, this γ2-biased internalization of membrane receptors could lead to enhanced loss of inhibitory transmission and may be responsible for the higher mortality rates observed in δKO during SE. While latency to first SRS and overall observation of seizure expression remains consistent within the WT mice, the δKO mice exposed to pilocarpine-SE exhibit wide variation. One potential explanation for the variance observed among δKOs is that higher mortality during SE eliminates a number of individuals before studies of epileptogenesis and chronic epilpesy may be carried out. Additionally, there is also ample data showing that in mice, age and sex both play a role in the sensitivty to pilocarpine- and mortality rates from SE (Buckmaster and Haney, 2012). Although age-

matched males were used for both WT and δKO studies, developmental differences in germline δKO mice could have a profound affect on their sensitvity to pilocarpine and their propensity for developing siezures following SE.

The hippocampus is a critical brain area for cognitive functions such as learning and memory, and often displays high susceptibility to damage in a number of neurological conditions such as epilepsy, stroke, and TBI. The hippocampus also displays remarkable plasticity in the face of insult/injury, and a number of changes involving aberrant neurogenesis and re-wiring of neuronal circuits have been implicated in clinical patients and experimental models. The highly structured circuits involved in hippocampal function rely on a balance of excitatory and inhibtory neurotransmisison, and thus loss of specific neuronal populations within the hippocampus is a key area of focus for therapeutic research concerned with improving functional outcomes in neurodegenrative pathologies. The pilocarpine-SE model is associated with significant neurodegenration in both principle, and interneuron populations across the hippocampal subfields and extrahippocampal areas, which is thought to play a major role in contributing to epileptogenic processes following SE. SE has also been shown to increase neurogenesis in the DG and promote excessive mossy fiber sprouting, which are hypothesized to contribute to hyperexctiability and seizure susceptibility. We therefore sought to characterize neuronal loss within the hippocampus and extrahippocampal areas associated with neurodegeneration in chronic epilepsy in WT and δKO mice 90 days after pilocarpine-SE. We investigated visual changes in cytoarchitecture (cressyl violet), as well as visualization and quantification of principle neuron (NeuN) and parvalbumin-positive (PV+) interneuron loss.

In comparing changes in overall cytoarchitecture as visualized with CV stain, chronic-epilepsy following pilocarpine-SE resulted in substantial cell loss across hippocampal subfields and extra-hippocampal areas of interest in both WT and δKO mice. Although qualitative in nature, the observations here show similar changes in WT and δKO mice, with the most prominent hippocampal losses in CA1 and CA3 subfields. These results

are consistent with reports of broad cell death due to repeated convulsive seizures and hyperexctitable states. Prinicipal neuron loss, a common observation in epileptic models, was extensive and not profoundly different between genotypes, although small region-specific differences suggest differntial effects of the germline loss of δ-GABA-ARs on seizure-induced degeneration in these areas. Contrasting this observation with the signifcantly greater degree of PV+ inihibtory interneuon loss observed in δKO mice points to varying degree of importance for δ-GABA-ARs and tonic inhibition in neuroprotection in SE and epileptogenesis: PV+ inhibitory interneuons, which are known to be particularly susceptible to epileptic damage and death (Kuruba et al., 2011), suffer significantly increased losses following pilocarpine induced SE and subsequent epilpeptogenesis follwing germline deletion of δ-subunit containing extrasynpatic GABA-ARs. As PV+ intenreurons are known to contirbute to the synchronization of oscillations (Kalusberger et al., 2005), and loss or reduction in PV+ has been shown to increase seizure susceptibility (Schwaller et al., 2004), the enhanced loss noted here in δKO mice suggests a greater propensity for seizures and epileptic acitivty, which may not be reflected in the SRS data due to compensational changes resulting from germline deletion, or exclusion of individuals due to enhanced mortality in δKOs. Follow-up studies with conditional knockout of δ-subunit containing GABA-ARs could provide for greater insight into the functional outcomes of this enhanced loss.    Mossy fiber sprouting was significantly increased in both WT and δKO mice expressing chronic epilpetic seizures. These increases were similar between genotypes, although higher baseline staining intensities were observed in control δKO mice, which display hyperexcitability even before exposure to pilocarpine and chornic siezures. These observations  suggest that WT and δKO mice undego similar axonal sprouting following chronic convulsive seizures.

Taken together, these results demonstrate that δ-containing receptors play an important role in the development of epileptogensis in models that do not induce significant neurodegeneration, such kindling. While several confounds remain regarding the

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development and incidence of epilepsy in post-SE δKO mice, the increased mortality and neuron-specific cell loss, as well as the variance in the development of SRS suggest a modulating role for δ-containing receptors in SE and subsequent epileptogenesis. Overall, these findings support the notion that extrasynaptic GABA-ARs and tonic inhibtion play a contributory role in neuroprotection and seizure susceptibility in epileptogenesis and may serve as a viable route for therapeutic intervention aimed at augmentation of inhibition. Loss of extrasynpatic δ-GABA-ARs resulted in striking differences in epileptogenesis and acute seizure susceptibility.

## V.3 The role of δ-subunit extrasynaptic GABA-A receptors in the antiepileptogenic actions of neurosteroids

The prinicpal findings of this study are that aumenting or inhibiting neurosteroid levels conveys significant modulation of kindling epileptogenesis in mice, and that chronic treatment with the neurosteroid ganaxolone following pilocarpine-SE provides led to stiriking amelioration of epileptogenesis and neurodegenerative pathologies associated with SE and chronic epilepsy. Furthermore, modification of neurosteroid levels conveyed antiepileptogenic effects even in mice lacking neurosteroid-sensitive δ–GABA-ARs, an intriguing observation that may provide cues to a more complete understanding of the mechanisms underlying neurosteorid modification of epileptogenesis. Consistent withour observations of accelerated kindling epileptogenesis in mice treated with finasteride, inhibition of neurosteroid synthesis following pilocarpine-SE decreased latency to expression of spontaneous seizures, although this effect did not lead to the exacerbation of neurodegeneration or cognitive impairment we observed in vehicle-treated cohorts.

Acute seizure activity and prolonged status epilepticus are known to induce a wide variety of changes in brain physiology, including changes in receptor expression and trafficking, increased production of ROS and inflammation, loss of balance between inhibitory and excitatory circuits, and neuronal death and abberant neurogenesis. A

number of previous reports have described various facets of modification of epileptogenesis following pilocarpine-SE using antiinflammatory or inhibtion-promoting interventions, but none so far have reported significant inhibition of all pathologies (Table 2 & 3). The "big picture" encompassing the mechanistic basis of epileptogenesis is complex and still far from being fully understood, and observations from our current study offer insight on the nature and extent to which these may contribute to epileptogenesis, as well as potential mechanisms by which neurosteroid-based therapies and δ-GABA-AR signaling modulate epileptogenesis in two distinct models of epileptogenesis.

Our initial investigation into the effect of germline loss of extrasynaptic δ-GABA-ARs revealed striking increases in acute seizure susceptibility and accelerated epileptogenesis in the hippocampal kindling model. Loss of extrasynaptic δ-GABA-ARs leads to reduced tonic inhibition and increased hippocampal excitability, an observation which suggests that accelerated kindling epileptogenesis in δKO mice may be due to increased stimulus intensity with each kindling stimulation. Indeed, WT mice exhibited lower AD durations than δKO mice at corresponding stimulations, and average AD duration for a given racine seizure score were similar between Wt and δKO mice. Fully kindled δKO mice also exhibted reduced sensitivity to the acute anticonvulsant effect of the nuerosteroid AP. Given this information, we sought to thoroughly investigate the potential of modifying neursteroid levels for disease modification in WT and neurosteroid-insensitive δKO mice. Previous studies have demonstrated that antiepileptogenic effects of progesterone treatment on kinding epileptogenesis were due to increased circulating levels of its steroid derivative AP (Reddy et al., 2010). Using our sequential gonadotropin protocol, we measured the rise of AP concetration in plasma, and found that exogenous treatment with 0.5 mg/kg AP provided for similar levels which would serve as our baseline in establishing a dose-response characterization of this effect. Concurrent treatment with finasteride abolished the increase in circulating AP

in gonadotropin treated mice, but had no effect on circulating levels of AP in animals receiving AP injections.

Gonadotropin treatment resulted a significant delay in kindling epileptogenesis in WT and δKO mice, with both genotypes displaying similar reductions in kindling rate proportionate to the rates of untreated cohorts. Interestingly, AP treatment had no effect on the acute seizure severity in WT mice, as indicated by similar AD durations in treated and untreated cohorts. The observation of decreased seizure stage scores relative to AD duration during late-phase kindling in WT mice suggests that AP may be conveying its antiepileptogenic effects through mechanisms beyond blunting of the kindling stimuli. However, δKO mice did not display lower seizure scores relative to AD duration, with similar AD durations observed in both groups from stages 2-5. While this observation supports reduced stimulus intensity as a partial driving force underlying the observed antiepileptogenic effects of GN treatment, it also suggests a route independent of δ-GABA-ARs. Treatment with finasteride resulted in a powerful acceleration of kindling epileptogensis in WT and δKO mice, characterized by greatly increased AD durations and higher seizure scores at respective stimuli. This study provides substantial evidence for the role of endogenous neurosteroids in limiting acute seizure activity, and a correlation between AD duration and hippocampal kindling rate. It also suggest that even in δKO mice, endogenous neurosteroids provide for augmentation of GABAergic inhibition. Finasteride treatment also abolished the disease-modifying effect of Gn treatment in kindling epileptogenesis in WT and δKO mice. In WT mice, GN + FN treatment resulted in an accelration of kindling rate, but to a lesser degree than with FN treatment alone. In δKO mice, GN + FN treatment resulted in kindling rates similar to that of control cohorts, despite treated mice displaying shorter AD durations for the duration of kindling. Clearly the relationship between kindling rate and AD duration is more complex than would be assumed under the notion that they follow a linear relationship.

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Having demonstrated a powerful and likely neurosteroid-dependent effect of Gn and FN on kindling epileptogenesis, we sought to examine the effect of treatment with the exogenous neurosteroid AP, which we hypothesized would produce robust inihibtion of kindling epileptogenesis similar to that of Gn treatment. Chronic injection of AP 0.5 mg/kg resulted in reduced AD durations and lower seizure scores as respective kindling stimulations in both WT and δKO mice. More specifically, AP treatment conveyed this affect to a similar extent in both genotypes, although δKO mice showed greater variation in late-stage seizure expression than did WT mice. From this observation we gather more evidence for a relationship between AD duration and kindling rate, as well as support for the actions of neurosteroids through δ-GABA-AR independent mechanisms., likely via augmentation of other types of GABA-ARs.

To more conclusively examine this effect, we repeated hippocampal kindling in WT mice treated daily with AP 0.5 mg/kg and 50 mg/kg finasteride. Indeed, finasteride treatment failed to inhibit the disease-modifying effect of AP treatment in kindling epileptogenesis. When considered with our previous findings on modulating neurosteroids in kindling epileptogenesis, this strongly supports a neurosteroid-centric mechanism and led us to investigate the effect of higher-doses of AP in the kindling model. Increasing daily AP treatment to 3.0 mg/kg resulted in near-complete inhibition of kindling epileptogenesis in WT mice. Although kindling progression was similar to that of vehicle treated controls until the $10^{th}$ stimulation, high-dose AP cohorts did not progress beyond stage 2 seizures, even after a greatly extended kindling protocol. Commensurate with the reduction of seizure stage scores beyond the $10^{th}$ stimulation, high-dose AP mice also displayed lower AD durations than control counterparts, providing powerful evidence that reduced stimulus intensity may be correlated with reduction in kindling epileptogenesis. Having established a significant body of evidence for the neurosteroid-specficity of the antiepileptogenic observations in these studies, we utilized 3β–AP, which has been shown to have significantly reduced efficacy on synaptic currents (Kokate et al., 1994) as well as tonic currents (Carver and Reddy, 2016), to investigate the potential of AP to modify epileptogenesis through a GABA-

AR-independent mechanism. As expected, chronic high-dose (3.0 mg/kg) 3β-AP treatment had no effect on kindling rate, although treated animals did experience increased AD duration during the final (stage 5) phases of kindling. This observation provides strong evidence for a GABA-AR-dependent mechanism of neurosteroid modification of kindling epileptogenesis.

As the focus of this study was to investigate translational potential of neurosteroid modulating interventions in epileptogenesis, we sought to replicate these studies using ganaxolone, a synthetic analog of AP that has shown increased bioavailability and has undergone clinical trials in epileptic patients. To investigate potential sex-differences in response to neurosteroid-based intervention in kindling epileptogenesis, male WT and δKO mice were used for these studies. Hippocampal kindling was carried out in 3 cohorts, using our baseline dose of 0.5 mg/kg in one, our high-dose of 3.0 mg/kg, and an intermediate dose of 1.0 mg/kg. GX led to a robust and dose-dependent retardation of kindling epileptogenesis in both WT and δKO mice. The decreased sensitivty to neurosteroid treat in δKO was evident in our 0.5 mg/kg cohort, in which WT mice experienced a significantly greater delay in kindling epileptogenesis than δKO counterparts. Neither genotype displayed significantly lower AD duration respective to seizure stage scores at this dose, with increases in AD duration observed as seizure stages progressed beyond level 2.

The genotype-specific difference in sensitivity was no longer apparent at 1.0 mg/kg and 3.0 mg/kg doses of GX, indicating that this protocol results in neurosteroid concentration adequate to augment GABAergic inhibition independent of δ-GABA-ARs. Of interest, little difference in efficacy between 0.5 mg/kg and 1.0 mg/kg doses was observed in WT mice, whereas δKO mice receiving 1.0 mg/kg displayed a greater extent of kindling retardation than 0.5 mg/kg counterparts, with similar overall kindling rates to WT counterparts. High-dose GX conveyed a near-complete inhibition of kindling epileptogenes in both WT and δKO mice, similar to observed in our studies utilizing AP. AD durations in this group were similar between genotypes and remained constant

throughout the kindling process, further supporting the effect of acute stimulus reduction on kindling epileptogenesis. As a final investigation into potential mechanisms and physiological outcomes of neurosteroid modulation of kindling epileptogenesis, we examined the extent of mossy-fiber sprouting in fully-kindled WT and δKO mice from vehicle, low GX, and intermediate GX cohorts. Fully kindled animals of both genotypes displayed significantly increased mossy fiber sprouting in the DG granule layer and DH. In both genotypes, GX treatment significantly reduced the increase in mossy fiber sprouting in the DG granule layer in a dose-dependent fashion, but did not significantly alter sprouting in the DH. While a hallmark of epileptic pathology, mossy fiber sprouting has been altered or blocked in experimental models of epilepsy that still generate seizures, and our data suggests that kindling induces mossy fiber sprouting, and that the extent to which these abberant connections are made may be reduced in neurosteroid treated mice.

While our kindling studies provide a great deal of insight on neurosteroid modulation epileptogenesis, the hipoocampal kindling model lacks several key characterisics of clinical epilepsy, and leaves many questions regarding the nature of the underlying mechanisms. Is the acute reduction of seizure stimuli primarily responsible for the apparent antiepileptogenic effects of neurosteroids in the kindling model? Utilizing the pilocarpine-SE model of TLE to examine the effects of aumenting or inhibiting neurosteroid levels on epileptogenesis provides for an opportunity to address these questions and more. SE is known to induce inflammation and activation of glial cells, interupt BBB permeability, and result in neurodegeneration and abberant neurogenesis. It also leads to SRS and cognitive impairments that more closely replicate clinical epilepsy in a more accurate fashion than the kindling model. Following our characterization of post-SE epileptogenesis in WT and δKO mice, we repeated these studies in WT male mice, utilizing chronic application of high-dose GX (5.0 mg/kg, s.c., 2x daily) or finasteride (50 mg/kg, i.p.) following SE. GX treatment resulted in profound inhibition of epileptogenesis following SE. Prevalence of epilepsy was reduced, with 75% of our cohort remaining SRS-free for the duration of the study. In animals which

experienced SRS, the latent period was significantly increased, and the number of SRS was redcued compared to control post-SE mice.

GX treatment also amleriorated neurodegeneration following SE, reduced astrocyte activity (but did not affect microglia activation as measured by IBA1(+) staining), and resulted in improvement in object memory in mice 90 days after SE. Taken together, these observations suggest a powerful multi-faceted mechanism of GX in interupting epileptogenesis following SE. In stark contrast to the antiepileptogenic effect observed with GX treatment, chronic inhibition of neurosteroid synthesis with finasteride resulted in accelerated expression of SRS and greater overall seizure prevalence compared to control post-SE mice. Latency to first SRS in FN treated mice was half that observed in controls, and SRS were detected in all mice in this cohort. FN treatment also resulted in increased seizure expression over the 90 day study, but did not result in increased average single seizure duration. FN treated animals also displayed levels of neurodegeneration and inflammation, as well as cognitive deficits similar to that of untreated post-SE cohorts.

To establish a context in which to evaluate these findings, we first look to previous reports that have shown P450scc, the rate-limiting enzyme in steroid synthesis, is upregulated in hippocampal glial following SE, and that prolonged SE was associated with greater increases in P450scc and long latent periods (Biagini et al., 2006). Moreover, the same authors observed a significant decrease in latent period duration following treatment with finasteride. Our observations are in aligment with these findings, and support the idea that neurosteroid levels may regulate the latent period following SE. SE has been shown to induce significant alterations in GABA-AR expression and trafficking, whereby internalization of synaptic GABA-ARs in increased (indeed, efficacy of benzodiazepines reduced in SE due to decreased availability of benzodiazepine-sensitive synaptic GABA-ARs), while extrasynpatic $\delta$-GABA-ARs are preserved or upregulated during and immediately after SE (Yu, et al., 2013). It is therefore possible that neurosteroid treament would have an increased inhibtory effect

immediately following SE, inhibiting seizure expression. The reduction in latent period in FN treated mice is in agreement with this idea. However, this notion does not fully address the full range of pathologies associated with SE. In examining neurodegeneration following SE, we must examine the regional and temporal aspects of neuronal loss, as well as the glial and other environmental factors. The timeline of neuronal loss following SE ranges from hours to weeks, with loss of hilar neurons occuring most rapidly (within 3 hours of SE), while pyramidal cells in CA1 and CA3 reach greatest level of cell death between 1 and 3 weeks post-SE (Nascimento et al., 2012).

Excitotoxicity and inflammation are indicated as contributors to neurodegeneration following SE, and studies utilizing antiinflammatory treatments have demonstrated reduced cell death and decreased seizure severity in the pilocarpine-SE model (Jung et al., 2006; Saha and Chakrabarti, 2014; Mishra et al., 2015). Interestingly, AP has been shown to reduce inflammation following TBI through induction of CD55 (an inhibitor of inflammatory cascade activators), as well as reduce BBB permeability following stroke (VanLandingham et al., 2007, Ishrat et al., 2010). Taken together, these observations begin to paint a picture by which neurosteroids are in a prime position to simultaneously reduce neuroinflammation and increase inhibition following SE. Our 20-day treatment window would provide for these beneficial effects during both acute and delayed phases of neurodegenration, and at the dose given, would be expected to result in brain concentrations sufficient for augmentation of synpatic and extrasynaptic GABAergic inihibtion. By restoring inhibitory balance while simulatenously aiding in reducing inflammation, neurosteroid treatment could significantly alter the factors implicated in driving neuronal death follow SE.

The significant rescue of neuronal populations in the hippocampus are likely to underlie the improvement in object memory in GX treated mice, although the observations of persistent increases in anxiety-like behvaior across all post-SE groups suggests that the reduction in neuronal loss and inflammation seen with GX treatment is insufficient for

complete prevention of cognitive deficits. When interpeting the results of finasteride treatment in post-SE mice, we must consider the proposed mechanisms as distinct but inter-related, and therein we may draw several conclusions regarding the nature of neurosteroids' role in modulating epileptogenesis and related pathologies. First, evidence from kindling and SE models suggests that inhibiting neurosteroid synthesis leads to acute increases in excitability and seizure susceptibility, and that augmenting neurosteroid levels reverses this effect. This effect appears particularly important to the modulation of acute stimuli, such as kindling-induced seizure expression, and may likewise contribute to the modification of the latent phase following SE. Second, the driving forces of the generation of SRS and neuronal death following SE are partially established during SE, but may be modified by immediate treatment. GX treatment led to considerable rescue of hippocampal neuronal populations compared to control or finasteride-treated mice, but was not capable of eliminating these losses altogether. Third, despite the modulatory role endgoenous neurosteroids appear to play in mediating epileptogenesis following SE, the loss of this mediation has a greater affect on immediate seizure activity than it does on the neuronal pathologies and subsequent functional implications. Put simply, enhancing or increasing neurosteroid activity shows significant potential for modifying or ameliorating a wide spectrum of acute and chronic epileptogenic pathologies, while eliminating neurosteroids has a greater affect on immediate excitability and seizure expression in epilepsy.

## CHAPTER VI
## CONCLUSIONS

Neurosteroids are important endogenous modulators of many neural functions. One of the primary molecular targets for endogenous neurosteroids is the neuronal GABA-A receptor chloride channel. Allopregnanolone and other structurally similar neurosteroids are potent allosteric agonists as well as direct activators of both synaptically and extrasynaptically located GABA-A receptors in the brain. Therefore neurosteroids can serve to enhance synaptic (phasic) and extrasynaptic (tonic) inhibitory actions. The resulting chloride current conductance generates a type of shunting inhibition that play a key role in network excitability, seizures, and behavior. Dysfunction of extrasynaptic GABA-A receptors may cause profound impact in many CNS conditions. Catamenial epilepsy, a form of pharmacoresistant epilepsy in women in which seizures are clustered around specific points in the menstrual cycle, most often around the perimenstrual (C1) or periovulatory (C2) period. Although menstrual cycle-related neurosteroid fluctuations play a key role in the pathophysiology of catamenial epilepsy, the precise role of extrasynaptic GABA-A receptors is poorly understood. Similarly, the neuroendocrine role of neurosteroids in epileptogenesis and their therapeutic potential for disease-modification of epilespy remains unclear. We addressed these major questions in this dessertation research.

In studies in Aim 1, we utilized the δKO mice to identy the role of extrasynaptic δGABA-A receptors in catamenial-like seizure exacerbation. Using mouse catamenial epilepsy model, we demonstrated a striking increase in seizure exacerbation in δKO mice in the NSW model of perimenstrual catamenial epilepsy, which strongly supports the role of extrasynaptic δGABA-A receptor-mediated tonic inhibition in catamenial epilepsy. As expected, δKO mice exhibited reduced antiseizure activity to benzodiazepines but not to neurosteroids. The heightened seizure susceptibility and altered antiseizure drug responses are consistent with the relative deficiency of δGABA-

A receptors in the brain. These studies provide further support for the tonic inhibition-based neurosteroid replacement therapy for catamenial epilepsy.

In studies in Aim 2, we investigated role of extrasynaptic δGABA-A receptors in susceptibility to limbic epileptogenesis. We demonstrated that δ-containing receptors play an important role in the development and persistence of limbic epileptogensis in models that do not induce significant neurodegeneration, such kindling. Our findings support the premise that extrasynaptic GABA-ARs and tonic inhibtion play a contributory role in neuroprotection and seizure susceptibility in epileptogenesis and may serve as a viable route for therapeutic intervention aimed at augmentation of inhibition. Lack of extrasynpatic δGABA-ARs resulted in striking differences in kindling epileptogenesis and acute seizure susceptibility, suggesting that these receptors may play in pathologic neuronal excitability and epileptogenesis.

In studies in Aim 3, we investigated the role of extrasynaptic δGABA-A receptors in the antiepileptogenic or disease-modifying activity of neurosteroids. Using kindling model of epileptogenesis, we identified the functional role of neurosteroids in antiepileptogenic interventions during kindling epileptogenesis. The results paint a vivid picture characterizing the nature of neurosteroid-based disease-modification during kindling. Enhancing neurosteroid activity through endogenous or exogenous means consistently inhibited kindling epileptogenesis. Inhibiting neurosteroid synthesis with finasteride resulted in accelerated epileptogenesis and exacerbated seizures. Moreover, increasing the dose of synthetic neurosteroid GX produced a near-complete inhibition of kindling progression. In the pilocarpine-SE model of epileptogenesis, we found that augmenting or inhibiting neurosteroid levels has led to stiriking changes in epileptogenesis, as evident from alterations in the frequency of spontaneous seizures as well as changes in neurodegenerative pathologies associated with SE-induced chronic epilepsy. However, neither therapetuic or pathologic interventions had a significant effect on the cognitive deficits observed in vehicle-control mice following pilocarpine-SE. Finally, administration of the synthetic neurosteroid GX resulted in antiepileptogenic effects and

neuroprotection in the pilocarpine model. Overall, these results indicate a critical contributory and potentially multifaceted role for neurosteroids and extrasynaptic δ–GABA-A receptors in the development of epileptogenssis, with intriguing therapeutic implications for desinging novel antiepileptogenic or disese-modifying interventions for epilepsy.

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