COGNITIVE IMPAIRMENT AND ABERRANT PLASTICITY IN THE KINDLING MODEL OF EPILEPSY

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Saskatoon, Saskatchewan

By
Justin James Botterill

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ABSTRACT

Epilepsy is a neurological disorder that affects approximately 1% of the population worldwide. Although motor seizures are the best known feature of epilepsy, many patients also experience severe interictal (between-seizure) behavioral and cognitive comorbidities that have a greater negative influence on quality of life than seizure control or frequency. To study the characteristics of these interictal comorbidities and the neural mechanisms that underlie them, I use the kindling model of epilepsy. Kindling refers to the brief electrical stimulation of a discrete brain site that produces a gradual and permanent increase in the severity of elicited seizure activity. The repeated seizures associated with kindling induce robust structural and functional plasticity that appears to be primarily aberrant. Importantly, the aberrant plasticity evoked by repeated seizures is thought to contribute to the pathophysiology of epilepsy and its associated behavioral and cognitive comorbidities. Unfortunately, the relationship between aberrant plasticity and cognition dysfunction following repeated seizures remains poorly understood.

The aim of this dissertation is to gain a better understanding of the effects of repeated convulsions on aberrant neural plasticity and interictal behavior. In Chapter 2, I will examine the effect of short- and long-term amygdala kindling on amygdala- and hippocampal-dependent forms of operant fear conditioning. To evaluate whether kindling alters neural circuits important in memory, I will analyze post-mortem measures of neural activity following the retrieval of fearful memories. In Chapter 3, I will evaluate whether deficits in operant fear learning and memory are a general consequence of convulsions induced by kindling stimulations or whether these deficits occur following kindling of specific brain regions. To evaluate whether aberrant plasticity following kindling of different brain regions contributes to learning and memory deficits, I will make post-mortem examinations of the inhibitory neurotransmitter neuropeptide Y and its Y2 receptor. In Chapter 4, I will investigate the relationship between hippocampal neurogenesis and cognition. Specifically, I will determine whether kindling of different brain regions induces an aberrant form of hippocampal neurogenesis that contributes to cognitive dysfunction. In Chapter 5, I will investigate whether kindling of different brain regions alters different subpopulations of hippocampal GABAergic interneurons, in terms of number and morphological features. Finally, Chapter 6 will provide preliminary evidence that the cognitive impairments associated with kindling can be ameliorated through intrahippocampal infusions of

recombinant reelin. The collection of studies in this dissertation improves our understanding of the relationship between aberrant plasticity and cognitive impairments associated with repeated convulsions.

ACKNOWLEDGEMENTS

DEDICATION

I dedicate this work to my mother (Dianne) and father (Michael).

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LIST OF ABBREVIATIONS

AD   after-discharge

AED   antiepileptic drugs

AMPA   α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ANOVA   analysis of variance

APV   D,L-2-amino-5-phosphonovaleric acid

AVG CS   average freezing during tone presentations

AVG PS   average freezing during post-shock periods

AVG PT   average freezing during post-tone intervals

BDNF   brain-derived neurotrophic factor

BLA   basolateral amygdala

BLBP   brain lipid binding protein

BNST   bed nucleus of the stria terminalus

BrdU   Bromodeoxyuridine

BSA   bovine serum albumin

CA   Cornu Ammonis

CEA   central amygdala

CeL   central amygdala lateral division

CeM   central amygdala medial division

CG   cingulate gyrus

CN   caudate nucleus

CNS   central nervous system

CR   calretinin

CS   conditioned stimulus

DAB   3,3'-diaminobenzidine

DCX   doublecortin

DG   dentate gyrus

dHip   dorsal hippocampus

DISC1   disrupted in schizophrenia 1

DNA	deoxyribonucleic acid
ECT	electroconvulsive therapy
EEG	electroencephalogram
endoN	endo neuraminidase
ELISA	enzyme-linked immunosorbent assay
Ent	entorhinal cortex
GABA	γ-aminobutyric acid
GAD	glutamatic acid decarboxylase
GCL	granule cell layer
GCV	ganciclovir
GFAP	glial fibrillary acidic protein
GPe	globus pallidus external
HBD	hilar basal dendrite
HDAC	histone deacetylase
HEGC	hilar ectopic granule cell
Hil	hilus
HIPP	hilar perforant path-associated
HVc	hyperstriatum ventralis par caudalis
IEG	immediate early gene
ILAE	International League Against Epilepsy
i.p.	intraperitoneal
ir	immunoreactive
LA	lateral amygdala
LTD	long-term depression
LTP	long-term potentiation
MAM	methylaxoxymethnaol
mBDNF	mature brain-derived neurotrophic factor
MEA	medial amygdala
MGN	medial geniculate nucleus
mRNA	messenger RNA

MSN	medium spiny neuron
NCAM	neural cell adhesion molecule
NeuN	neuronal nuclei
NGS	normal goat serum
NHS	normal horse serum
NMDA	N-methyl-D-aspartate
NPC	neural progenitor cell
NPY	neuropeptide Y
NPY2R	neuropeptide Y 2 receptor
PAG	periaqueductal gray
PB	phosphate buffer
PBS	phosphate-buffered saline
PNN	perineuronal net
PrH	perirhinal cortex
Prox1	prospero homeobox 1
PS	post-shock
PT	post-tone
PTEN	phosphatase and tensin homolog
PV	parvalbumin
PvP	polyvinylpyrrolidone
PYY	peptide YY
RTF	regulatory transcription factor
s.c.	subcutaneous
SGZ	subgranular zone
SL	stratum lucidum
SLM	stratum lacunosum moleculare
SNr	substantia nigra pars reticulate
SOM	somatostatin
SO	stratum oriens
SP	stratum pyramidale

SR	stratum radiatum
STN	subthalamic nuclei
SVZ	subventricular zone
TBS	tris-buffered saline
TLE	temporal lobe epilepsy
TrkB	tyrosine receptor kinase B
US	unconditioned stimulus
VIP	vasoactive intestinal polypeptide
VPA	valproic acid

CHAPTER 1

General Introduction[1]

1.1 Outline of the Thesis

The term plastic is of Greek origin (*plastos, plastikos*) and refers to a substance that is malleable or able to take on a variety of shapes (Pascual-Leone, Amedi, Fregni, & Merabet, 2005). Although the brain was once perceived as a relatively static organ incapable of change following its elaborate developmental period, it is now clearly established that the brain constantly changes in response to experience or injury (Kolb, Mychasiuk, & Gibb, 2014). The concept of neural plasticity was first described by William James in *The Principles of Psychology* in an attempt to explain behavior modifications and habit formation in living organisms (James, 1890). In the late 1890s and early 1900s, Santiago Ramón y Cajal further posited that learning and behavioral flexibility must have an underlying anatomical basis in the brain, presumably involving the formation of new connections between neurons (Berlucchi & Buchtel, 2009; Pascual-Leone et al., 2005). In the arguably one of the most influential neuroscience works of the 20th century, *The Organization of Behavior*, Donald Hebb proposed that the strength or effectiveness of individual synapses can change in response to activity (i.e., synaptic plasticity) (Hebb, 1949). The concepts of plasticity proposed by James, Cajal and Hebb provided the foundation for our modern understanding of neural plasticity, which can be defined as the ability of the nervous system to respond to intrinsic or extrinsic stimuli through structural and functional neuronal reorganization.

The propensity for change is the defining characteristic of the nervous system. Indeed, neural plasticity occurs in response to a myriad of factors, including environmental stimuli, learning, drugs, hormones, maturation, aging, diet, and disease (Kolb & Muhammad, 2014). The plasticity of the nervous system is generally perceived as an adaptive mechanism that serves to improve the survival of an organism by providing flexibility in cognition and thus behavior. In this regard, learning and memory represents one of the most widely studied examples of adaptive

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[1] Portions of this chapter have been published in: Botterill, J.J., Guskjolen, A.J., Kalynchuk, L.E., Caruncho, H.J. (2012) Rodent models as tools for discovering novel therapeutic targets in the brain: the case of epilepsy. In: Botana, L.M., Loza, M editors. <u>Therapeutic Targets: Modulation, Inhibition, and Activation</u>. John Wiley & Sons Inc.

neural plasticity. Experimental models of learning and memory primarily focus on the short- and long-term changes in the strength of efficacy of synaptic transmission, collectively referred to as long-term potentiation (LTP) (Bliss & Gardner-Medwin, 1973) or long-term depression (LTD) (Ito, Sakurai, & Tongroach, 1982). Many of the experimental studies of LTP and LTD involve the hippocampus because of its dense innervation of excitatory neuronal circuits and critical roles in learning and memory. Remarkably, the mechanisms involved in the activity-dependent synaptic refinement and the stability of neural circuits underlying nervous system plasticity appear to be the same as those that are recruited in learning and memory paradigms. In particular, both of these processes involve the stimulation of neurotransmitters and receptors on the surface membrane of cells, the activation of intracellular signal transduction pathways, and the synthesis of new proteins that influence the physical shape of neurons and the density of synapses (Johnston, 2003; Mayford, Siegelbaum, & Kandel, 2012). The ability of the nervous system to reorganize neuronal circuits or form entirely new ones is becoming increasingly recognized as a potential panacea for disease (Kolb & Muhammad, 2014).

The incredible ability of the nervous system to undergo broad neural plasticity comes with great risk. This caveat is best exemplified in the case of the neurological disorder epilepsy, whereby the inherent tendency of the nervous system to refine existing synapses and form new connections becomes exaggerated. The exaggerated plasticity associated with seizures promotes the formation of recurrent excitatory circuits that cause excessive and synchronous neural activity (Scharfman, 2002). The hippocampus appears to be particularly prone to aberrant seizure-induced plasticity, presumably owing to its large number of excitatory circuits that undergo rapid activity-dependent refinement (Yasuda et al., 2011). In addition, the hippocampus is one of only two areas in the adult brain where new cells are generated and integrate into the existing circuitry, a process known as neurogenesis (Ming & Song, 2011). Although the formation of new neurons in the epileptic brain was once hypothesized to serve as an adaptive repair mechanism (Parent & Lowenstein, 2002), recent evidence indicates that aberrant seizure-induced neurogenesis further contributes to hippocampal dysfunction and cognitive decline (Cho et al., 2015). Unfortunately, our understanding of the mechanisms involved in aberrant plasticity associated with repeated convulsions remains poorly understood. This issue is important to investigate, as improving our understanding of brain plasticity can inform scientists about normal and abnormal brain function.

This dissertation includes a series of experiments that attempt to further our understanding of the relationship between aberrant seizure-induced neural plasticity and cognitive impairment. To study these issues, I will use the kindling model of epilepsy, whereby seizures are repeatedly induced by electrical stimulation of a discrete brain region. Initially, I will characterize the behavioral and cognitive effects of short- and long-term amygdaloid kindling on amygdala- and hippocampal-dependent learning and memory tasks. In subsequent experiments, I will investigate the effect of kindling different brain regions on measures of learning and memory, aberrant hippocampal neurogenesis, and plasticity of inhibitory interneurons. Finally, I will also provide preliminary evidence that the seizure-induced impairments of cognition can be rescued using a novel therapeutic approach.

The remaining sections of this chapter will provide an overview of epilepsy, neuroanatomy, operant conditioning, neurogenesis, and aberrant neural plasticity associated with repeated seizure activity. The section on epilepsy will focus on its clinical features, neuropathology, and current treatment options. I will then discuss widely used animal models of epilepsy that allow researchers to study the neurobiological and behavioral changes associated with repeated seizure activity. This chapter will also provide an overview of neuroanatomy relevant to the current thesis, with particular emphasis towards the amygdala, hippocampus, and caudate nucleus. I will also discuss the use of operant fear conditioning as a paradigm to study the neural circuits involved in learning and memory. I will then provide a detailed summary of the history, stages, and function of adult hippocampal neurogenesis. Following this, I will discuss plasticity associated with seizures, with particular emphasis towards the creation of aberrant circuits that promote seizure activity and cognitive dysfunction. This chapter will conclude with a discussion of specific topics that remain to be answered in the field of epilepsy and how the experimental chapters of this thesis attempt to resolve these issues.

Chapters 2, 3, 4, 5, and 6 will provide experimental data that address experimental questions outlined in this chapter. Chapter 7 will provide a general overview of my experimental research, the implications of my research findings and the limitations of my research results. Chapter 7 will conclude with a brief discussion on the specific areas where further research is required.

1.2 Introduction to Epilepsy

Epilepsy is a neurological disorder that affects approximately 1% of the population. Current estimates indicate that one in ten people with have at least one seizure in their lifetime and that one-third of those individuals will develop epilepsy (Engel, Jr. & Pedley, 2008). Epilepsy is characterized by an enduring predisposition to generate seizures (Fisher et al., 2014). Seizures are defined as paroxysmal alterations of neurologic function resulting from the excessive and hypersynchronous discharge of neurons in the brain (Stafstrom & Carmant, 2015). Although seizures are most commonly associated with motor disturbances, they can also manifest as sensory, cognitive, or autonomic disturbances. The current diagnosis criteria for epilepsy requires a patient to have two unprovoked seizures occurring at least 24 hours apart (Fisher et al., 2014). It is important to clarify that epilepsy is not a single syndrome or disorder. Instead, epilepsy is an umbrella term that describes a broad spectrum of seizure disorders. In some instances, the term epilepsy syndrome is used, which refers to groups of clinical features that typically occur together. An example of clinical features covered by the term epilepsy syndrome include the seizure phenotype, age of onset, electroencephalogram (EEG) record, provoking factors (i.e., triggers), genetic factors, prognosis, and response to treatment (Stafstrom & Carmant, 2015).

In addition to the significant personal burdens experienced by individuals afflicted with epilepsy, there are also substantial global economic costs associated with this disorder (Begley & Beghi, 2002; Shafer & Begley, 2000). The current estimates suggest that the annual economic cost of epilepsy is between 10 (Yoon, Frick, Carr, & Austin, 2009) to 12.5 (Begley et al., 2000) billion dollars in United States and 14.8 billion dollars in Europe (Gustavsson et al., 2011). The variation in these reports is partially attributable to the use of different factors that are considered in the cost analysis (e.g., direct vs indirect costs). Direct medical costs associated with the diagnosis, treatment, prevention, or rehabilitation account for as little as 14% of the total economic burden, whereas indirect costs associated with unemployment and decreased productivity account for up to 86% of the total economic burden (Begley et al., 2000). The substantial personal and global costs associated with epilepsy clearly illustrate the need for better diagnoses, treatments, and patient outcomes.

1.2.1 Classification of the Epilepsies

The International League Against Epilepsy (ILAE) has created a classification system of

seizures and epilepsy syndromes based on their clinical features and electroencephalogram recordings (Table 1-1). The development of such a classification system is important, as it provides clinicians a clear criterion for diagnosis, prognosis, and treatment. Broadly speaking, epilepsy can be divided into three major categories: generalized, focal, and unknown (e.g., epileptic spasms). Generalized seizures consist of rapid electrical discharges (e.g., polyspikes on the EEG record with slow-wave discharges at 3-5 Hz) that occur simultaneously and symmetrically in both cerebral hemispheres (Seneviratne, Cook, & D'Souza, 2012; Smith, 2005). Generalized tonic-clonic seizures typically last between 1 to 3 minutes and comprise bouts of rigidity (i.e., tonus) followed by jerking movements (i.e., clonus) that coincide with a loss of consciousness. Following the ictal (i.e., seizure) period, patients often enter a post-ictal period characterized by confusion and fatigue (Hamelin, Kahane, & Vercueil, 2010). Due to the uncontrollable nature of generalized tonic-clonic seizures, these type of seizures pose an increased risk for personal harm and accidents. For example, individuals that experience generalized tonic-clonic seizures are more likely to accidentally bite their tongue, become incontinent, or suffer head trauma and/or bodily injury from falling onto hard surfaces (Engel, Jr. & Pedley, 2008).

Absence seizures represent the second major subcategory of generalized seizures. Absence seizures are characterized by a brief loss and return of consciousness associated with 3-Hz spike-wave discharges on the EEG (Smith, 2005). The category of absence seizures is further divided into typical and atypical categories. Typical absence seizures usually last less than 30 seconds and involve the abrupt cessation of ongoing activities that are characterized by a blank or "absent" stare and occasional eye blinking or head nodding. Upon cessation of the seizure, the individual has no recollection of the seizure and returns to normal functioning. Atypical absence seizures have similar features, except they develop more slowly and last for a longer duration of time (e.g., several minutes). A minor loss of muscle tone such as slumping is common. Upon cessation of the seizure, the individual may appear confused.

The third major subcategory of generalized seizures includes myoclonic seizures. This subcategory is characterized by seizures that typically only last for a few seconds. Myoclonic seizures consist of rapid involuntary muscle contractions that are not associated with loss of consciousness. Myoclonic atonic seizures involve the acute loss of muscle tone associated with

Table 1-1. International Classification of Seizures and Epilepsies

I. **Generalized Seizures**
   a. Tonic-Clonic (in any combination; *formerly known as Grand Mal*)
   b. Absence
      i. Typical (*formerly known as Petit Mal*)
      ii. Atypical
      iii. Absence with special features
          1. Myoclonic absence
          2. Eyelid myoclonia
   c. Myoclonic
      i. Myoclonic
      ii. Myoclonic atonic
      iii. Myoclonic tonic
   d. Clonic
   e. Tonic
   f. Atonic

II. **Focal Seizures**
   a. Without impairment of consciousness of awareness.
      i. With observable motor or autonomic components. This roughly corresponds to the concept of a "simple partial seizure". "Focal motor" and "autonomic" are terms that may adequately convey this concept depending on the seizure manifestations.
      ii. Involving subjective sensory or psychic phenomena only. This corresponds to the concept of an aura, a term endorsed in the 2001 Glossary.
   b. With impairment of consciousness or awareness.
      i. This roughly corresponds to the concept of "complex partial seizure". "Dyscognitive" is a term that has been proposed for this concept.
   c. Evolving to a bilateral, convulsive seizure (involving tonic, clonic, or tonic and clonic components). This expression replaces the term "secondarily generalized seizure".

III. **Unknown**
      i. Epileptic spasms

Adapted from: Commission on Classification and Terminology, ILEA (2010).

dropping or falling referred to as *drop-attacks* (Oguni, Uehara, Imai, & Osawa, 1997). Immediately following a myoclonic seizure, the individual can return to their normal function without any lingering effects.

Focal seizures represent the second major category of seizures. As implied by their name, focal seizures consist of epileptiform discharges that arise in a specific locus of the brain and typically remain localized within discrete regions of one cerebral hemisphere. The clinical presentation of focal seizures is variable and depends on the region of epileptogenic focus and the recruitment of adjacent neural circuits. For example, seizures that originate in the occipital lobe may produce symptoms associated with visual phenomena (Stafstrom & Carmant, 2015), whereas seizures that originate in limbic structures such as the amygdala often produce intense fearful emotions (Biraben et al., 2001). Focal seizures are further subdivided on whether there is a loss of consciousness. In focal seizures where consciousness is not lost (*formerly simple partial seizures*), the patient may describe motor, sensory, autonomic, or psychic symptoms (Stafstrom & Carmant, 2015). In focal seizures where consciousness is lost, the seizure is considered dyscognitive (*formerly complex partial seizures*) (Berg et al., 2010). Patients that experience dyscognitive seizures often engage in behavioral automatisms, become unresponsive to external stimuli, and experience periods of memory loss. In some instances, a focal seizure may spread into both hemispheres and produce tonus, clonus, or tonic-clonic seizures. These seizures were previously referred to as secondarily generalized seizures.

1.2.2 Temporal Lobe Epilepsy

Temporal lobe epilepsy (TLE) is the most common manifestation of adult focal-onset epilepsy and accounts for approximately 60% of all cases (Tellez-Zenteno & Hernandez-Ronquillo, 2012). TLE seizures are characterized by rhythmic spikes in the EEG that are either synchronous or asynchronous and localized within the temporal lobes of the brain. Although TLE seizures are defined by a partial focal onset, these seizures can manifest into secondarily generalized tonic, clonic, or tonic-clonic seizures. Epidemiological studies indicate that TLE can develop for many reasons, including benign tumors, vascular malformations, cortical developmental malformations, and post-traumatic head injuries that are often associated with reactive gliosis (Al Sufiani F. & Ang, 2012; Curia et al., 2014; Kraemer & Awad, 1994). The

numerous factors involved in the acquisition of TLE demonstrates the heterogeneity of this disorder and the challenges in developing successful treatment options.

TLE can be further divided into limbic and neocortical forms, with limbic TLE recruiting the mesial temporal lobes (mTLE) and neocortical TLE (nTLE) recruiting the lateral temporal lobes (Bercovici, Kumar, & Mirsattari, 2012). Induction of seizures in these discrete portions of the temporal lobes differ in their prevalence and clinical features. Specifically, nTLE is quite rare compared to mTLE and represents less than 10% of all TLE cases (Schramm, Kral, Grunwald, & Blumcke, 2001). Furthermore, nTLE seizures typically last less than one minute, whereas mTLE seizures usually last more than one minute (Foldvary et al., 1997). However, both mTLE and nTLE usually involve some form of sensory phenomena or aura that precedes seizure onset. In patients with mTLE, epigastric and visceral sensations are most common. In contrast, vertiginous and auditory sensations are more prevalent in patients with nTLE (Bercovici et al., 2012). Repetitive manual (e.g., picking, fumbling) or oral (e.g., lip smacking, chewing) behaviors known as automatisms are also more commonly found in patients with mTLE. Moreover, patients with mTLE are also more likely to experience limb movements or dystonic posturing whereas nTLE patients are likely to engage in brief whole body movements. Lastly, mTLE patients often experience post-ictal (after seizure) confusion and this is rare in nTLE patients.

Seizure control is essential in patient outcome. However, treating epileptiform activity in the brain represents just one of many challenges that clinicians face. Indeed, TLE is often complicated by psychiatric, cognitive, and social comorbidities that are becoming increasingly recognized as critical factors in long-term patient care (Hamiwka & Wirrell, 2009; Hermann, Seidenberg, & Jones, 2008) and independent risk factors for poor quality of life (Cramer, 2002; Garcia-Morales, de la Pena, & Kanner, 2008; Johnson, Jones, Seidenberg, & Hermann, 2004). In fact, comorbid factors correlate more strongly with overall quality of life than seizure frequency (Boylan et al., 2004; Perrine et al., 1995; Pulsipher, Seidenberg, Jones, & Hermann, 2006). One possible explanation for this finding is that seizure activity generally occurs in discrete temporal episodes whereas behavioral and cognitive comorbidities seem to persist during interictal periods. The heterogeneity among patients with TLE complicates any generalizations regarding its specific interictal comorbidities; however, these comorbidities can be roughly divided into three categories: psychiatric, cognitive, and social.

Approximately 6% of patients with epilepsy experience a comorbid psychiatric disorder, and this number rises upwards of 10-20% in patients with TLE (Gaitatzis, Trimble, & Sander, 2004). Of these comorbidities, mood disorders are reported most frequently (24-74% of patients) followed by anxiety disorders (10-25% of patients), psychoses (10-20% of patients), and personality disorders (15-40% of patients). Depression is the most common mood disorder comorbid with epilepsy and can affect up to 50% of patients with TLE during their lifetime (Gaitatzis et al., 2004). The prevalence of comorbid depression in epilepsy patients is substantially higher than in any other neurological disorder (Kogeorgos, Fonagy, & Scott, 1982). This troubling finding may partially explain why the suicide rate in patients with epilepsy, especially those with TLE, is 5 fold higher than the general population (Barraclough, 1987). In addition to depression, many patients with TLE experience severe fear and anxiety. Indeed, feelings of fear are the most common affective manifestation induced by seizures with a temporal focus (Cendes et al., 1994; Pegna, Perri, & Lenti, 1999). However, it is important to note that fear and anxiety are not necessarily related to anticipated seizure activity. Rather, the onset of these emotions can be unexpected, intense, nonspecific, and fully debilitating (Biraben et al., 2001). The fear and anxiety experienced by patients with TLE often present as panic attacks, and many researchers postulate a close neurological link between these two disorders (Beyenburg, Mitchell, Schmidt, Elger, & Reuber, 2005; Kalynchuk, 2000; Trimble & van Elst, 2003).

As the medial temporal lobes are intimately involved in learning and memory abilities (Squire & Zola-Morgan, 1991), it is not surprising that patients with TLE often experience cognitive dysfunction. Although the prevalence of memory impairments is variable between reports, as many as 70% of patients with TLE are predicted to demonstrate some degree of memory impairment (Helmstaedter & Kockelmann, 2006; Zhao et al., 2014). The deficits appear to impinge on several facets of cognition, particularly aspects of memory mediated by the hippocampus. Deficits on tasks that involve the hippocampus, including spatial learning, declarative (e.g., episodic), and visuospatial memory are frequently reported in patients with TLE (Carreno, Donaire, & Sanchez-Carpintero, 2008; Haag et al., 2010; Schwarcz & Witter, 2002). Patients with TLE also show deficits in identifying the faces of celebrities, in remembering faces, and in recognizing emotional facial expressions (Carreno et al., 2008), as well as impairments in long-term memory consolidation, remote declarative memory, and

autobiographical memory (Bell & Giovagnoli, 2007; Haag et al., 2010; Howard et al., 2010). Such deficits highlight the fact that the memory impairments associated with TLE often extend beyond the standardized neuropsychological testing used in hospital settings.

When considering how essential learning, memory, and language abilities are in daily life, it is perhaps not surprising that patients with TLE often also present with social comorbidities that negatively affect their quality of life. These social dysfunctions can, for instance, limit one's potential in intellectually demanding jobs and professions (Carreno et al., 2008). Indeed, TLE is correlated with lower education, lower incomes, and higher rates of unemployment (Hermann et al., 2008). Patients with TLE also have abnormalities in advanced social functioning (e.g., in recognizing a social *faux pas*) and a higher probability of remaining or becoming unmarried (Schacher et al., 2006). Patients with TLE often experience feelings of powerlessness, loss of independence, social withdrawal, and/or marginalization (Cornaggia, Beghi, Provenzi, & Beghi, 2006; Hamiwka & Wirrell, 2009). The stigma that often accompanies seizures can have a significant negative impact on self-esteem (Cornaggia et al., 2006). This latter finding is problematic, because self-esteem plays an important role in long-term social outcome.

Patients with mTLE often display a distinct pattern of neuropathology in hippocampal and extrahippocampal regions (Thom, 2014). In particular, hardening or sclerosis of the hippocampus is the most common pathology in patients with refractory mTLE. The first evidence of hippocampal sclerosis in post-mortem samples of epilepsy patients was documented in 1825 by Bouchet and Cazauvieilh, followed by a much more comprehensive study in 1880 by Wilhelm Sommer (Thom, 2009). The ILAE commission report defines hippocampal sclerosis as neuronal loss and gliosis in either the hippocampal *Cornu Ammonis* (CA) sectors (*Cornu Ammonis sclerosis*) or the hilus of the dentate gyrus (*end folium sclerosis*) (Wieser, 2004). With *Cornu Ammonis* sclerosis, damage to pyramidal cells in the CA1 is often extensive, although damage to pyramidal cells in other CA areas such as CA3 has also been reported (Malmgren & Thom, 2012). End folium sclerosis is restricted to cases involving the dentate hilus and is characterized by gliosis, the loss of hilar mossy cells and γ-aminobutyric acid (GABA) interneurons, and dispersion of the dentate gyrus (Al Sufiani F. & Ang, 2012; Curia et al., 2014).

Whether hippocampal sclerosis is a causal factor or a consequence of chronic epilepsy

remains a topic of vigorous debate. It is well known that hippocampal sclerosis increases the risk for developing refractory TLE and cognitive impairment as the disorder progresses (Fuerst, Shah, Shah, & Watson, 2003; Marques et al., 2007). In addition, the number of patients who undergo surgical removal of the epileptic focus are also disproportionately affected by TLE and represent approximately 73% of all resection cases (Curia et al., 2014). This finding emphasizes the fact that TLE is particularly challenging to manage and often refractory. Importantly, hippocampal sclerosis is found in over 60% of samples from surgical resections, which suggests it may represent a major factor contributing to chronic epilepsy (Thom, 2014). Indeed, removal of the sclerotic hippocampus is typically a positive post-surgical predictor of improved seizure control and quality of life (Lowe et al., 2004; Ozkara et al., 2008; Wiebe, Blume, Girvin, & Eliasziw, 2001). Collectively, these results provide a strong association between hippocampal sclerosis and chronic mTLE. However, conflicting evidence also indicates that hippocampal sclerosis is more closely linked to initial precipitating injuries (e.g., head trauma) that is typical of acquired epilepsies (Laxer et al., 2014; Mathern, Adelson, Cahan, & Leite, 2002). In line with this, a subset of post-mortem examinations on patients that experienced decades of severe untreated seizures, including generalized status epilepticus seizures, were reported to show no signs of hippocampal sclerosis or neuronal loss (Thom, Zhou, Martinian, & Sisodiya, 2005). This later finding suggests that severe chronic epilepsy does not inevitability cause hippocampal sclerosis or damage to the hippocampus. This last point is particularly relevant for animal models of epilepsy, where there is a variable degree of cell loss reported across the different models.

1.2.3 Current Treatments for Epilepsy

Approximately 60-70% of patients suffering from epilepsy are anticipated to experience some degree of seizure relief with antiepileptic drugs (AEDs). However, approximately one-third of patients experience intractable or pharmacoresistant epilepsy and do not respond to any of the ~20 currently available AED drugs (Table 1-2). Individuals who experience intractable epilepsy may undergo invasive surgical resection of the epileptic focus as mentioned above. However, some individuals may not be eligible to undergo surgery, especially if their epileptic focus includes or is adjacent to structures that are critical for other functions (e.g., those involved in language). Although there is considerable variability in the mechanisms of action for the currently available AED drugs, there are minimal differences in their effectiveness and every

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Table 1-2. Antiepileptic Drugs (AEDs) and their mechanisms of action

AEDs	Mechanism of Action
Phenytoin Carbamazepine Oxcarbazepine Lamotrigine Topiramate	Blocks repetitive activation of sodium channels
Phenobarbital Benzodiazepines Clobazam	Increases activity of $GABA_A$ receptors
Perampanel Topiramate Felbamate	Blocks AMPA or NMDA receptors
Valproate Ethosuximide	Blocks T-Type calcium channels
Lamotrigine Topirmate Zonisamide Valproate	Blocks N- & L-Type calcium channels
Gabapentin Lamotrigine	Regulates hyperpolarization-activated cation currents (H-Channels)
Retigabine	Increases potassium channel function [Kv7]
Tiagabine	Inhibits GABA reuptake

Modified from (Stafstrom & Carmant, 2015).

AED is associated with side effects (Kwan & Brodie, 2003; St Louis, 2009). For example, AEDs often produce fatigue, dizziness, blurred vision, headaches, cognitive impairments, rash, weight gain or loss, and altered white or red blood cell counts (Bachmann et al., 2011; St Louis, 2009; Stafstrom & Carmant, 2015). The decision to start a particular AED depends on the clinical presentation, cost of medication, and the desire to avoid specific side effects. AEDs doses are therefore gradually increased to achieve the greatest level of seizure control at the lowest possible dose to avoid many of the aforementioned side effects.

AEDs can be further divided into two major categories: narrow and broad-spectrum. Narrow-spectrum AEDs work well for focal and myoclonic epilepsies. Alternatively, broad-spectrum AEDs work well for the majority of seizure types, including generalized seizures. The specific mechanisms of action of each AED varies substantially, but their general purpose is to reduce neural activity. For example, many AEDs block sodium or calcium channels to reduce neuronal depolarization or increase potassium channel function to enhance inhibition (Loscher, 2011). Furthermore, some AEDs work by inhibiting the major excitatory neurotransmitter glutamate through antagonism of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) or N-methyl-D-aspartate (NMDA) receptors. In contrast, other AEDs potentiate the actions of the major inhibitory neurotransmitter GABA (e.g., GABA$_A$ receptor agonists).

Unfortunately, AEDs are generally not effective against treating the behavioral and cognitive comorbidities strongly associated with TLE and in some cases AEDs may even exacerbate the symptoms (Eddy, Rickards, & Cavanna, 2011; Park & Kwon, 2008). Therefore, even patients with some degree of seizure control often continue to experience debilitating cognitive or behavioral problems that affect daily function. The behavioral comorbidities associated with TLE are poorly understood and difficult to study in clinical populations due to the heterogeneity inherent in the disorder itself. Animal models provide possibilities for studying behavioral and cognitive comorbidities under more controlled conditions. A greater understanding of the neurobiological mechanisms that contribute to epileptogenesis and interictal comorbidities will aid in the discovery of novel therapeutic targets that improve patient outcome.

1.3 Animal Models of Temporal Lobe Epilepsy

Although clinical studies of human epileptic populations can provide information about the nature and prevalence of epilepsy, they are often not helpful in determining how seizures

alter brain function to produce comorbid changes in behavior. Animal models provide a degree of experimental control that cannot be achieved in patient populations, and they also provide a way to avoid potential confounds related to psychosocial factors and variable drug treatments. In this section, I will focus on two experimental models that are used most frequently to study the behavioral comorbidities associated with TLE: chemoconvulsant-induced status epilepticus and electrical kindling. This section will discuss relevant findings from both of these models, including the strengths and weaknesses of each model for furthering our understanding of the nature and neurobiological mechanisms of epilepsy.

Chemoconvulsant models of TLE are created in rodents through single injections of an excitotoxic substance to produce status epilepticus. The most commonly used chemoconvulsants are pilocarpine and kainic acid (Tremblay & Ben-Ari, 1984; Turski, Czuczwar, Kleinrok, & Turski, 1983). Pilocarpine is a muscarinic receptor agonist, whereas kainic acid is an ionotropic glutamate receptor agonist. Although these chemoconvulsants act on different receptor systems, systemic injection of these excitatory substances readily produces epileptiform activity evidenced through EEG recordings. Within about 30 minutes of the injection, chemoconvulsants produce seizure activity that gradually intensifies over the course of several hours. Pilocarpine, which is the most commonly used chemoconvulsant, is initially characterized by a theta rhythm within the hippocampus with low-voltage and rapid activity throughout the cortex (Curia, Longo, Biagini, Jones, & Avoli, 2008). The EEG pattern then changes to high-voltage rapid-activity spikes within the hippocampus, spreads throughout the cortex, and eventually induces status epilepticus. Following the period of status epilepticus, there is generally a latent period of non-seizure activity. During the latent period, synaptic reorganization within the hippocampus and other brain regions proceeds rapidly, resulting in the emergence of spontaneously recurring seizures that appear several weeks later (Curia et al., 2008). Chemoconvulsants are an appealing experimental model as the subjects develop spontaneously recurring seizures that resemble the onset of epilepsy in human patients. A major advantage of using chemoconvulsant models is the ability to reproduce the progression of human mTLE in the laboratory animal.

There are some disadvantages of using chemoconvulsants to model human epilepsy. One problem is that chemoconvulsants produce relatively high mortality rates (e.g., 30-40%) (Curia et al., 2008). Researchers often attempt to minimize the rate of mortality by administering

anticonvulsants such as diazepam soon after the onset of status epilepticus. However, this can be an issue if the status epilepticus is terminated too soon, because the animals may not develop spontaneous recurring seizures. A second problem is that although chemoconvulsant-induced seizures resemble symptomatic etiologies of epilepsy resulting from a traumatic brain injury (Pitkanen et al., 2007), the animals used in these experiments do not actually experience any form of insult themselves. Therefore, these models exclude the study of potential interactions between a brain insult and subsequent epileptiform activity. A third issue is that the effects of chemoconvulsants occur rapidly, thus providing a short time interval to investigate the progression of epileptogenesis (McIntyre & Gilby, 2009). Finally, a fourth limitation of chemoconvulsants is that they produce overt damage within numerous structures of the brain (Turski et al., 1983). Because spontaneous seizures can occur in absence of obvious brain damage, these observations suggest that brain damage per se is not necessary for the emergence of spontaneous seizures (McIntyre & Gilby, 2009). Unnecessary brain damage associated with chemoconvulsant status epilepticus therefore adds extra confounds to consider when interpreting results.

Despite these issues, chemoconvulsants provide one of the few ways to induce spontaneously recurring seizures in laboratory animals, and as such, they are important tools for understanding the impact of seizures on the brain and behavior. A number of researchers have shown that chemoconvulsants produce significant alterations in behavior during the interictal period that parallel many of the behavioral comorbidities observed in human patients with TLE. The most consistent and convincing data are related to comorbid symptoms of cognitive impairment. Administration of either pilocarpine or kainic acid produces deficits in delay fear conditioning (Cardoso, Carvalho, Lukoyanova, & Lukoyanov, 2009; Kemppainen, Nissinen, & Pitkanen, 2006) and spatial learning on a Morris water maze task (Gayoso et al., 1994; Groticke, Hoffmann, & Loscher, 2007; Groticke, Hoffmann, & Loscher, 2008; Hort, Brozek, Mares, Langmeier, & Komarek, 1999; Liu, Gatt, Werner, Mikati, & Holmes, 1994; Muller, Groticke, Bankstahl, & Loscher, 2009; Rice, Floyd, Lyeth, Hamm, & DeLorenzo, 1998; Sarkisian et al., 1997; Sartori et al., 2009; Stafstrom, Chronopoulos, Thurber, Thompson, & Holmes, 1993). Pilocarpine also results in spatial memory performance deficits on the radial arm maze task (Detour, Schroeder, Desor, & Nehlig, 2005; Harrigan, Peredery, & Persinger, 1991; Wu et al., 2001) and increases errors on a delayed-match–to-sample object recognition task (Kleen, Scott,

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Holmes, & Lenck-Santini, 2010). There is also evidence that chemoconvulsants induce alterations in depression and anxiety-like behavior. For example, pilocarpine increases the amount of time spent immobile in a forced swim test and decreases consumption of a sucrose solution in rats, which are widely used measures of learned helplessness and anhedonia, respectively (Cryan, Valentino, & Lucki, 2005; Katz, 1982; Mazarati et al., 2008; Willner, Towell, Sampson, Sophokleous, & Muscat, 1987). However, it is important to note that other studies have reported a decrease in depression-like behavior in mice treated with either pilocarpine or kainic acid (Groticke et al., 2008; Muller, Bankstahl, Groticke, & Loscher, 2009). Similar inconsistent findings are reported with respect to anxiety. There is evidence that kainic acid increases anxiety-induced defensive behavior in cats (Griffith, Engel, Jr., & Bandler, 1987) and that pilocarpine increases anxiety in the open field in mice (Muller et al., 2009). However, there is also conflicting evidence that neither pilocarpine or kainic acid increases interictal anxiety in the open field (Cardoso et al., 2009; Groticke et al., 2008; Liu et al., 1994) or elevated plus maze (Groticke et al., 2007; Muller et al., 2009) in mice and rats.

One factor that could explain some of these discrepant results is the fact that there is considerable methodological variability from study to study in the use of chemoconvulsants. Some of this variability includes inter-experiment differences in species, strain within species, and gender, as well as differences in the dose of chemoconvulsant used and the duration of status epilepticus produced by each chemoconvulsant. These methodological differences need to be carefully considered when interpreting results. However, the fact that chemoconvulsants seem to produce clear impairments in several different learning and memory tasks despite these methodological differences suggests that these models may be particularly well suited to study the cognitive comorbidities associated with TLE. How well these models represent comorbidities that are more related to anxiety and depression will await further study.

1.3.1 Characteristics of Kindling

Kindling is a model of TLE that has been used extensively to study epileptogenesis (Racine, 1978). Much like the process of creating a fire, "kindling" refers to the gradual development and intensification of elicited motor seizures that result from daily electrical stimulation of discrete brain regions (Fig. 1-1). To deliver the electrical stimulations, rodents used in kindling experiments are subjected to stereotaxic surgery to implant an indwelling
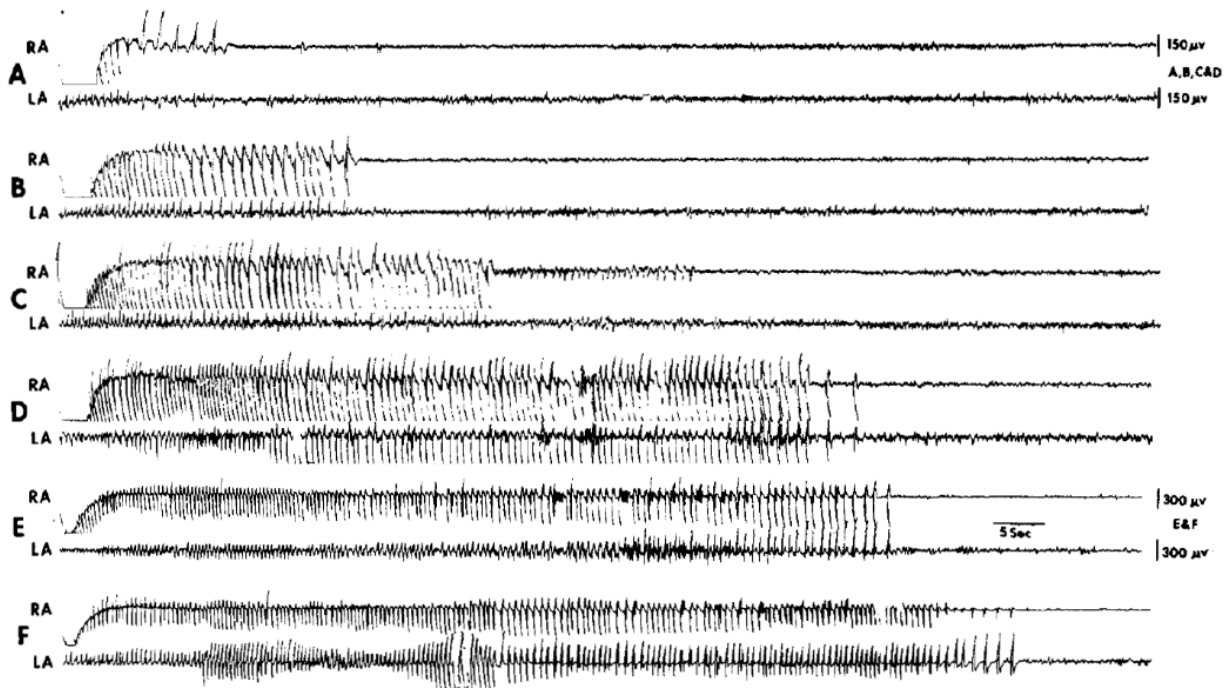
Figure 1-1. The progressive nature of kindling. Development of afterdischarge evoked by suprathreshold electrical stimulation of the right amygdala. **(A)** 2nd AD; 50 µa stimulation. **(B)** 4th AD; 40 µa stimulation. **(C)** 5th AD; 30 µa stimulation. **(D)** 8th AD; 30 µa stimulation with class 1 seizure. **(E)** 10th AD; 25 µa stimulation with class 3 seizure. **(F)** 14th AD; 25 µa stimulation with class 5 seizure. Note the increased duration and spike amplitude with consecutive stimulations. LA, left amygdala; RA, right amygdala. Figure from (Racine, 1972) under copyright license agreement #3784390321123.

electrode into a discrete brain region (Goddard, 1967; Goddard, McIntyre, & Leech, 1969). Historically, kindling research has focused on limbic structures within the temporal lobes due to the relatively low seizure thresholds in these regions and pronounced behavioral effects (McIntyre & Gilby, 2008; McIntyre & Gilby, 2009). A hallmark of kindling is the gradual intensification of electrographic seizure activity around the site of stimulation, known as an afterdischarge, which is accompanied by a similar intensification of convulsions. At first, the electrical stimulations produce a short period of behavioral arrest with focal epileptiform brain activity (Goddard, 1967; Goddard et al., 1969). However, after several electrical stimulations, the epileptiform activity (i.e., afterdischarge) becomes more pronounced and persists for a longer period of time, and it eventually spreads away from the site of stimulation toward adjacent brain regions (e.g., secondary foci). The subjects of kindling studies are typically rats, because kindling in mice can be difficult due to the large size of the electrode apparatus that must be mounted on the animal's skull.

Soon after the discovery of kindling by Graham Goddard, Ronald Racine developed an objective classification system for scoring the progression of convulsions produced by repetitive electrical stimulation (Racine, 1972). The classification system is as follows: Class 0 convulsions are classified as a brief behavioral arrest; class 1 convulsions are characterized by orofacial automatisms; class 2 convulsions are defined as orofacial automatisms and repetitive head nodding; class 3 convulsions comprise unilateral forelimb clonus and mastication with salivation; class 4 convulsions involve generalized convulsions with rearing and forelimb clonus; and finally, class 5 convulsions involve rearing with rapid bilateral forelimb clonus followed by a loss of equilibrium. Within this classification system, rats are considered to be "kindled" once they experience three consecutive class 5 convulsions (Pinel & Rovner, 1978). The process of kindling induces permanent changes in synaptic and morphological plasticity with limbic brain circuits such that once a rat is "kindled", usually only one stimulation is necessary to induce a class 5 generalized motor convulsion, even after an extended seizure free period (Goddard et al., 1969). However, kindling stimulations can be continued past "kindled" criteria, and with continued stimulation, synaptic and morphological brain changes become greater and spontaneously recurring seizures eventually develop (Pinel & Rovner, 1978). The profound reorganization of neural circuits associated with kindling has led some researchers to use this paradigm as a model to study aberrant neural plasticity (Scharfman, 2002).

As with the chemoconvulsant models described above, kindling offers several advantages to researchers who want to study the behavioral comorbidities associated with TLE. One advantage is that kindling offers a level of experimental control over such factors as the focal site of seizure initiation (Fig. 1-2), the number of seizures experienced by the subjects, and the time period between seizure activity and behavioral testing that is not possible with chemoconvulsant models. Kindling is also relatively easy to implement in a laboratory setting, and the manner in which it develops and alters brain function and behavior is very consistent from experiment to experiment and lab to lab. Another advantage is that kindling has good predictive validity, in that anticonvulsant drugs that are effective against kindled seizures are also effective clinically, and those that work poorly against kindling tend to also show poor therapeutic efficacy in human patients (Loscher, 2011; Loscher, Klitgaard, Twyman, & Schmidt, 2013). Finally, kindling produces an intensification of seizure activity with minimal overt brain damage. Researchers are therefore able to investigate behavioral and/or cognitive disruptions without concern for the secondary brain damage often found in chemoconvulsant methods. However, kindling also presents a few disadvantages. The major limitation of kindling is that kindling does not produce spontaneously recurring seizures unless the subjects experience a large number of stimulations (i.e., between 200 - 300) (Pinel & Rovner, 1978). This last point is the major caveat posed by critics who question the clinical relevance of kindling (Bertram, 2007). Another disadvantage of kindling is that it can be very time-consuming, with weeks of daily stimulations often necessary, and it requires the animal to endure invasive surgery to implant stimulating and recording electrodes.

1.3.2 Cognitive and Behavioral Changes Associated with Kindling

Kindling has been extensively used to study the behavioral comorbidities associated with TLE (Kalynchuk, 2000). The vast majority of experiments with kindling employ either amygdaloid or hippocampal kindling, because these brain regions are often focal sites for seizure initiation in human patients and mediate many of the behaviors that are altered in patients with TLE. Indeed, kindling of either the amygdala or hippocampus produces behavioral and cognitive disturbances not seen in control animals or in animals that have been kindled in a non-limbic brain site such as the caudate nucleus (Kalynchuk, Pinel, & Treit, 1998). Amygdaloid kindling has the greatest effect on behaviors related to emotion, with several reports indicating enhanced
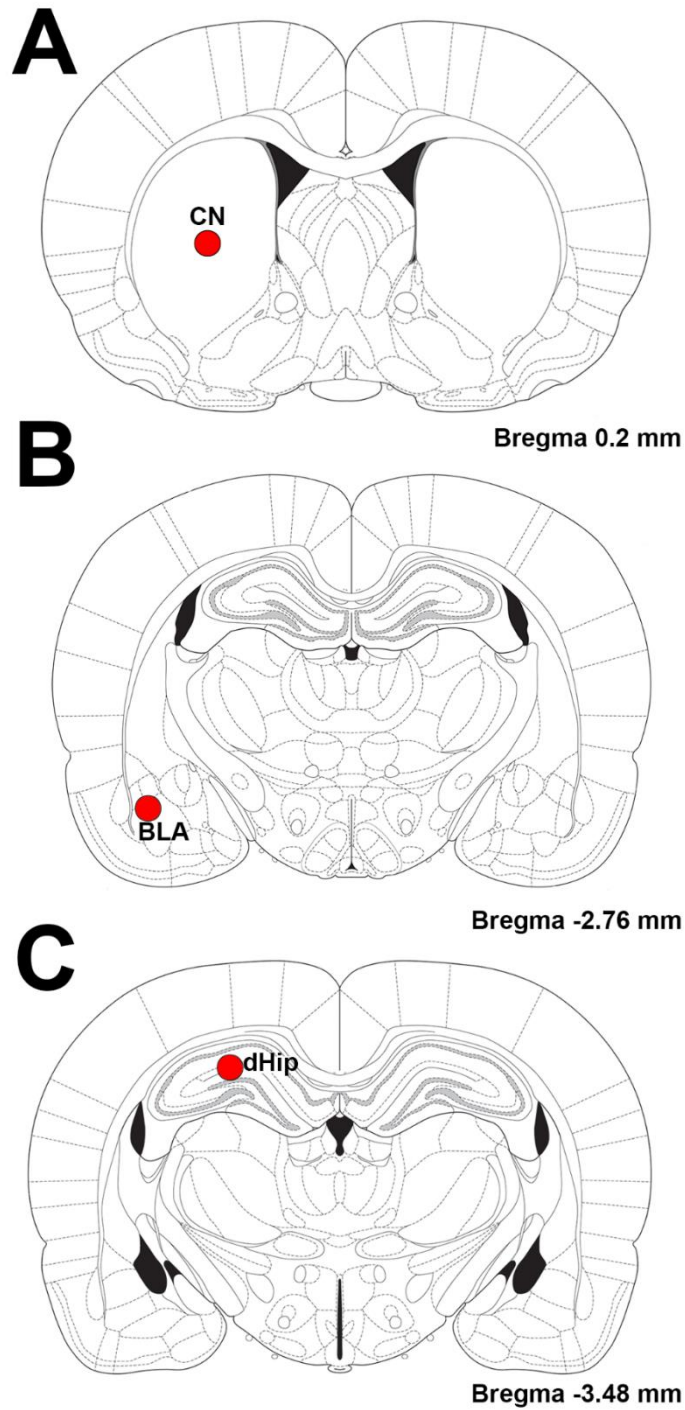
Figure 1-2. Stereotaxic coordinates of commonly kindled brain regions. **(A)** Caudate nucleus **(B)** basolateral amygdala and **(C)** dorsal hippocampus. In addition to experimental control over the focal site of stimulation, kindling allows researchers to control factors such as the number of seizures and stimulation intensity. CN, caudate nucleus; BLA, basolateral amygdala; dHip, dorsal hippocampus. Adapted from (Paxinos & Watson, 1998).

fear and anxiety-like behaviors (Depaulis, Helfer, Deransart, & Marescaux, 1997; Fournier, Darnbrough, Wintink, & Kalynchuk, 2009; Kalynchuk, Pinel, Treit, & Kippin, 1997; Kalynchuk et al., 1998; Kalynchuk, Pinel, & Treit, 1999; Kalynchuk, 2000; Kalynchuk & Meaney, 2003; Wintink, Young, Davis, Gregus, & Kalynchuk, 2003). In contrast, hippocampal kindling has greater effects on mnemonic function related to spatial learning and memory. For example, hippocampal kindling impairs spatial memory performance in the Morris water maze (Gilbert, Hannesson, & Corcoran, 2000; Hannesson et al., 2001; Sherafat et al., 2013). Moreover, several studies have investigated the effect of hippocampal kindling on spatial cognition in the radial arm maze test. Lopes da Silva and colleagues found that hippocampal kindling impaired both working and reference memory while testing concurrently with the kindling procedure, but upon cessation of kindling, only reference memory was impaired (Lopes da Silva, Gorter, & Wadman, 1986). Several other studies have further validated that partial or full hippocampal kindling produces long-lasting memory impairments on the radial arm maze test (Leung, Boon, Kaibara, & Innis, 1990; Leung, Brzozowski, & Shen, 1996; Leung & Shen, 2006).

Many of the studies that have investigated the behavioral effects of kindling have used partial (i.e., kindling elicited ADs without generalized motor seizures) or short-term (i.e., three class 5 convulsions) kindling. However, the behavioral comorbidities associated with kindling increase as the subjects receive more kindling stimulations. Over several experiments, the behavioral consequences of 20, 30, 50, 60, 75, 99, and 100 amygdala kindling stimulations have been evaluated. The results clearly show that amygdala kindling produces a progressive increase in fear and anxiety-like behavior, with 20-30 stimulations producing mild fear, 50-75 stimulations producing intermediate fear, and 99-100 stimulations producing pathological levels of fear (Kalynchuk et al., 1997; Wintink et al., 2003; Young, Wintink, & Kalynchuk, 2004). In line with this, whereas amygdala kindling to three class 5 convulsions does not disrupt hippocampal-dependent behaviors (Hannesson et al., 2008), long-term kindling of the amygdala does (Cammisuli et al., 1997; Fournier, Botterill, Marks, Guskjolen, & Kalynchuk, 2013; Kalynchuk et al., 2001). These observations suggest that the greater behavioral changes associated with long-term kindling likely result from more extensive neural reorganization of the focal site of stimulation and the adjacent structures where epileptiform activity propagates.

Finally, similar to chemoconvulsant models, kindling does not appear to produce the full

range of behavioral comorbidities seen in human TLE patients. Although long-term kindling reliably increases anxiety-like behavior and memory impairments, one study reported that amygdala kindling decreased immobility behavior in the forced swim test and increased sucrose preference, which are measures opposite to a depressive phenotype (Wintink et al., 2003). This is somewhat surprising given the high incidence of depression in human TLE patients (Gaitatzis et al., 2004). One possibility to explain these findings is that the behavioral indices of depression used in kindling studies are not sensitive to the type of depression seen in the clinic. A second possibility is that amygdaloid kindling does not affect brain structures that contribute to depressive symptomology in human patients but kindling of other brain structures, such as the hippocampus, may be more susceptible to depressive-like behavior. At present, it is not possible to come to definitive conclusion on this issue. However, it is clear that kindling produces substantial changes in interictal behavior that parallel many aspects of the behavioral comorbidities seen in human TLE patients. Therefore, kindling provides numerous benefits to further our understanding of the brain mechanisms involved in these comorbidities.

1.4 Neuroanatomy

The temporal lobes have long been implicated in epilepsy. In particular, the amygdala and hippocampus have received substantial research focus because these brains areas are frequently sites of seizure initiation and propagation (Bertram, Zhang, Mangan, Fountain, & Rempe, 1998). Moreover, the hippocampus and amygdala undergo rapid neural plasticity in response to seizures, including the formation of new synaptic connections, neuronal death, alterations to neurotransmitter systems, and reorganization of existing synapses (Scharfman, 2002). Although these changes are well documented, the significance of these changes remains elusive. It is possible that many or all of these changes contribute to the pathogenesis and maintenance of epilepsy. In addition, these neural changes likely contribute to interictal behavioral and cognitive comorbidities often seen in rodent models and clinical cases of epilepsy. In addition to the temporal lobes, the basal ganglia is also broadly implicated in epilepsy because of its role in governing motor function. The basal ganglia comprise a series of nuclei in the forebrain and midbrain that include the caudate nucleus and putamen (collectively referred to as the striatum), globus pallidus, entopenduncular nucleus, subthalamic nucleus, and substantia nigra that collectively influence motor activity (Engel, Jr. & Pedley, 2008).

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1.4.1 The Amygdaloid Complex

The amygdaloid complex is an almond shaped cluster of heterogeneous nuclei that differ in their cytoarchitecture, connectivity, and functionality (Fig. 1-3). The amygdala is most well-known for its involvement in the acquisition, storage, and expression of fear memories (LeDoux, 2000). Indeed, the amygdala has been strongly implicated in fear behavior since the seminal work by Kluver and Bucy in 1937 that reported bilateral anterior temporal lobectomies in rhesus monkeys impair normal fear responses (Goscinski, Kwiatkowski, Polak, Orlowiejska, & Partyk, 1997). Following Kluver and Bucy's report, Paul MacLean described a series of anatomical structures underlying the visceral brain, including the amygdala, which he collectively named the "limbic system" (Maclean, 1952). The role of the amygdala in emotion was further demonstrated by the finding that amygdalar lesions impair the expression of previously learned fear associations (Weiskrantz, 1956). Collectively, these seminal studies provided strong support for the notion that the amygdala is critical for fear learning and expression.

The nuclei of the amygdala involved in fear learning and memory are divided into two major areas: the basolateral (BLA) and central amygdala (CeA). In addition to this subdivision, intercalated cell masses border the internal and external capsule of the amygdala and regulate its function by gating amygdalar input and output (Nitecka & Ben-Ari, 1987; Pare & Smith, 1993; Royer, Martina, & Pare, 1999). The basolateral complex includes the lateral, basolateral, and basomedial amygdala. The basolateral complex resembles a cortical-like structure and primarily consists of glutamatergic spiny projection neurons that comprise approximately 80% of the cell population (Duvarci & Pare, 2014; Sah, Faber, Lopez De, & Power, 2003). Interestingly, the dendrites of BLA projection neurons are not arranged in parallel as is typical of other structures (e.g., cortex, hippocampus). Instead, the dendrites of BLA projection neurons are randomly organized (Sah et al., 2003). The majority of BLA projection neurons have electrophysiological properties that resemble regular spiking neurons with variable degrees of spike frequency adaptation, owing to differential distributions of voltage-gated and Ca^{2+}-activated potassium channels (Faber & Sah, 2002; Sah et al., 2003).

The remaining 20% of cells in the BLA are GABAergic interneurons. Similar to cortical structures, the interneurons of the BLA are characterized by a fast-spiking phenotype with brief action potentials (Spampanato, Polepalli, & Sah, 2011). Two major non-overlapping interneuron
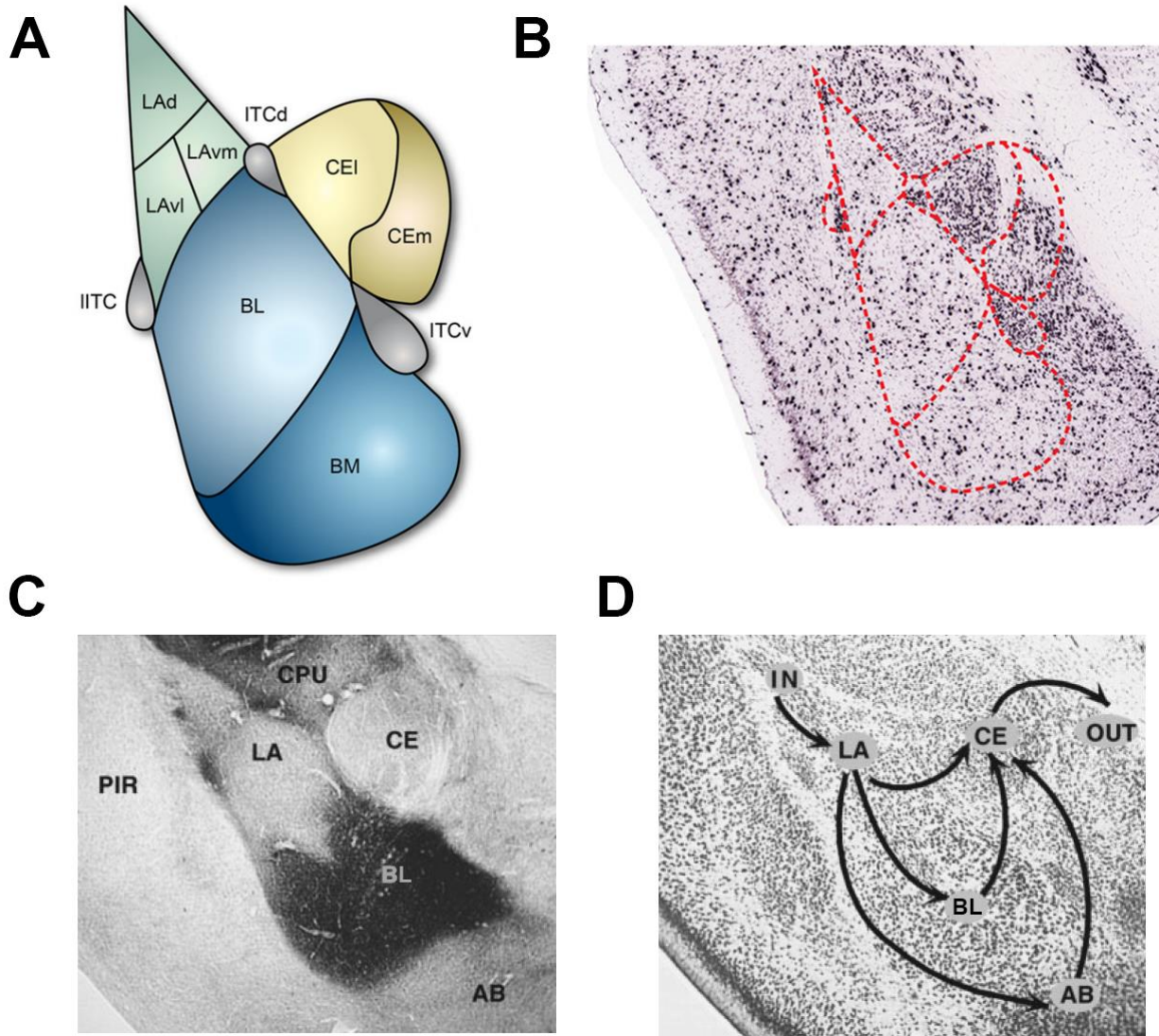
Figure 1-3. Anatomy of the amygdala. **(A)** The major divisions of the amygdala. Cortical-like subnuclei are primarily found in the lateral and basolateral amygdala (green and blue, respectively). In contrast, the central amygdala primarily consists of GABAergic interneurons (yellow). Intercalated cell masses border the internal and external amygdalar capsule (grey). **(B)** Representative photomicrograph of in-situ hybridization for the GABA-synthesizing enzyme GAD67. Note the intense staining in the CeA. **(C)** A representative coronal amygdala section stained for acetylcholinesterase clearly shows the borders of the different amygdalar subdivisions. **(D)** The basic neural pathways of the amygdala. Briefly, somatosensory information converges onto the lateral portion of the amygdala, which then projects to the central amygdala directly, or indirectly through the basolateral and accessory basal nuclei of the amygdala. The central amygdala is the main output of the amygdala. LA, lateral amygdala; BL, basolateral nucleus; BM, basomedial nucleus; AB, accessory basal nucleus; CE, central amygdala; ITC, intercalated cell mass; CPU, caudate-putamen; PIR, piriform cortex. Figure adapted from (LeDoux, 2000; Lee, Kim, Kwon, Lee, & Kim, 2013).

groups in the BLA express either calbindin (CB) or calretinin (CR), with the majority expressing CB and fewer than 20% expressing CR (McDonald & Mascagni, 2001). Of the interneurons that express calbindin, the two most prevalent non-overlapping interneuron subclasses include those that express parvalbumin (PV) or somatostatin (SOM) (McDonald & Mascagni, 2001; McDonald & Mascagni, 2002). PV- and SOM-expressing interneurons in the BLA have received significant research focus because differences in their inputs and postsynaptic targets have broad implications for regulating amygdala function during the formation of fear associations (Wolff et al., 2014). Specifically, PV-expressing interneurons primarily receive inputs from principal cells and form inhibitory synapses with the soma, axon initial segment, and proximal dendrites of projection cells to regulate feedback inhibition (Freund & Buzsaki, 1996; Duvarci & Pare, 2014). In contrast, SOM-expressing interneurons receive cortical inputs and target the distal dendrites of principal cells to regulate feedforward inhibition (Freund & Buzsaki, 1996).

The second major division of the amygdaloid complex is the CeA, which consists primarily of GABAergic interneurons. The CeA is further subdivided into the lateral (CeL) and medial (CeM) nuclei. Neurons in the CeL are characterized by a small soma, multiple primary dendrites with extensive arborization, and dense dendritic spines that resemble medium spiny neurons of the striatum (Duvarci & Pare, 2014). In contrast, the neurons of the CeM contain a large soma with fewer dendritic arborizations than CeL neurons and a medium density of dendritic spines (Duvarci & Pare, 2014). Intercalated cells on the border of the internal capsule send projections from the lateral and basolateral amygdala to the CeL and CeM, respectively to regulate feedforward inhibition. The electrophysiological properties of CeA neurons differ by species. In neurons of the rat CeA, the three major subtypes include regular spiking, low-threshold bursting, and late firing (Dumont, Martina, Samson, Drolet, & Pare, 2002).

The connectivity of the amygdala is complex and consists of numerous afferent and efferent projections, as well as numerous inter- and intra-nuclear connections. The lateral amygdala is a locus of convergence for all sensory modalities: olfactory, gustatory and visceral, somatosensory, visual, and auditory. The glutamatergic neurons of the lateral amygdala convey sensory information by projecting into the BLA, the CeL, and intercalated cells of the dorsal internal capsule (Pape & Pare, 2010). Furthermore, neurons of the BLA project into the CeM via intercalated cells of the ventral intermediate capsule (Pape & Pare, 2010). The BLA also sends

numerous efferent projections to the striatum, hypothalamus and cortex. Interestingly, the ventral hippocampus and prelimbic cortex form reciprocal connections with the BLA that contribute to fear learning and expression (Little & Carter, 2013; Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000). Within the CeA, the CeL projects into the CeM, but this projection is not reciprocated. The CeM represents the major output zone of the CeA and sends projections to numerous brainstem sites, including the periaqueductal gray (PAG), parabrachial nuclei, solitary nucleus, and dorsal vagal complex (Duvarci & Pare, 2014; Tovote, Fadok, & Luthi, 2015). The BLA and CeM both project to the bed nucleus of the stria terminalus (BNST), an area often considered part of the extended amygdala for its role in regulating anxiety (Davis, Walker, Miles, & Grillon, 2010).

1.4.2 The Hippocampal Formation

The term hippocampus, derived from Greek words for horse and sea monster, was first used by the 16th century anatomist Arantius who noticed the resemblance of this brain region with that of a seahorse (Amaral & Lavenex, 2006). The hippocampal formation extends along the floor of the inferior horn of the lateral ventricle and consists of several discrete anatomical structures, including the hippocampus proper, the dentate gyrus, subicular complex (subiculum, presubiculum, and parasubiculum), and entorhinal cortex (Fig. 1-4) (Amaral & Lavenex, 2006). The hippocampus is widely known for its critical roles in learning and memory, which is best illustrated by the famous clinical case with the patient Henry Molaison (H.M). In an attempt to cure H.M.'s severe epilepsy, surgeons carried out a bilateral medial temporal lobectomy that removed his hippocampi, parahippocampal cortices, entorhinal cortices, piriform cortices, and amygdalae. Although the surgery was a success in the sense that H.M.'s seizures were better controlled, the surgery rendered him unable to form new explicit memories (Scoville & Milner, 1957). This seminal finding provided the basis for much of the neuropsychological studies on the role of the hippocampus in learning and memory. Studies in the early 1970's by John O'Keefe further expanded the importance of the hippocampus to include spatial navigation and exploration. In an elegant series of studies, O'Keefe conducted *in vivo* recordings of freely moving rats and discovered place cells that depolarize in specific zones of an environment (O'Keefe & Dostrovsky, 1971). The seminal work by O'Keefe fueled the discovery of similar spatially-oriented grid cells in the main input of the hippocampus, the entorhinal
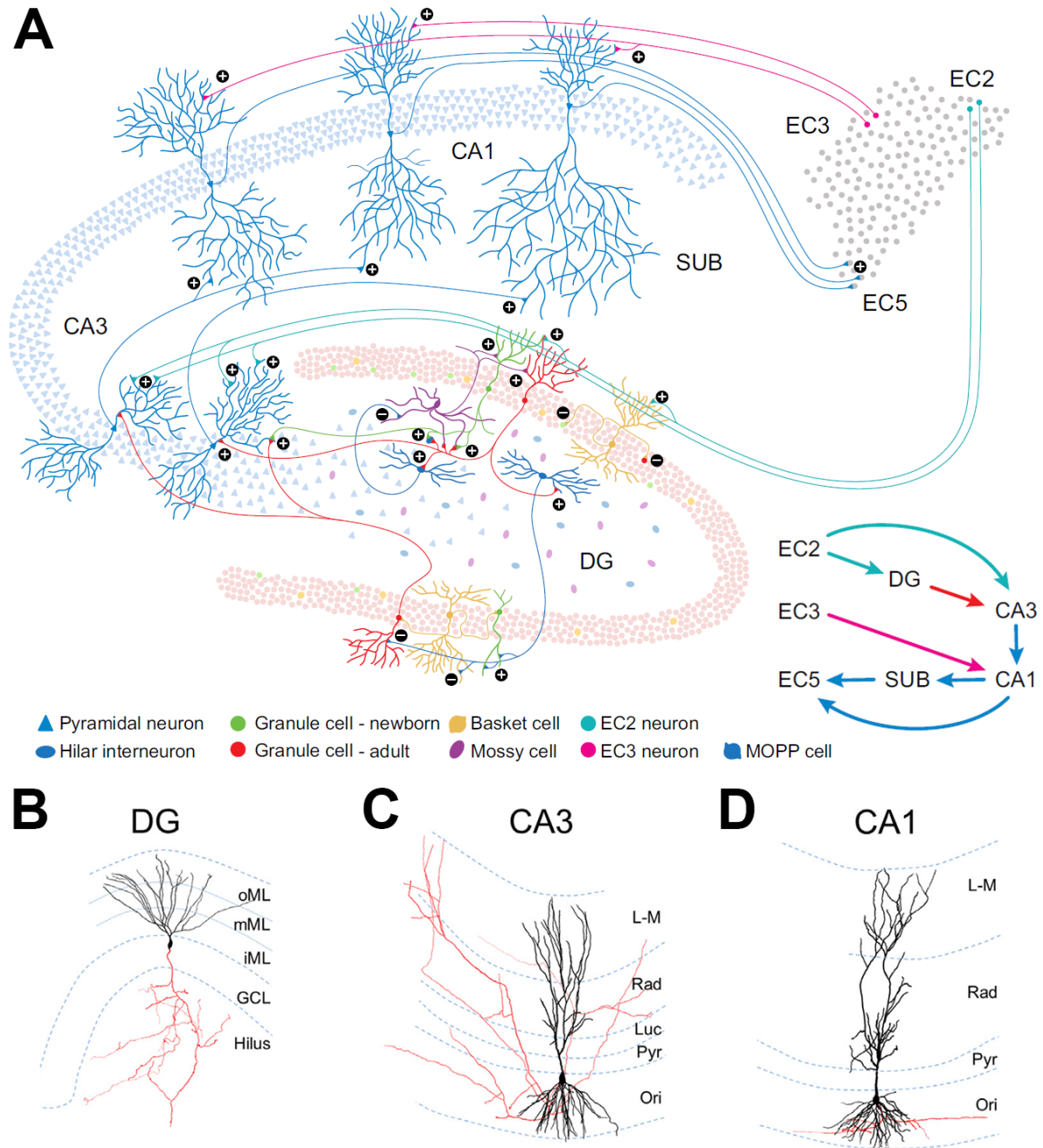
Figure 1-4. Anatomy of the rat hippocampus. **(A)** The hippocampus and related circuitry. The entorhinal cortex (EC) innervates the apical dendrites of dentate gyrus (DG) granule cells. The DG granule cells then project to the CA3 and in turn innervate the CA1 via the Schaffer collaterals. The CA1 projects to the entorhinal cortex and subiculum to complete the trisynaptic circuit. **(B-D)** The laminar distribution of hippocampal granule cells **(B)** and pyramidal cells **(C-D)**, with the soma and dendrites depicted in black and the axon in red. DG, dentate gyrus; EC, entorhinal cortex; SUB, subiculum; ML, molecular layer; GCL, granule cell layer; L-M, lacunosum-moleculare; Rad, stratum radiatum; Luc, stratum lucidum; Pyr, stratum pyramidale; Ori, stratum oriens. Adapted from (Aimone et al., 2014; Degro, Kulik, Booker, & Vida, 2015).

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cortex, by Edvard and Britt-May Moser (Hafting, Fyhn, Molden, Moser, & Moser, 2005). Collectively, the discovery of place and grid cells was awarded the 2014 Nobel Prize in Physiology or Medicine.

The hippocampus proper, also known as *Cornu Ammonis* or *Ammon's horn*, is named after the ram shaped horns of the Egyptian God Amun (Pearce, 2001). The hippocampus proper is comprised of the tightly packed CA1 and the more loosely packed CA2 and CA3 cell layers. The CA cell layers in all subfields primarily consist of glutamatergic excitatory pyramidal neurons. Hippocampal pyramidal neurons are characterized by two opposing dendritic trees, with prominent apical dendrites that emerge from the apex of the soma and shorter basal dendrites that emerge from the base of the soma (Amaral & Lavenex, 2006). Immediately below the stratum pyramidale is the stratum oriens, a relatively cell-free layer where the short basal dendrites of CA pyramidal neurons ramify and receive innervation from several classes of GABAergic interneurons. The apical dendrites of CA pyramidal neurons branch extensively throughout the remaining strata. The stratum lucidum is a narrow acellular zone located directly above the CA3 pyramidal cell layer that is occupied by mossy fibers terminals that project from the dentate gyrus (Amaral & Lavenex, 2006). The stratum radiatum and stratum lacunosum-moleculare are located adjacent to the stratum lucidum and above the stratum pyramidale in layers CA2 and CA1. The stratum radiatum represents the strata where the major CA3 to CA1 Schaffer collateral projections are located. The stratum lacunosum-moleculare is the most superficial layer of the hippocampus and represents the terminal field for extrahippocampal inputs, collectively referred to as the temporoammonic pathway.

The hippocampal formation is widely used as a model system in neurobiology due to its highly organized principal cell layers and laminar distribution of inputs (Amaral, Scharfman, & Lavenex, 2007). An interesting feature of the hippocampal formation is that its projections are almost exclusively unidirectional and transmitted via the trisynaptic circuit (Andersen, Bliss, & Skrede, 1971). In particular, neurons in layer II of the entorhinal cortex convey the majority of cortical information to the dentate gyrus through fibers collectively referred to as the perforant pathw. The axons of dentate granule cells, known as mossy fibers, then project to the somata of CA3 pyramidal cells. The pyramidal cells of CA3, in turn, project to the apical dendrites of CA1 pyramidal cells via the Schaffer collaterals (Lopes da Silva, Witter, Boeijinga, & Lohman, 1990).

The CA1 pyramidal cells then project to the subiculum. The CA1 and subiculum also project back to the deep layers of the entorhinal cortex to complete the circuit and convey information back to the cortex (Canto, Wouterlood, & Witter, 2008). Although the largely unidirectional nature of the trisynaptic circuit appears simple at a superficial level, every hippocampal principal cell layer receives feedback and feedforward inhibition from local interneurons that dynamically regulate hippocampal network activity (Freund & Buzsaki, 1996).

1.4.3 The Dentate Gyrus

The dentate gyrus is the primary target of cortical input to the hippocampal formation. The dentate gyrus is comprised of three layers, the stratum moleculare (molecular layer), the stratum granulosum (granule cell layer; GCL) and the polymorphic layer (hilus). The stratum moleculare is a relatively cell-free zone at the most superficial layer of the dentate gyrus and it is comprised of inner, middle and outer subregions. The molecular layer primarily contains the dendrites of granule cells, pyramidal basket cells, and polymorphic layer cells (Amaral et al., 2007). In addition, the projections of the perforant path terminate in the middle and outer molecular layer and form excitatory axospinous terminations on the distal dendrites of granule cells (Amaral & Lavenex, 2006; Nafstad, 1967).

The stratum granulosum or GCL is the densely packed principal cell layer of the dentate gyrus. Dentate granule cells are characterized by an elliptical cell body, a diffusely branched dendritic tree that extends into the molecular layer, and an axon that projects into the hilus. Each granule cell is substantially smaller (10 µm wide) than the pyramidal neurons (20 - 30 µm wide) located in other principal layers of the hippocampus (Amaral et al., 2007). The GCL is approximately 4 to 8 cells thick and estimates indicate that there are approximately 1.2 million neurons in the GCL of a normal rat (Rapp & Gallagher, 1996). Immediately below the GCL is a 2-cell wide germinal layer called the subgranular zone (SGZ). The SGZ has received significant research focus over the past several decades as it is one of the few locations in the brain where stem cells produce new neurons throughout adulthood (Ming & Song, 2011). The involvement of the SGZ in hippocampal neurogenesis will be discussed further in section 6.

The polymorphic layer or hilus is the deepest layer of the dentate gyrus and contains a variety of neurons and axonal projections. Dentate granule cells extend large unmyelinated axons known as mossy fibers through the hilus and into the CA3. The large boutons of the mossy fibers

form en passant (i.e., non-terminal) synapses with hilar mossy cells and CA3 pyramidal neurons (Amaral et al., 2007). In addition, each mossy fiber produces several thinner collaterals within the hilus that preferentially terminate on the dense population of hilar GABAergic interneurons that dynamically regulate hippocampal function (Acsady, Kamondi, Sik, Freund, & Buzsaki, 1998). Pyramidal basket cells are the most common inhibitory interneuron in the dentate gyrus and reside between the GCL and hilus. Each pyramidal basket cell contains a single apical apsiny dendrite that projects into the molecular layer and branches diffusely. The terminals of pyramidal basket cells form inhibitory symmetric contacts on the proximal apical dendrites and cell bodies of dentate granule cells (Amaral et al., 2007). Golgi analyses of the pyramidal basket cell axonal plexus revealed that a single pyramidal basket cell innervates upwards of 10,000 dentate granule cells (Amaral & Lavenex, 2006). In addition, each pyramidal basket cell contains numerous basal dendrites that ramify throughout the hilus. A second notable interneuron subtype in the hilus is the atypical long-spined multipolar class known as hilar perforant path-associated (HIPP) cells (Han, Buhl, Lorinczi, & Somogyi, 1993). HIPP cells commonly express the neuropeptide SOM and form inhibitory symmetric synapses on the proximal dendrites of granule cells in the middle and outer molecular layers to regulate dentate gyrus activity (Savanthrapadian et al., 2014).

The glutamatergic excitatory mossy cell is the most common cell type in the hilus. Mossy cells have a large triangular or multipolar cell body approximately 25 - 35 µm wide and feature several thick dendrites that emanate from the cell body (Scharfman & Myers, 2012). The proximal dendrites of mossy cells are their most distinctive feature and contain large, complex spines called thorny excrescences that are the terminal sites of mossy fiber axons (Scharfman & Myers, 2012). Mossy cells provide excitatory projections to the inner molecular layer by innervating the proximal dendrites of dentate granule cells (Scharfman, 1995). Interestingly, mossy cells also innervate monosynaptically-coupled GABAergic interneurons that project to the middle and outer molecular layer and inhibit the distal dendrites of dentate granule cells that receive projections from the perforant path (Scharfman, 1995). Mossy cells are therefore in a unique position to regulate the activity of dentate granule cells through direct excitatory and indirect inhibitory projections.

The complex pattern of neural connections within the hippocampus is thought to be

particularly conducive for pattern separation. Pattern separation is a theoretical network process that generates output firing patterns that are less similar to another than its firing inputs (Deng, Aimone, & Gage, 2010). The decorrelation of cortical (input) and hippocampal (output) firing patterns is thought to reduce interference during the encoding similar events or places. Pattern separation is therefore thought to represent a major factor involved in the formation of discrete memories. The phenomenon of pattern separation is hypothesized to occur in the dentate gyrus for two major reasons. First, the GCL contains a large number of principal cells relative to its major inputs and outputs, the entorhinal cortex and CA3, respectively. Specifically, the 1.2 million granule cells in dentate gyrus greatly outnumbers the density of principal cells in the entorhinal cortex (5:1) and CA3 (3:1) (Amaral, Ishizuka, & Claiborne, 1990). A substantially greater ratio of cells in the dentate gyrus compared to the entorhinal cortex is thought to facilitate the sparse coding functions of the dentate gyrus and therefore reduce the interference or overlap of similar information. Second, feed-forward and feedback inhibition from local GABAergic interneurons causes the dentate gyrus to be notoriously quiescent in vivo and therefore well suited for sparse coding (Jung & McNaughton, 1993). Recent evidence has implicated hilar mossy cells in pattern separation, as these cells can regulate the function of the dentate gyrus by innervating both dentate granule cells and GABAergic interneurons (Myers & Scharfman, 2009)

1.4.4 The Dorsal Striatum & Caudate Nucleus

The striatum is divided into dorsal and ventral aspects based on cortical, thalamic, and dopaminergic inputs. The dorsal striatum consists of the caudate nucleus and putamen, whereas the ventral striatum includes the nucleus accumbens and olfactory tubercle. The striatum receives substantial glutamatergic innervation from the cortex and thalamus and is considered the major source of input for the basal ganglia (Fig. 1-5). The primary outputs of the dorsal striatum include the globus pallidus (entopeduncular nucleus), subthalamic nuclei, and substantia nigra pars reticulata (SNr), which collectively contribute to higher-order motor functions (Gerfen, 1985). The SNr is the major output of the basal ganglia, and projects to the anterior thalamus, which in turn innervates the cortex.

The medium spiny neuron (MSN) belongs to the class of GABAergic inhibitory interneurons and accounts for approximately 95% of all neurons that are located in the striatum (Tepper, Tecuapetla, Koos, & Ibanez-Sandoval, 2010). MSNs are characterized by a medium

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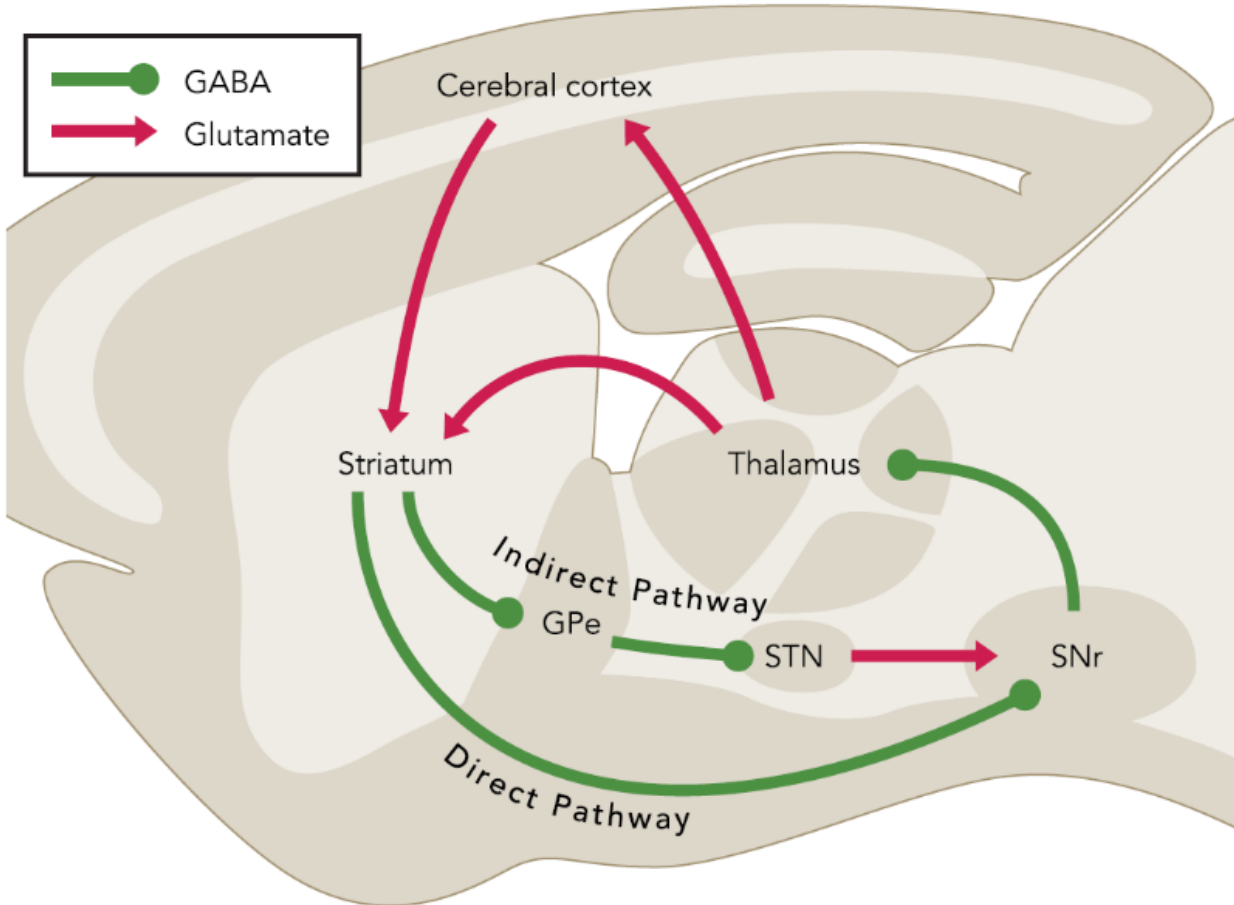
Figure 1-5. A sagittal schematic of the major projections of the basal ganglia. Excitatory glutamatergic inputs reach the main input of the basal ganglia, the striatum, which consists of dorsal and ventral aspects. The striatum projects directly to the substania nigra pars reticulata (SNr) and indirectly via the external globus pallidus (GPe) and subthalamic nuclei (STN). These projections are primarily GABAergic. The substantia nigra pars reticulata projects to the thalamus, which subsequently innervates both the striatum and cerebral cortex with glutamatergic projections. GPe, globus pallidus external; STN, subthalamic nuclei; SNr, substantia nigra pars reticulate. Adapted from (Kravitz & Kreitzer, 2012)

diameter soma (15 µm) with extensive dendritic arborizations. MSNs are divided into direct or indirect classes, dependent on neurochemical profiles and anatomical projections. Specifically, direct MSNs express D1 receptors and project from the dorsal striatum to the SNr and internal globus pallidus. In contrast, indirect MSNs of the dorsal striatum express D2 receptors and project to the SNr indirectly via the external globus pallidus and subthalamic nuclei (Kravitz & Kreitzer, 2012). These differences in pathways are important, as the direct MSN pathway facilitates motor function, whereas the indirect MSN pathway inhibits motor function (Kravitz & Kreitzer, 2012). In addition to MSNs, the function of the dorsal striatum is further regulated by cholinergic interneurons (<1% of cells) and several GABAergic interneuron subpopulations (~4% of cells) (Tepper et al., 2010).

The caudate nucleus has received significant attention in the field of epilepsy for its possible roles in the generation or prevention of seizure activity. Several studies have reported that activation of the caudate nucleus through electrical stimulation or NMDA microinjection potently inhibits seizure activity (La Grutta, Amato, & Zagami, 1971; Turski et al., 1987). Further support for the anticonvulsant properties of the caudate nucleus was demonstrated by the observation that lesions of this region increase the severity of pentamethylenetetrazol-induced seizures (Kirkby, 1977). However, compelling evidence for the role of the caudate nucleus in seizure generation has come from studies that report electrical kindling of this structure rapidly produces seizures (Pinel, Treit, & Rovner, 1977; Saucier & Corcoran, 1992; Timofeeva, 1989). Moreover, destruction of the caudate-putamen with ibotenic acid prior to amygdala kindling was reported to delay seizure development (Yamada et al., 2002). This latter finding suggests that the caudate-putamen may play a critical role in the generalization of limbic seizures.

The caudate nucleus is also known to play an important role in hippocampal-independent learning. As described above, the hippocampus is involved in the formation of spatial memories using external cues (e.g., allocentric coordinates) to form cognitive maps of where learning occurred (e.g., place-learning) (Morris, Garrud, Rawlins, & O'Keefe, 1982). In contrast, the caudate nucleus is important for non-hippocampal dependent learning using body-centered cues (e.g., egocentric coordinates). This latter type of learning is preferentially seen in repetitive stimulus-response learning associated with habit formation (Brasted, Humby, Dunnett, & Robbins, 1997; Cook & Kesner, 1988). Although the hippocampus and caudate nucleus show

clear dissociations and independent contributions to learning, these two systems can work in tandem if the desired behavioral outputs are compatible (White & McDonald, 2002).

1.5 Pavlovian Fear Conditioning

Pavlovian or classical fear conditioning is a useful tool that allows neuroscientists to study the neural circuitry underlying emotion, learning, and memory. Fear conditioning involves the pairing of an innocuous conditioned stimulus (CS) with an aversive unconditioned stimulus (US) that initially produces an unconditioned fear response. In most fear conditioning experiments, the CS can be a tone, light, or context, whereas the US is typically an aversive footshock. However, after repeated pairings of the CS and US, the CS elicits a conditioned response that shares similar characteristics with innate fear responses. In rodents, typical conditioned responses include defensive freezing (Blanchard & Blanchard, 1972; Bolles, 1970), ultrasonic distress vocalizations (Blanchard, Blanchard, Agullana, & Weiss, 1991; Lee, Choi, Brown, & Kim, 2001), and alterations in autonomic nervous system functions such as an increase in heart rate or blood pressure (Iwata, Chida, & LeDoux, 1987; Stiedl & Spiess, 1997).

Over the past two decades, fear conditioning has become an increasingly popular paradigm to study emotion, learning, and memory. The surge in popularity of fear conditioning experiments are partially attributable to its simple experimental design and robust effects. First, the operant conditioning chambers are standardized between laboratories and typically feature a sound-attenuated chamber, a grid floor, a shock source, and a stimulus generator (e.g., speaker). The operant chambers are connected to a computer that allows the user to control CS-US pairings via computer software. This approach is scalable and therefore researchers can operate multiple operant chambers simultaneously for high throughput data collection. Second, fear conditioning procedures produce rapid and long-lasting fear associations that induce robust cellular and molecular plasticity (Schafe, Nader, Blair, & LeDoux, 2001). For example, a single CS-US pairing can produce conditioned fear responses that last for several months (Maren, 2008). Moreover, fear conditioning is consistent with other learning and memory paradigms whereby LTP is required for short-term memory and protein synthesis is required for long-term memory consolidation. For example, acquisition of fear learning induces LTP, while blockade of LTP impairs the acquisition of fear learning without affecting fear expression (Campeau, Miserendino, & Davis, 1992; Kim, DeCola, Landeira-Fernandez, & Fanselow, 1991; Rogan,

Staubli, & LeDoux, 1997). In contrast, protein synthesis inhibitors such as anisomycin have no effect on the acquisition of fear conditioning, but they significantly impair the consolidation and subsequent retrieval of fear memories (Schafe & LeDoux, 2000). Finally, one of the greatest benefits of fear conditioning is the dissociable contribution of discrete brain areas involved in the acquisition and/or expression of fear memories. These dissociable contributions allow researchers to evaluate the function of discrete brain regions and subsequently determine if specific neural circuits are disrupted following experimental treatments. The discrete brain areas involved in fear learning and memory will be discussed in more detail in the following two subsections.

1.5.1 Delay Fear Conditioning

Delay fear conditioning is an amygdala-dependent form of operant fear conditioning that involves pairing a CS presentation with a co-terminating US. The most common example of delay fear conditioning involves the presentation of an auditory tone (CS) that co-terminates with an aversive footshock (US) to produce a conditioned response, such as freezing (Fig. 1-6A). In this form of fear learning, the amygdala is the locus of sensory convergence, with auditory and somatosensory information projecting directly to the lateral portion of the amygdala. The lateral amygdala then relays this information to the major output structure of the amygdala, the CeA, via direct and indirect (e.g., BLA) projections. The CeA then projects to several structures described earlier (e.g., BNST, PAG, lateral hypothalamus) to produce behavioral and autonomic fear responses.

Several lines of research have provided compelling evidence for the involvement of the amygdala in associative delay fear conditioning. Some of the greatest evidence has come from observations that lesions or temporary inactivation of the amygdala profoundly disrupts the acquisition and expression of conditioned responses (e.g., no changes in blood pressure, heart rate, or conditioned freezing) (Davis & Whalen, 2001). In line with this, amygdalar lesions also reduce sensitivity to aversive footshocks (Hitchcock, Sananes, & Davis, 1989). Lesions of structures that convey information to or from the amygdala also impair conditioned fear responses. For example, auditory stimuli project to the lateral amygdala indirectly from auditory association areas of the cortex and directly from the medial geniculate nucleus (MGN) of the thalamus. Lesions of the MGN, but not the auditory association cortex impairs the formation of
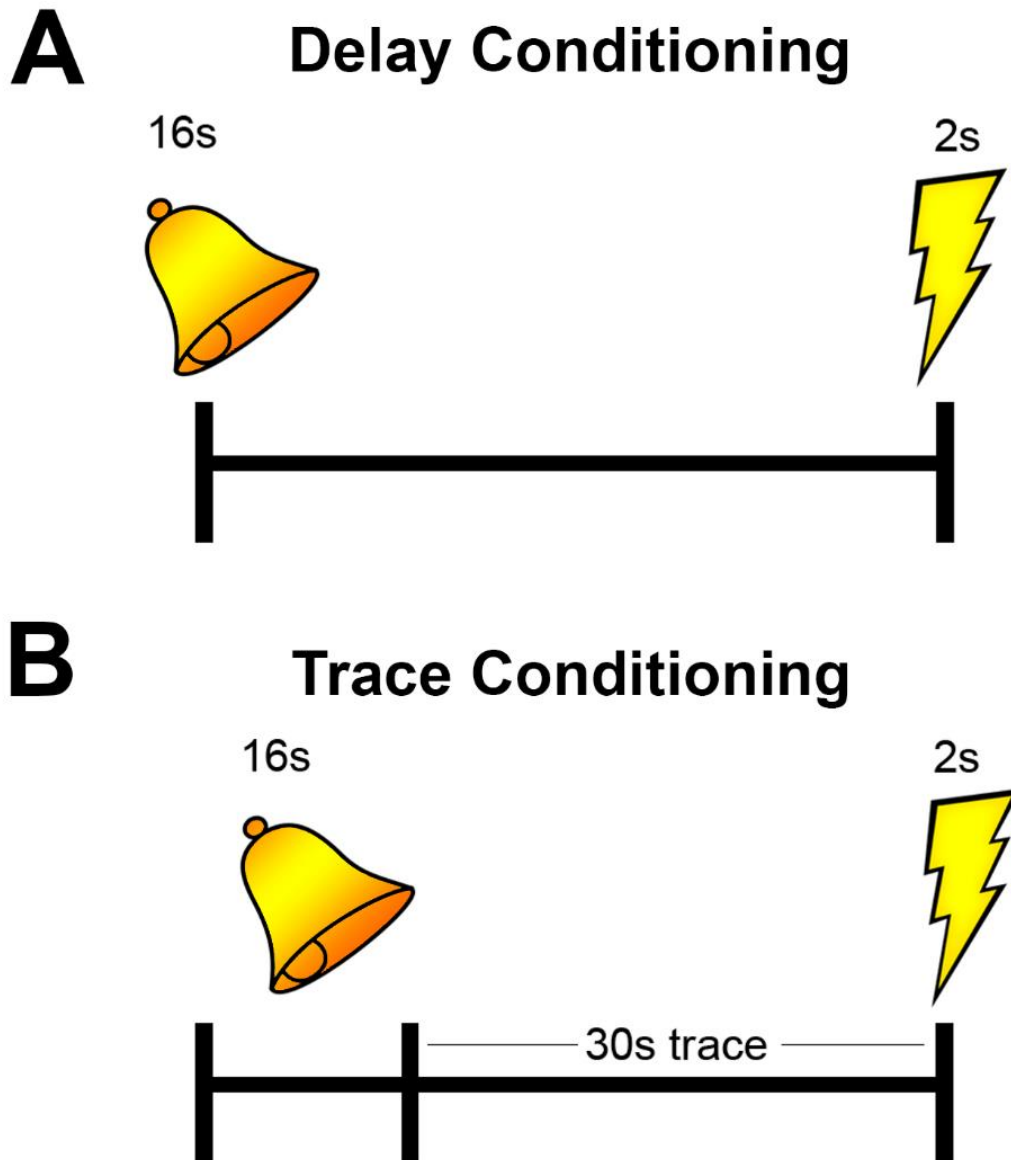
Figure 1-6. Overview of delay and trace fear conditioning. **(A)** Delay fear conditioning involves pairing an auditory tone (conditioned stimulus; CS)with a co-terminating footshock (unconditioned stimulus; US) to produce a conditioned response such as freezing. This simple association is amygdala-dependent (see text) **(B)** Auditory trace fear conditioning involves the presentation of an auditory tone, followed by a temporal gap (trace interval) that co-terminates with a footshock. The CS-US association in trace conditioning is more cognitively challenging and involves the recruitment of additional brain regions such as the hippocampus, prelimbic cortex, anterior cingulate cortex, and the entorhinal and perirhinal cortices. The recruitment of additional brain regions in auditory trace fear conditioning renders this task amygdala-independent.

tone-footshock associations (Campeau & Davis, 1995). Similarly, projections of the amygdala to the PAG mediate conditioned freezing and lesions of the PAG impairs conditioned freezing without affecting other conditioned autonomic responses (LeDoux, Iwata, Cicchetti, & Reis, 1988). Several electrophysiological studies have further supported the involvement of the amygdala in fear learning (LeDoux, 2000). For example, delay fear conditioning drastically increases the magnitude of tone-elicited responses in the lateral portion of the amygdala and converts previously unresponsive cells into tone-responsive cells (Quirk, Repa, & LeDoux, 1995). Moreover, tone-elicited responses of conditioned subjects mimic the induction of associative LTP and do not occur if the tone and footshock remain unpaired (Rogan et al., 1997). Most recently, several optogenetic gain or loss of function experiments have demonstrated that specific amygdalar microcircuits gate fear learning and memory (Li et al., 2013; Tovote et al., 2015; Wolff et al., 2014). Collectively, the evidence from lesion, pharmacological inactivation, electrophysiological, and optogenetic experiments unequivocally demonstrate the critical role of the amygdala in fear learning and memory.

Although delay conditioning is best known for the critical role of the amygdala in fear learning and memory to discrete auditory tones, there is also evidence that the hippocampus forms fear associations of the training context (Kim & Fanselow, 1992; Quinn, Oommen, Morrison, & Fanselow, 2002). A clear dissociation of the relative contribution of the amygdala and hippocampus in delay fear conditioning is best illustrated in two testing paradigms following the initial delay training session. Specifically, a previously fear conditioned subject will not show any behavioral or physiological response to an innocuous novel environment. However, this same subject will immediately display conditioned fear responses upon the presentation of training tone in the novel environment. In contrast, subjects that are returned to the original training environment several days after their training session will immediately display conditioned fear responses in absence of training tones. These behavioral effects demonstrate the relative contributions of the amygdala and hippocampus to cued and contextual aspects of delay fear conditioning, respectively (Quinn, Wied, Ma, Tinsley, & Fanselow, 2008).

1.5.2 Auditory Trace Fear Conditioning

Auditory trace fear conditioning is a hippocampal-dependent form of operant fear conditioning that includes a temporal gap between the presentation of the tone (CS) and

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footshock (US) (Fig. 1-6B). The duration of the temporal gap is variable between laboratories and consist of either short (e.g., 2 – 5 seconds) or long (e.g., 30 – 60 seconds) trace periods. The temporal discontiguity between the CS and US in auditory trace conditioning is inherently more challenging and requires more trials to learn the fear association compared to delay conditioning (Beylin et al., 2001). The increased difficulty of trace fear conditioning is thought to recruit additional brain structures, including the dorsal hippocampus, prelimbic cortex, anterior cingulate cortex, and the entorhinal and perirhinal cortices (Esclassan, Coutureau, Di, & Marchand, 2009; Kholodar-Smith, Boguszewski, & Brown, 2008a; Misane et al., 2005; Raybuck & Lattal, 2011; Runyan, Moore, & Dash, 2004). The recruitment of these additional structures is hypothesized to render trace fear conditioning amygdala-independent (Raybuck & Lattal, 2011).

Several studies have clearly established a critical role of the hippocampus in auditory trace fear conditioning. The first study that demonstrated the necessity of the hippocampus in auditory trace fear conditioning reported that hippocampal lesions disrupt auditory trace, but not delay fear conditioning in rats (McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998). Subsequent lesion studies revealed that discrete lesions of the dorsal hippocampus impaired the acquisition of auditory trace conditioning, whereas more comprehensive lesions that extend into the ventral hippocampus also disrupted the retrieval of trace fear memories (Burman, Starr, & Gewirtz, 2006). Furthermore, selective knockout of NMDA receptors in the CA1 was found to disrupt auditory trace, but not delay fear conditioning (Huerta, Sun, Wilson, & Tonegawa, 2000). Similarly, infusions of the NMDA receptor antagonist $_{D,L}$-2-amino-5-phosphonovaleric acid (APV) into the dorsal hippocampus was found to impair the acquisition and expression of conditioned fear in rats that underwent auditory trace fear conditioning (Quinn, Loya, Ma, & Fanselow, 2005). Finally, inactivation of the amygdala with the $GABA_A$ receptor agonist muscimol was reported to impair delay fear conditioning, but had no effect on auditory trace fear conditioning (Raybuck & Lattal, 2011). Collectively, these studies provide strong support for the notion that auditory trace fear conditioning is hippocampal-dependent and amygdala-independent.

1.6 Adult Neurogenesis: Mechanisms and Purpose

1.6.1 History of Neurogenesis

A long-held dogma in neuroscience was that mammalian species receive their full

complement of neurons during embryonic development and that no new neurons are generated during adulthood. Distinguished anatomists in the late 1800's and early 1900's (e.g., Koelliker, His, and Cajal) advocated for the rigidity of the postnatal mammalian brain because of the consistency in its architecture following development periods (Gross, 2000). Despite the position that embryonic and early postnatal phases were the only periods of cell birth, a few studies in the early 20th century provided evidence of mitotic figures in rats between the ages of 4 to 120 days old (Allen, 1912; Bryans, 1959; Hamilton, 1901; Sugita, 1918). Unfortunately, these observations were largely ignored by the scientific community because the crude methods of the time prevented neuroscientists from reliably detecting cell division and determining whether the divided cells differentiated into glia or neurons.

However, a plethora of studies over the past 50 years have unequivocally demonstrated that new neurons are generated in discrete regions of the mammalian central nervous system (CNS) during adulthood, a process known as adult neurogenesis. A major advance in the study of adult neurogenesis was the development of immunohistological methods for labelling dividing cells with nucleotide analogues. In particular, the pioneering use of [^{3}H]-thymidine autoradiography provided reliable methods for labelling dividing cells to specify the region of proliferation and time of birth (Hughes et al., 1958; Sidman, Miale, & Feder, 1959). [^{3}H]-thymidine is incorporated into the DNA of cells during the synthesis phase of the cell cycle and therefore allows neuroscientists to study the progeny of dividing cells days, weeks, or months later using autoradiography. This technique was first used by Ian Smart to detect newly divided cells in three day old mice (Smart, 1961). Shortly thereafter, Joseph Altman reported [^{3}H]-thymidine labelling of glia and presumably neurons approximately 2 months after bilateral electrolytic lesions of the lateral geniculate body (Altman, 1962). In subsequent reports, Altman further described neurogenesis in the dentate gyrus (Altman & Das, 1965) and olfactory bulb (Altman, 1969) of adult rats. Altman referred to these [^{3}H]-thymidine labelled cells as microneurons; granule or stellate cells containing short axons. Despite these exciting findings, a major hindrance to the acceptance of Altman's findings by the general scientific community was that the small nuclei of microneurons made it difficult to distinguish them from glial nuclei (Gross, 2000).

In the 1970's and early 1980's, Michael Kaplan developed a series of studies that further

provided support for postnatal neurogenesis. In particular, Kaplan reported that complex environments (e.g., balls, toys, running wheels) increased the presence of [^{3}H]-thymidine labelled cells in layer IV of the adult rat visual cortex (Kaplan, 2001). Kaplan and colleagues also ran a series of studies using electron microscopy and light autoradiographs to demonstrate that the [^{3}H]-thymidine labelled cells in the dentate gyrus and olfactory bulb of adult rats displayed the ultrastructural features of neurons, including dendrites and synapses (Kaplan & Hinds, 1977; Kaplan & Bell, 1984). Through the use of [^{3}H]-thymidine labelling and electron microscopy, Kaplan also provided the first evidence of non-human primate neurogenesis in the subventricular zone (SVZ) of an adult macaque monkey (Kaplan, 1983). Despite these promising findings, Kaplan met strong opposition. Pasko Rakic, a prominent primate researcher at Yale, argued that Kaplan's criteria of a neuroblast (Kaplan & Bell, 1984) did not meet Yale's criteria of a mitotic neuroblast and published a series of reports that directly contradicted Kaplan's work. Specifically, Rakic reported that he was unable to detect a single heavily labelled [^{3}H]-thymidine cell in the adult rhesus monkey and therefore concluded that all neurons of the rhesus monkey are generated during prenatal and early postnatal life (Rakic, 1985).

While the role of neurogenesis in rodents and non-human primates was at a stalemate, Fernando Nottebohm's group published a series of important papers on avian neurogenesis. Nottebohm and colleagues found that the volume of two song-related nuclei (e.g., hyperstriatum ventralis pars caudalis, HVc; and the robust nucleus of the archistriatum) were influenced by hormonal and seasonal changes in adulthood (Nottebohm, 1985). These findings led to the hypothesis that volume changes in these regions may be due to fluctuations in the number of neurons within these structures. Indeed, Nottebohm and colleagues demonstrated that approximately 1.5% of the total HVc neuronal pool is generated daily using [^{3}H]-thymidine labelling (Goldman & Nottebohm, 1983) and that the ultrastructural features of [^{3}H]-thymidine labelled cells were identified as neurons that received synapses (Burd & Nottebohm, 1985). Most interestingly, Nottebohm and colleagues demonstrated that [3H]-thymidine labeled cells were recruited into functional circuits, as shown by intracellular recordings of action potentials in response to auditory stimuli (Paton & Nottebohm, 1984). Despite considerable evidence for the generation and integration of adult generated neurons in the avian brain, there remained considerable opposition that adult neurogenesis did not occur in the mammalian brain at this time.

The last hurdle to resolve the issue of adult neurogenesis in the mammalian brain involved the development of new methods that could reliably detect cell division and determine cell fate. Reynolds and Weiss were the first researchers to isolate and identify neural stem cells as the precursors to neurons and glia in the adult brain (Reynolds & Weiss, 1992). In this seminal report, isolated cells from the adult mouse striatum supplemented with epidermal growth factor were shown to proliferate *in vitro* and develop characteristics consistent with neurons and astrocytes (Reynolds & Weiss, 1992). The introduction of the synthetic thymidine analogue 5-bromo-3'-deoxyuridine (BrdU) also played a significant role in the acceptance of adult neurogenesis by the scientific community. Similar to [^{3}H]-thymidine labelling, BrdU is incorporated into cells during the S-phase of mitosis and can label proliferating cells and their progeny. However, the major advantage of BrdU is that it can be visualized with immunocytochemistry and therefore allows neuroscientists to do stereological investigations of adult generated neurons. Furthermore, in combination with neuronal-specific antibodies (e.g., neuronal nuclei; NeuN), researchers could finally confirm the neuronal fate of adult generated neurons. The use of these new methods confirmed the presence of two major neurogenic niches in the adult mammalian brain: the SVZ of the lateral ventricles and the SGZ in the dentate gyrus of the hippocampus (Ming & Song, 2011). In the SVZ, newborn neurons migrate through the rostral migratory stream to become interneurons; granule neurons and periglomerular neurons of the olfactory bulb. In contrast, newborn neurons in the SGZ differentiate into excitatory granule cells and integrate into the local hippocampal network. Arguably the most important evidence of adult mammalian neurogenesis was the discovery of hippocampal neurogenesis in the brains of terminal cancer patients that received injections of BrdU (Eriksson et al., 1998). The concept of adult hippocampal neurogenesis was further confirmed through retrospective birth dating of hippocampal cells based on atmospheric-derived levels of ^{14}C in genomic DNA. This study found a subpopulation of hippocampal cells with elevated atmospheric-derived levels of ^{14}C in genomic DNA that paralleled atmospheric ^{14}C levels associated with nuclear bomb weapon testing during the Cold War (1955-63). This study reported that a typical adult human generates 700 neurons per day, per hippocampus, representing an annual turnover of 1.75% of cells (Spalding et al., 2013). The discovery of adult neurogenesis in the adult human brain has broad implications for learning, memory, health, and disease.

1.6.2 Stages of Neurogenesis in the Dentate Gyrus

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Over 9000 newborn neurons are generated daily in the young adult rat hippocampus (Cameron & McKay, 2001). Interestingly, less than 25% of newborn neurons survive this process and eventually integrate into the local hippocampal network (Christian, Song, & Ming, 2014). The large number of neurons generated daily and their regulation by various physiological and pathological stimuli suggests that these new neurons must play a critical role in brain function and dysfunction. However, it is important to emphasize that adult neurogenesis is much more complex than simply counting the number of proliferating cells. Adult neurogenesis represents a dynamic process that occurs over several weeks to months. Indeed, the process of adult neurogenesis is characterized by several distinct steps: the proliferation of progenitor cells, neuronal differentiation, migration, and the integration of these cells into the existing hippocampal network (Fig. 1-7).

In the hippocampal dentate gyrus, neural progenitor cells (NPCs) are exclusively located in the SGZ, a germinal layer approximately 2-3 nuclei wide that borders the inner granule cell layer and hilus. Although there remains considerable debate on the identity of the "true" neural stem cell population in the SGZ (Aimone et al., 2014), the most widely accepted candidate is slowly dividing radial glia-like cells (type 1) (Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001). Radial glia-like cells are characterized by a bipolar soma located in the SGZ, thick apical processes that extend through the GCL and reach the molecular layer, and positive expression of several biomarkers indicative of neural stem cells (Kempermann, Jessberger, Steiner, & Kronenberg, 2004). For example, radial glia-like cells express biomarkers such as glial fibrillary acidic protein (GFAP), the transcription factor sex-determining region Y-box 2 (Sox 2), the intermediate filament protein nestin, and brain lipid binding protein (BLBP) (Ming & Song, 2011). Radial glia-like cells in the SGZ are notoriously quiescent in vivo (Lugert et al., 2010; Steiner et al., 2006), but they divide asymmetrically to produce highly proliferative transit-amplifying progenitor cells (type 2).

Type 2 cells are characterized by an ovoid shaped dense nucleus and brief plump tangential cytoplasmic extensions (Filippov et al., 2003). Type 2 cells are thought to represent the transition between glial and neuronal lineage (Steiner et al., 2006). Indeed, several markers of radial-glia like neural stem cells, including Sox2, BLBP, and nestin are present in type 2 cells. However, this class of cells is further subdivided dependent on the absence (type 2a) or presence
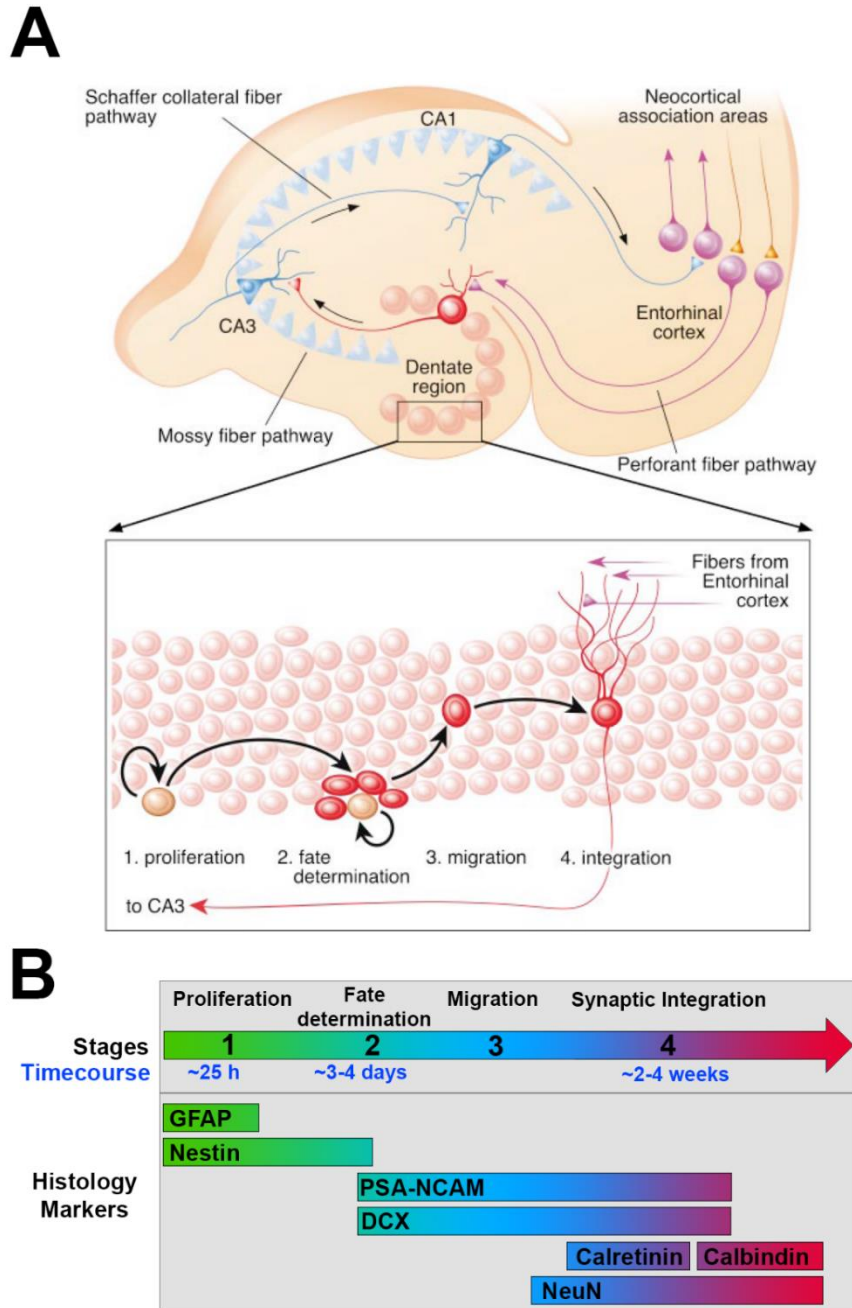
Figure 1-7. Hippocampal neurogenesis. **(A)** The location and stages of hippocampal neurogenesis. Neural progenitor cells residing in the germinal layer of the hippocampus, the subgranular zone, give rise to transit-amplifying cells that commit to a neuronal fate and migrate into the GCL. After several weeks, adult generated neurons integrate into the existing hippocampal circuitry. The apical dendrites of adult generated neurons extend into the molecular layer to receive innervation from the entorhinal cortex and extend their axonal projections towards CA3. **(B)** The stages of neurogenesis. The approximate time course and the histological markers that used to identify adult-generated neurons at different developmental stages. Adapted from (Lie, Song, Colamarino, Ming, & Gage, 2004).

(type 2b) of the immature neuronal marker doublecortin (DCX) (Filippov et al., 2003). Furthermore, type 2 cells also express the transcription factors NeuroD1 and prospero homeobox 1 (Prox1) that are involved in the maintenance of transit-amplifying progenitor cells and the maturation of dentate granule cells (Lavado, Lagutin, Chow, Baker, & Oliver, 2010; Miyata, Maeda, & Lee, 1999). The presence of NeuroD1 and Prox1 are essential in the survival of this cell population, as their absence results in cell death and abnormal dentate gyrus morphology. Collectively, type 2b cells represent cells that have committed to a neuronal fate.

Type 2 cells give rise to migratory neuroblasts (type 3) that are characterized by a round nucleus and low proliferative ability under normal physiological conditions (Kronenberg et al., 2003). Type 3 neuroblasts retain the expression of DCX, but nestin expression is absent. Type 3 neuroblasts gradually exit the cell cycle and represent the population of immature postmitotic neurons that gradually mature into granule neurons of the dentate gyrus over the next several weeks to months. The majority of recently divided cells reach the postmitotic stage within 3 days after the initial division (Aimone et al., 2014; Kempermann et al., 2004). These immature granule cells transiently express the calcium binding protein CR (Brandt et al., 2003) and DCX. As these immature neurons mature, the expression of DCX and CR is replaced with the mature granule cell markers calbindin and NeuN (Kempermann et al., 2004). This transition in marker expression parallels morphological changes in adult generated neurons that make them indistinguishable from the rest of the mature granule cell population. However, as discussed below, although the morphological features of recently matured adult generated neurons is indistinguishable from the preexisting granule cell population, the electrophysiological properties exhibit a greater degree of synaptic plasticity.

1.6.3 Morphological and Physiological Maturation of Adult Generated Hippocampal Neurons

It takes approximately 2 months for adult generated neurons to reach morphological maturity. Within the first week after birth, immature neurons migrate radially and begin integrating into local GCL circuitry following an outside-in pattern similar to developmental periods (Kempermann, Gast, Kronenberg, Yamaguchi, & Gage, 2003). Briefly, cells born during embryonic and early postnatal stages are typically found in the outer GCL, whereas adult generated neurons are primarily located in the inner and middle GCL layers (Mathews et al.,

2010). The migratory process is dependent upon both intrinsic and extrinsic molecular mechanisms. Specifically, the susceptibility gene disrupted-in-schizophrenia-1 (DISC1) represents an intrinsic mechanism that relays positional signals during cell migration. Downregulation of DISC1 causes accelerated integration of immature neurons, resulting in abnormal migration, dendritic morphology, and firing patterns characteristic of mature neurons (Duan et al., 2007). Furthermore, the extracellular matrix protein reelin represents an extrinsic guidance mechanism that prevents adult generated neurons from migrating into the hilus (Gong, Wang, Huang, & Parent, 2007). Downregulation of reelin results in granule cell migration deficits (Frotscher, Haas, & Forster, 2003), whereas mutant mice null for reelin display abnormal lamination of the hippocampus (Forster, Zhao, & Frotscher, 2006).

Much of our initial understanding of the morphological and electrophysiological properties of adult generated neurons was determined by studying a limited number of granule cells that were labelled with histological stains (e.g., biocytin). Since these initial investigations, the morphological maturation of adult generated neurons has been precisely characterized using retroviral methods that birth date and label adult generated neurons and their processes with green fluorescent protein (GFP) (van Praag et al., 2002; Zhao, Teng, Summers, Jr., Ming, & Gage, 2006). As adult generated neurons mature, they extend apical dendrites through the GCL and into the molecular layer, the main postsynaptic site of glutamatergic input for granule cells. The first apical dendritic spines are detected 16 days post division, dramatically increase between 21 to 28 days, and eventually peak by 56 days post division (Zhao et al., 2006). The axonal projections of immature adult generated neurons reach the CA3 pyramidal cell layer within 4 to 10 days post division, which indicates that these fibers reach the CA3 before the formation of the first dendritic spines (Hastings & Gould, 1999; Zhao et al., 2006). At the ultrastructural level, electron microscopy has revealed that the axons of adult generated neurons synapse with dendritic shafts and thorny excrescences of postsynaptic targets in the hilus and CA3, respectively, as early as 17 days post division (Toni et al., 2008). Two main features of adult neurogenesis distinguish this process from embryonic or perinatal neurogenesis. Firstly, adult generated neurons mature at a slower rate compared to neonates (Overstreet-Wadiche, Bensen, & Westbrook, 2006). Secondly, adult generated neurons display a preference to synapse on preexisting boutons (Toni et al., 2007), which may facilitate their integration into local hippocampal circuitry.

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The morphological transition from an immature to mature adult generated neuron parallels changes in the electrophysiological properties of these cells. The physiological integration of adult generated neurons into the dentate gyrus circuitry recapitulates embryonic development, which is characterized by a silent synapse, an initially depolarizing GABA response, a transition to excitatory glutamatergic inputs, and finally an inhibitory GABA response (Esposito et al., 2005). Recently proliferated cells commit to a neuronal fate (e.g., type 2b) typically within 3 days post division, yet they lack synaptic connections at this timepoint as indicated by the failure to evoke postsynaptic responses following extracellular stimulation of the GCL at high stimulus strengths (Esposito et al., 2005). However, these immature neurons have functional GABA and glutamate receptors on their plasma membrane by 4 days post division, demonstrated by their responsiveness to focal application of GABA or glutamate (Esposito et al., 2005). These immature neurons initially have high intracellular chloride (Cl$^-$) concentrations, owing to the presence of the Cl$^-$ importer NKCC1. Because of the initially high levels of intracellular Cl$^-$ in immature neurons, tonic activation by ambient GABA results in depolarization of these cells instead of hyperpolarization (Ge et al., 2006). This initially depolarizing GABA response is critical for immature neuron survival, as knockdown of NKCC1 causes deficits in dendritic growth and the formation of GABAergic and glutamatergic synaptic inputs (Ge et al., 2006). The expression of the NKCC1 Cl$^-$ importer decreases with a parallel increase of the Cl$^-$ exporter KCC2 as adult generated neurons mature. The developmental transition of Cl$^-$ transporter expression reduces the high intracellular concentrations of Cl$^-$ and therefore gradually converts the depolarizing actions of GABA to hyperpolarizing. In addition to tonic GABA activation of immature neurons, synaptic input from GABAergic interneurons also occurs during this time. Numerous interneuron subtypes innervate immature neurons, including PV-expressing basket cells, SOM-expressing HIPP cells, hilar interneuron with commissural-associational pathway-related cells and molecular layer perforant pathway cells (e.g., ivy and neurogliaform cells) (Christian et al., 2014).

Immature neurons continue to be depolarized by ambient and synaptic GABA for approximately 2-3 weeks prior to becoming responsive to glutamate. Interestingly, the GABA-glutamate transition period occurs when glutamatergic spines are first detected on the apical dendrites of immature adult generated neurons. As the immature neurons transition from the depolarizing actions of GABA to the depolarizing actions of glutamate, there exist two critical

periods for the survival and integration of immature neurons. Initially, the NR1 subunit of the NMDA receptor is required for competitive integration and survival of immature neurons between 2-3 weeks post division (Tashiro, Sandler, Toni, Zhao, & Gage, 2006). At 4-6 weeks, a second critical integration period occurs, where adult generated neurons exhibit enhanced LTP, a greater potentiation amplitude, and lower induction threshold; characteristics that are dependent on the NR2B subunit of the NMDA receptor (Ge, Yang, Hsu, Ming, & Song, 2007). The exaggerated synaptic plasticity at this late stage of maturation is hypothesized to promote experience-dependent integration of adult generated neurons (Christian et al., 2014; Ge et al., 2007). Finally, as adult generated neurons reach a mature stage, their mossy fiber axons contact inhibitory interneurons of the GCL, hilus, and CA3 (Acsady et al., 1998; Toni et al., 2008). These local inhibitory interneurons provide fast perisomatic inhibition of mature adult generated neurons that is comparable to the existing mature granule cell population (Esposito et al., 2005).

1.6.4 Regulation of Adult Neurogenesis

Adult neurogenesis is a dynamic process that is regulated by numerous interacting intrinsic and extrinsic factors across its numerous stages (i.e.., from proliferation to integration). A critical area where much of this regulation occurs is the local microenvironment of the SGZ, also known as the "neurogenic niche". The neurogenic niche incorporates numerous interacting regulatory factors, including local vasculature, secreted molecules (e.g., growth factors, neuropeptides and neurotransmitters) and local circuit activity.

Several lines of evidence support the notion that local vasculature contributes to adult neurogenesis. In particular, endothelial cells, which line the blood vessels, secrete soluble factors that induce self-renewal of neural stem cells and progenitor cell proliferation (Shen et al., 2004). Furthermore, astrocytes also secrete growth factors and cytokines that regulate neural progenitor cell differentiation (Barkho et al., 2006). This point is particularly relevant in the context of vasculature, as the endfeet of astrocytes wrap around blood vessels and therefore represent a potential source of secretory molecules with regulatory effects on neurogenesis. Support for this notion is demonstrated by the finding that clusters of neural progenitors are typically found in close proximity to blood vessels within the SGZ (Palmer, Willhoite, & Gage, 2000). The link between vasculature and neurogenesis was further demonstrated by the finding that recombinant adeno-associated viral vector (rAAV) gene transfer of the vascular endothelial growth factor

(VEGF) in the hippocampus dramatically increases both angiogenesis and neurogenesis (Cao et al., 2004).

The findings described above highlight the importance of secreted molecules in regulating neurogenesis. The most widely studied secreted growth factor involved in adult neurogenesis is brain derived neurotrophic factor (BDNF). Within the brain, BDNF expression is greatest within the hippocampus and in dentate granule cells in particular (Katoh-Semba, Takeuchi, Semba, & Kato, 1997). The neurogenic effects of BDNF are well illustrated by the finding that local infusion of BDNF into the hippocampus significantly increases neural progenitor proliferation (Scharfman et al., 2005). Tyrosine receptor kinase B (TrkB), a high affinity receptor of BDNF resides on neural progenitors and mediates the neurogenic effects of BDNF (Li et al., 2008). Indeed, deletion of TrkB on neural progenitors impairs adult hippocampal neurogenesis. Furthermore, heterozygous BDNF mice (BDNF $^{+/-}$) expressing a truncated dominant-negative form of TrkB have reduced survival of adult generated neurons (Rossi et al., 2006). More recently, it was reported that BDNF derived from local granule cells promotes differentiation and maturation of progenitor cells by increasing GABA release through interactions with PV-expressing interneurons (Waterhouse et al., 2012). Interestingly, external factors including environmental enrichment, physical exercise, and antidepressant drugs all induce BDNF expression (Zhao, Deng, & Gage, 2008). The neurogenic effects of these external factors will be discussed shortly.

Neuropeptide Y (NPY) is a peptide neurotransmitter with diverse functions in the CNS. NPY is considered a pleiotropic growth factor because of its broad involvement in angiogenesis and neural proliferation (Kitlinska, Kuo, & Pons, 2005). The neurogenic effect of NPY involves increasing neural proliferation through activation of its Y1 receptor (Howell et al., 2003; Howell et al., 2005). The critical role of Y1 receptor activation in adult neurogenesis was demonstrated by the reduction of cell proliferation and DCX immature neuron survival rates in homozygous Y1 receptor knockout mice (Howell et al., 2005). Furthermore, intracerebroventricular (ICV) infusion of exogenous NPY has also been reported to increase cell proliferation and differentiation in vivo (Decressac et al., 2011). Interestingly, a long lasting overexpression of NPY can be induced by intrahippocampal infusions of BDNF (Reibel et al., 2000). These findings suggest that interactions between BDNF and NPY may enhance the proliferation and

differentiation of adult generated neurons.

In addition to the previously described intrinsic factors, adult neurogenesis is dynamically regulated by numerous extrinsic factors. The most well documented external factors that alter adult neurogenesis include environmental enrichment, physical exercise, and pathological states. Enriched environments, characterized by social housing, complex environments (e.g., multiple levels) and toys have been shown to increase the survival of adult generated neurons (Kempermann, Kuhn, & Gage, 1997). Furthermore, voluntary physical exercise involving running wheels is common in environmental enrichment studies and promotes proliferation of neural progenitors (van Praag, Kempermann, & Gage, 1999). Interestingly, running has local effects on SGZ vasculature, increasing cerebral blood volume (Pereira et al., 2007) and the surface area of blood vessels (van Praag, Shubert, Zhao, & Gage, 2005). Enriched environments and running are thought to have an additive effect on adult neurogenesis, whereby running promotes proliferation and environmental enrichment increases the survival of the recently proliferated cells (Fabel et al., 2009). Lastly, pathological states bi-directionally influence adult neurogenesis. In particular, depression, chronic stress, and schizophrenia are associated with reduced hippocampal neurogenesis (Gould & Tanapat, 1999; Jacobs, van, & Gage, 2000; Reif, Schmitt, Fritzen, & Lesch, 2007). In contrast to other pathological states, seizures robustly increase adult hippocampal neurogenesis. In particular, seizures influence all stages of adult neurogenesis, including proliferation, maturation, migration, and integration (Parent & Murphy, 2008). These findings will be discussed in more detail in section 7.

1.6.5 The Role of Hippocampal Neurogenesis in Learning and Memory

The environments in which we interact with are far from static. As we progress through our daily lives, we are constantly bombarded with novel information (e.g., names, locations, ideas). Because adult neurogenesis is a highly dynamic process that is responsive to and regulated by numerous external factors, one attractive hypothesis is that the generation of new neurons in the adult brain represents a mechanism to encode and store novel information associated with the challenges of daily life. Numerous studies have provided support for this idea by demonstrating positive correlations between the rate of neurogenesis and cognitive ability or proficiency. For example, considerable strain differences exist in adult hippocampal neurogenesis of adult rats, and those with the lowest rate of adult hippocampal neurogenesis

demonstrate the slowest learning on the Morris water maze task (Kempermann & Gage, 2002). Furthermore, factors that are known to reduce adult hippocampal neurogenesis, including chronic stress and aging are associated with impaired learning and memory (Drapeau et al., 2003; Klempin & Kempermann, 2007; Mirescu & Gould, 2006). In contrast, factors that increase neurogenesis, including environmental enrichment and physical exercise demonstrate numerous cognitive enhancing effects (van Praag et al., 2005). Despite the seemingly plausible relationship between neurogenesis and memory, it is an oversimplification to argue that correlation indicates causation (Lazic, 2010). Indeed, although there is substantial evidence that neurogenesis can influence learning and memory, there is also evidence that neurogenesis is limited to certain hippocampal functions and behaviors (Cameron & Glover, 2015). These caveats will be discussed in more detail throughout this section.

The main supporting evidence for a role of neurogenesis in learning and memory has come from manipulations that directly increase (e.g., learning, exercise) or reduce (e.g., focal cranial irradiation, cell division inhibitors, and inactivation or deletion using modern genetic tools) the number of adult generated neurons. Altman and colleagues were the first to implicate postnatally generated cells in learning and behavior by demonstrating that focal cranial irradiation of postnatal hippocampal granule cells increased locomotor activity in an open field test and impaired spontaneous alternation in the Y maze (Bayer, Brunner, Hine, & Altman, 1973). Altman and colleagues studied young rats and therefore did not attribute the reported behavioral phenomena to a reduction in hippocampal neurogenesis. The first major claim that neurogenesis was important for memory was made by Fernando Nottebohm's group. Nottebohm and colleagues proposed that adult neurogenesis was important for the acquisition of new spatial memories in chickadees (Barnea & Nottebohm, 1994).

Major support for the role of adult neurogenesis in learning and memory came from studies that demonstrated hippocampal-dependent learning paradigms increase hippocampal neurogenesis in rodents. In particular, hippocampal-dependent learning tasks such as trace eyeblink conditioning and place (spatial) learning in the Morris water maze significantly increase the number of adult generated neurons (Gould, Beylin, Tanapat, Reeves, & Shors, 1999). In contrast, tasks that may activate the hippocampus but are considered hippocampal-independent, including delay eyeblink conditioning and cue testing in the Morris water maze have no effect on

the number of adult generated neurons. Further support for a role of adult generated neurons in learning was demonstrated by reducing neurogenesis with the cell division inhibitor methylazoxymethnaol (MAM). MAM treatment robustly impairs hippocampal-dependent trace eyeblink conditioning, but not hippocampal-independent delay eyeblink conditioning (Shors et al., 2001). Moreover, subjects that were provided a 21 day washout period after MAM treatment showed normal levels of neurogenesis, which interestingly coincided with normal acquisition of trace eyeblink conditioning (Shors et al., 2001). This same group further demonstrated that reducing hippocampal neurogenesis with MAM treatment drastically impaired hippocampal-dependent auditory trace fear conditioning (Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002), suggesting that this form of learning is also neurogenesis-dependent. Collectively, these seminal studies provided strong support for the involvement of adult neurogenesis in hippocampal-dependent learning and memory.

However, while these initial reports demonstrated clear associations between neurogenesis and memory, several studies have since indicated that this relationship is much less simple than initially thought. In particular, the relationship between the ablation of adult generated neurons and cognition has provided several inconsistent findings. These conflicting reports likely indicate that only some behavioral tasks are affected by a reduction of neurogenesis. As previously described, trace eyeblink and auditory trace fear conditioning are particularly sensitive to ablation of hippocampal neurogenesis. In contrast, contextual fear conditioning following focal cranial irradiation of adult generated hippocampal neurons is impaired in some studies (Snyder et al., 2009; Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006; Wojtowicz, Askew, & Winocur, 2008) but not others (Drew, Denny, & Hen, 2010; Groves et al., 2013; Shors et al., 2002). Furthermore, ablation of adult hippocampal neurogenesis with pharmacogenetic deletion or focal cranial radiation generally has little effect on learning or short term memory retention in the Morris water maze (Arruda-Carvalho, Sakaguchi, Akers, Josselyn, & Frankland, 2011; Ben Abdallah et al., 2013). These findings indicate that not all forms of learning are affected by ablation of hippocampal neurogenesis. One intriguing hypothesis is that cognitively challenging tasks (e.g., trace eyeblink conditioning and auditory trace fear conditioning) are most susceptible to ablation of hippocampal neurogenesis (Shors et al., 2002). In line with this idea, ablation of hippocampal neurogenesis has been reported to impair single trial, but not multiple trial contextual fear conditioning paradigms (Drew et al., 2010).

Although the studies above demonstrate some discrepancies in the relationship between hippocampal neurogenesis and learning, several lines of research support the role of adult neurogenesis in long-term memory consolidation and retrieval. For example, although ablation of hippocampal neurogenesis has little effect on acquisition or short-term memory in the Morris water maze, this same procedure dramatically impairs long-term memory retention (Deng, Saxe, Gallina, & Gage, 2009; Imayoshi et al., 2008; Jessberger et al., 2009; Snyder, Hong, McDonald, & Wojtowicz, 2005). Further evidence for this idea was demonstrated in a seminal study from Paul Frankland's group that found adult generated neurons between 4 to 6 weeks old are preferentially recruited into spatial memory circuits in the hippocampus (Kee, Teixeira, Wang, & Frankland, 2007). Specifically, 4 to 6 week old BrdU-labelled neurons had the greatest colocalization with the behaviorally relevant immediate early genes (IEGs) Arc and Fos following spatial memory recall tests on the Morris water maze (Kee et al., 2007). This time point of 4 to 6 weeks coincides with a period of enhanced excitability of adult generated neurons (Ge et al., 2007) and provides strong support for their role in long-term memory circuits. In line with this idea, Gu and colleagues recently reported that transient inactivation of 4 week old adult generated neurons with optogenetics impaired memory retrieval on the Morris water maze (Gu et al., 2012). In contrast, transiently silencing 2 or 8 week old adult generated neurons had no effect on memory retrieval. Collectively, these results support the idea that there is a time-dependent integration of adult generated neurons to support long-term memory networks.

A hypothesis that has gained popularity over recent years is that adult generated neurons serve as pattern separators in the dentate gyrus (see section 4.3). The addition of broadly tuned immature granule cells into a largely quiescent mature granule cell population is thought to direct learning towards adult generated neurons. Specifically, adult generated neurons are hypothesized to encode novel memories to reduce interference with older existing memories (Aimone, Wiles, & Gage, 2006). Unlike many of the inconsistent neurogenesis ablation studies described above, the relationship between neurogenesis and spatial discrimination appears much more consistent. In particular, selective ablation of the pro-apoptotic gene Bax in adult neural stem cells was found to increase adult hippocampal neurogenesis and enhance contextual fear-discrimination learning (Sahay et al., 2011). In contrast, reducing neurogenesis impairs the discrimination of highly similar contexts and results in generalized freezing to both contexts (Kheirbek, Klemenhagen, Sahay, & Hen, 2012; Nakashiba et al., 2012). In line with this, irradiation of

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hippocampal adult generated neurons was found to impair the discrimination of proximal but not distal spatial locations in the radial arm maze task (Clelland et al., 2009). Collectively, these findings suggest that adult hippocampal neurogenesis is important in cognitively challenging learning and memory tasks, such as those that require discrimination of similar stimuli.

1.7 Aberrant Hippocampal Plasticity Associated with Seizures

Acquired epilepsies such as those resulting from stroke, head trauma, or brain tumors develop over several months to years. This extended period precedes the onset of spontaneous seizures and is collectively referred to as the latent period. A variety of events occur during the latent period that are thought to contribute to the development of spontaneous seizures, including neuronal loss, reactive astrogliosis, inflammation, synaptic reorganization, mossy fiber axonal sprouting, and neurogenesis (Pitkanen & Sutula, 2002). Although it is widely recognized that each of these processes contributes to hippocampal plasticity and represent potential aberrant factors contributing to epileptogenesis and/or ictogenesis, this section will primarily focus on adult hippocampal neurogenesis and alterations of hippocampal GABAergic interneurons.

1.7.1 Adult Neurogenesis in the Epileptic Hippocampus

A massive increase in the number of adult generated neurons following experimentally-induced seizures has been reported in almost every animal model of epilepsy, including chemoconvulsant-induced status epilepticus (Parent et al., 1997), electrical kindling (Parent, Janumpalli, McNamara, & Lowenstein, 1998; Scott, Wang, Burnham, De, & Wojtowicz, 1998), electroconvulsive shock (Scott, Wojtowicz, & Burnham, 2000), flurothyl kindling (Ferland, Gross, & Applegate, 2002), and pentylenetetrazol kindling (Jiang et al., 2003). The generation of new neurons in the adult epileptic brain raises the perplexing question of their functional relevance. Is seizure-induced neurogenesis an endogenous repair mechanism to replace populations of dying or damaged cells in response to epileptic insults? Alternatively, is seizure-induced neurogenesis a mechanism that contributes to epileptogenesis and/or ictogenesis? Lastly, does seizure-induced neurogenesis contribute to behavioral and cognitive comorbidities? Although many of these questions remain topics of considerable debate, the following sections and experimental research of this thesis will attempt to address these issues.

Since the discovery of seizure-induced neurogenesis, the dominant theory in the field has been that adult generated neurons contribute to epileptogenesis (Kokaia, 2011; Parent & Lowenstein, 2002; Parent, Elliott, Pleasure, Barbaro, & Lowenstein, 2006). This idea is consistent with reports that anti-mitotic agents such as cytosine-b-D-arabinofuranoside (Ara-C) drastically reduce hippocampal neurogenesis and the development of spontaneously recurring seizures following pilocarpine-induced status epilepticus (Jung et al., 2004). The pro-epileptogenic theories of seizure-generated neurons is highly attributable to the well-described formation of aberrant recurrent circuits that feature a disproportionate number of excitatory synaptic inputs (Thind, Ribak, & Buckmaster, 2008; Wood et al., 2011; Zhan, Timofeeva, & Nadler, 2010). The following subsections will discuss the aberrant characteristics of seizure-generated neurons linked to epileptogenesis.

1.7.1.2 Aberrant Characteristics of Seizure-Generated Neurons: Hilar Ectopic Granule Cells

The majority of adult generated neurons born in the SGZ of the hippocampus commit to a neuronal fate, migrate into the GCL, and integrate into the existing circuitry over a course of several weeks. However, a subset of adult generated neurons known as hilar ectopic granule cells (HEGCs) migrate abnormally into the hilus following experimentally-induced seizures (Fournier et al., 2010; Jessberger et al., 2007b; Parent et al., 1997; Scharfman, Goodman, & Sollas, 2000). Interestingly, similar patterns of HEGC expression are found in postmortem hippocampal samples in human patients with epilepsy, which suggests that this phenomenon has clinical relevance (Houser, 1990; Parent et al., 2006). Although the reason why some adult generated neurons migrate ectopically remains elusive, several studies have implicated the extracellular matrix protein reelin. Reelin provides a stop signal to guide proper cell migration and its expression in the hippocampus is substantially reduced following seizures (Fournier et al., 2010; Frotscher et al., 2003; Gong et al., 2007; Heinrich et al., 2006). Although HEGCs are extremely rare in control subjects (i.e., <1% of cells), as many as 25% of seizure-generated neurons are reported to migrate ectopically, integrate into local circuitry, and survive for several months (Walter, Murphy, Pun, Spieles-Engemann, & Danzer, 2007). The long-term survival and integration of HEGCs into hippocampal networks has received significant research focus, as the novel circuits created by this ectopic cell population have broad implications for hippocampal function and excitability.

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HEGCs share many similarities with normotopic granule cells located in the GCL. Indeed, HEGCs functionally integrate into the local dentate gyrus circuitry, receive input from the perforant path, and terminate in the CA3 (Scharfman, Sollas, Berger, Goodman, & Pierce, 2003). Moreover, the membrane properties, action potential characteristics, and basic morphology (e.g., cell shape, diameter) of HEGCs are indistinguishable from normotopic granule cells (Scharfman et al., 2000). However, HEGCs have several aberrant characteristics not found in normotopic granule cells. For example, HEGCs form novel recurrent excitatory circuits in the inner molecular layer by synapsing on the apical dendrites of normotopic granule cells (Scharfman et al., 2000). HEGCs also spontaneously burst in synchrony with CA3 pyramidal cells, which initially led researchers to posit that they are innervated by the granule cell mossy fibers (Scharfman et al., 2000). Studies have since confirmed that mossy fibers are the major source of afferent input for HEGCs (Pierce, Melton, Punsoni, McCloskey, & Scharfman, 2005). At the ultrastructural level, HEGCs receive a greater number of asymmetric axosomatic excitatory synapses and fewer symmetric axosomatic inhibitory synapses compared to normotopic granule cells (Dashtipour, Tran, Okazaki, Nadler, & Ribak, 2001). Electrophysiological experiments also confirmed that HEGCs receive an abnormally high ratio of synaptic excitation to synaptic inhibition (Zhan et al., 2010). In line with these observations, cellular reconstructions of HEGCs further revealed that their dendrites are more complex and contain more branch points (Pierce, McCloskey, & Scharfman, 2011). Collectively, these studies indicate that HEGCs form novel recurrent circuits that promote excitability of the hippocampus and likely contribute to seizure activity. These findings are supported by the fact that a greater number of HEGCs are immunoreactive for neural activity markers (e.g., Fos) following spontaneous seizures compared to normotopic granule cells (Scharfman, Sollas, & Goodman, 2002). Collectively, these findings suggest HEGCs as hubs that are involved in the initiation or facilitation of seizure activity.

1.7.1.3 Aberrant Characteristics of Seizure-Generated Neurons: Dendritic Morphology

A second major aberrant characteristic of seizure-generated neurons relates to their altered dendritic morphology. Under normal physiological conditions, immature adult generated neurons transiently express basal dendrites that lack synaptic input and retract over a period of several days (Jones, Rahimi, O'Boyle, Diaz, & Claiborne, 2003). However, several experimental

models of epilepsy have reported that a subset of seizure-generated neurons form hilar basal dendrites (HBDs) that develop synapses and persist into maturity (Dashtipour, Wong, Obenaus, Spigelman, & Ribak, 2003; Jessberger et al., 2007b; Shapiro, Korn, & Ribak, 2005; Shapiro & Ribak, 2006; Spigelman et al., 1998; Thind et al., 2008). Remarkably, approximately 20-40% of seizure-generated neurons in rodents express HBDs, whereas this phenomenon is seen in less than 2% of adult generated neurons in the experimentally naïve brain (Shapiro et al., 2005; Walter et al., 2007). Several elegant retroviral labelling studies have shown that rodent HBDs are formed in a time-dependent manner. Specifically, HBDs are only formed in adult generated neurons that are immature at the time of seizure insults or in neurons generated following seizure activity (Jessberger et al., 2007b; Kron, Zhang, & Parent, 2010). In contrast, mature adult generated neurons do not spontaneously develop HBDs following seizures.

Although HBDs are quite rare in rodents under normal physiological conditions, approximately 10% and 25% of granule cells contain HBDs in monkeys and humans, respectively (Austin & Buckmaster, 2004; Seress & Mrzljak, 1987). Despite the greater prevalence of HBDs in the healthy human brain, an even greater number of HBDs are found in post-mortem samples of human mTLE patients compared to controls (Von Campe G., Spencer, & de Lanerolle, 1997). This suggests that in the human and rat, substantial plasticity of dendritic morphology occurs following seizures and may contribute to epileptogenesis.

Several lines of evidence have provided compelling evidence for an excitatory role of HBDs in seizure-generated neurons. In particular, HBDs of seizure-generated neurons are densely packed with spines (Spigelman et al., 1998), especially large mushroom body spines that are indicative of strong excitatory synapses (Jessberger et al., 2007b). Due to the location of HBDs in the hilus and evidence that HBDs are post-synaptic to small axon terminals, the mossy fiber axons were quickly identified as the major source of excitatory input onto HBDs (Ribak, Tran, Spigelman, Okazaki, & Nadler, 2000). The innervation of HBDs by mossy fiber axons is particularly problematic because it can lead to the formation of recurrent excitatory circuits that increase seizure susceptibility. Indeed, seizure-generated neurons with pronounced HBDs make more contacts with mossy fiber axons and receive approximately twice the amount of afferent input compared to seizure-generated neurons lacking HBDs (Murphy et al., 2011). Moreover, ultrastructural analyses revealed that less than 10% of HBDs synapses are GABA-positive axon

terminals (Thind et al., 2008). In this same work, serial electron micrograph reconstructions estimated that an average HBD receives 2140 excitatory synapses compared to 180 inhibitory synapses (Thind et al., 2008). In addition to the extension of HBDs, seizure-generated neurons also undergo an accelerated outgrowth of apical dendrites into the molecular layer (Overstreet-Wadiche, Bromberg, Bensen, & Westbrook, 2006). The apical dendrites of seizure-generated neurons appear thicker and contain more spines, suggesting they broadly integrate into local dentate gyrus circuitry (Jessberger et al., 2007b). The extensive dendritic plasticity and formation of novel circuits associated with seizure-generated neurons represent likely candidates involved in the pathophysiology of epilepsy.

Some of the strongest evidence supporting the role of aberrant neurogenesis in epileptogenesis was demonstrated by the selective deletion of phosphatase and tensin homolog (PTEN) from adult generated neurons of otherwise experimentally naïve mice (Pun et al., 2012). Deletion of PTEN causes excessive activation of the mammalian target of rapamycin (mTOR) pathway and is therefore epileptogenic. Interestingly, adult generated neurons that are negative for PTEN display aberrant features such as HEGCs, HBDs, and enhanced apical dendritic outgrowth (e.g., greater dendritic thickness and spine density) (Pun et al., 2012). Although PTEN deletion occurred in only 9-24% of adult generated neurons, the aberrant features associated with these neurons coincided with the development of spontaneous seizures. Collectively, this study provides strong support that the aberrant characteristics of even a small subset of adult generated neurons can dramatically shift network dynamics to promote epileptogenesis.

1.7.2 Does Aberrant Neurogenesis Contribute to Cognitive Deficits Following Seizures?

Relatively few studies have investigated the relationship between seizure-induced adult hippocampal neurogenesis and cognition. This is particularly surprising because adult generated neurons positively regulate learning and memory, but patients with epilepsy often experience debilitating interictal cognitive comorbidities. This raises the intriguing possibility that the aberrant characteristics of seizure-generated neurons described above may interfere with cognitive function. One possibility is that the formation of aberrant recurrent circuits may interfere with normal hippocampal functions (e.g., sparse coding). Moreover, although seizures are known to acutely increase neurogenesis, chronic seizures reduce neurogenesis below basal levels, possibly due to the depletion of, or damage to neural stem cell populations (Hattiangady,

Rao, & Shetty, 2004). The acute increase of hippocampal neurogenesis and subsequent decline may contribute to the progressive cognitive decline associated with chronic epilepsy (Fuerst et al., 2003; Hattiangady et al., 2004; Marques et al., 2007). However, more research is needed to elucidate the significance of adult neurogenesis in the epileptic human brain.

Few studies have utilized pharmacological approaches to investigate the relationship between seizure-induced adult neurogenesis and cognitive impairment. Jessberger and colleagues reported that the AED valproic acid (VPA) potently blocked the neurogenic effects of kainic acid by inhibiting histone deacetylases and normalizing the expression of neuron-restrictive silencing factor transcripts, including BDNF, GluR2, and synapsin 1 (Jessberger et al., 2007a). VPA also prevented the aberrant morphological features of adult generated neurons, such as HBDs. Interestingly, normalizing gene expression and the morphological features of seizure-generated neurons with VPA was found to restore cognitive performance on hippocampal-dependent object memory tests (Jessberger et al., 2007a). Another pharmacological study reported a similar outcome by targeting the polysialylated neural cell adhesion molecule (NCAM), a modulator of structural plasticity and adult hippocampal neurogenesis (Pekcec, Fuest, Muhlenhoff, Gerardy-Schahn, & Potschka, 2008). Enzymatic depolysialylation of NCAM via microinjection of endoneuraminidase (endoN) significantly reduced the magnitude of adult hippocampal neurogenesis following rapid amygdaloid kindling and restored cognitive performance in the Morris water maze to control levels (Pekcec et al., 2008). Despite these promising findings, VPA and endoN have numerous cellular targets, which makes it problematic to attribute cognitive and behavioral improvements solely to stabilization of adult hippocampal neurogenesis.

A recent genetic deletion study has provided the most compelling evidence for a role of aberrant seizure-induced neurogenesis in cognitive impairment. In this study, the ablation of dividing neural stem cells and progenitors was accomplished by administering ganciclovir (GCV) to nestin-thymidine kinase (Nestin-TK) transgenic mice (Cho et al., 2015). Importantly, unlike VPA and endoN, administration of GCV in Nestin-TK mice is selective to neural progenitors and has no adverse side effects (Niibori et al., 2012). Cho and colleagues ablated neurogenesis with GCV 4 weeks prior to a single pilocarpine challenge and 6 weeks prior to behavioral and post-mortem analyses. Pre-treatment with GCV significantly reduced the number of adult generated neurons in the SGZ and hilus. Interestingly, ablation of adult hippocampal

neurogenesis significantly reduced chronic seizure frequency and restored hippocampal-dependent object recognition memory (Cho et al., 2015). Cho and colleagues concluded that aberrant hippocampal neurogenesis contributes to epilepsy and cognitive decline. Taken together, the small number of studies using pharmacological or genetic approaches to normalize hippocampal neurogenesis following epileptic insults have provided strong support for the role of aberrant seizure-generated neurons in cognitive impairment.

1.7.3 The Effect of Seizures on Hippocampal GABAergic Interneurons

GABAergic interneurons represent a diverse population of cells that vary in respect to their axonal projections, termination zones, and neurochemical contents. The only major commonality of GABAergic interneurons is their tendency to express glutamic acid decarboxylase (GAD), the key enzyme involved in the synthesis of the inhibitory neurotransmitter GABA (Erlander, Tillakaratne, Feldblum, Patel, & Tobin, 1991). To simplify their diversity, interneurons are often divided into three broad functional groups (Bausch, 2005). Dendritic-targeting interneurons primarily innervate the distal dendrites of principal cells to control the generation of calcium spikes and synaptic plasticity (Miles, Toth, Gulyas, Hajos, & Freund, 1996). Interneurons that express the neuropeptides NPY or SOM are classified as dendritic-targeting interneurons. Perisomatic-targeting interneurons innervate the soma and proximal dendrites (basket cells) or axon initial segments (axo-axonic cells) of principal cells to regulate their output. The calcium-binding protein PV is most commonly found in perisomatic-targeting interneurons. Interneuron-selective interneurons contribute to the synchronization of dendritic inhibition through their preferential innervation of other interneurons (Gulyas, Hajos, & Freund, 1996; Toth & Magloczky, 2014). Cells that express the calcium-binding protein CR are generally classified as interneuron-selective interneurons. Despite the broad differences in neurochemical content and functional targets, each of these classes of interneurons show various degrees of susceptibility to neuronal death or plasticity in response to seizure activity. However, it is important to note that the relative susceptibility of these different interneuron classes appears to be at least partially dependent on the model of epilepsy that is used.

1.7.3.1 Neuropeptide Y

NPY is a 36 amino-acid neuropeptide that is expressed diffusely throughout the central and peripheral nervous systems. Within the hippocampus, NPY is expressed in a subset of GABAergic interneurons. The greatest density of NPY-expressing interneurons in the hippocampus is found in the dentate gyrus between borders of the granule cell layer and hilus (Freund & Buzsaki, 1996). In the central nervous system, NPY acts primarily through activation of its three major receptors, Y1, Y2, and Y5. NPY is best known in the field of epilepsy for its ability to inhibit excitatory neurotransmission. In particular, interactions between NPY and its Y2 receptor presynaptically inhibit glutamate release by reducing Ca^{2+} influx through inhibition of N-type, P/Q-type, and voltage-dependent calcium channels (Qian, Colmers, & Saggau, 1997; Schwarzer, Kofler, & Sperk, 1998; Silva et al., 2003). Interestingly, NPY and its Y2 receptor are dramatically upregulated in hippocampal granule cell mossy fibers after chronic seizures in several rodent models and human patients with epilepsy (Furtinger et al., 2001; Vezzani, Sperk, & Colmers, 1999). The long-lasting upregulation of NPY following seizures provides a potent ability to inhibit excitatory neurotransmission. Indeed, NPY has been shown to attenuate epileptiform activity and protect against seizure-induced excitotoxicity (Colmers & El, 2003; Parent & Lowenstein, 2002; Silva et al., 2003; Woldbye et al., 2010), whereas NPY knockout mice have an increased susceptibility to seizures (Baraban, Hollopeter, Erickson, Schwartzkroin, & Palmiter, 1997). Taken together, these observations have led researchers to posit that NPY represents a potent endogenous anticonvulsant mechanisms within the brain (Vezzani & Sperk, 2004).

1.7.3.2 Somatostatin

SOM is a neuropeptide that is expressed in the dendritic-targeting class of GABAergic interneurons. SOM is most densely distributed in the dentate gyrus, where it accounts for approximately 26% of all interneurons and over 50% of all GABAergic hilar interneurons (Buckmaster & Jongen-Relo, 1999; Houser & Esclapez, 1996). The axon collaterals of hilar SOM interneurons terminate in the middle to outer molecular layer to synapse with the distal dendrites of granule cells (Katona, Acsady, & Freund, 1999; Leranth, Malcolm, & Frotscher, 1990). A reduction in the number of hippocampal SOM interneurons is well documented in resected tissue samples from human epilepsy patients (de Lanerolle, Kim, Robbins, & Spencer, 1989; Sundstrom, Brana, Gatherer, Mepham, & Rougier, 2001) and in rodent models of epilepsy

(Houser & Esclapez, 1996; Magloczky & Freund, 1993; Schwarzer, Williamson, Lothman, Vezzani, & Sperk, 1995; Sloviter, 1991; Sperk et al., 1992; Sun, Mtchedlishvili, Bertram, Erisir, & Kapur, 2007). SOM interneurons in the hilus appear particularly sensitive to excitotoxic insults, as more than 50% of these cells are reported to die following pilocarpine-induced seizures (Buckmaster & Wen, 2011; Zhang et al., 2009). The selective reduction of SOM following seizures is hypothesized to reduce dendritic inhibition onto granule cells and therefore contribute to the pathogenesis of epilepsy (Cossart et al., 2001).

1.7.3.3 Parvalbumin

PV represents the class of perisomatic targeting interneurons and accounts for 20-24% of all interneurons in the hippocampus (Freund & Buzsaki, 1996). PV is expressed in pyramidal basket cells or axo-axonic cells in the dentate gyrus and the strata oriens or pyramidale in the CA3-CA1 subfields of the hippocampus (Freund & Buzsaki, 1996; Kosaka, Katsumaru, Hama, Wu, & Heizmann, 1987). PV interneurons are characterized by a fast-spiking phenotype that provides a major source of perisomatic inhibition onto hippocampal principal cells (Freund & Buzsaki, 1996). Moreover, the fast synaptic inhibition of PV interneurons contributes to hippocampal network oscillations critical for information processing (Bartos, Vida, & Jonas, 2007; Klausberger et al., 2005). A reduction in the number of hippocampal PV pyramidal basket cells reduces inhibition of dentate granule cells (Kobayashi & Buckmaster, 2003; Sloviter, 1987), which facilitates hypersynchronous neural activity and consequently increases the susceptibility to generate behavioral and electrographic seizures (Schwaller et al., 2004). This evidence is further supported by the finding that fewer PV-expressing interneurons are detected in resected hippocampal samples in human patients with intractable TLE (Andrioli, Alonso-Nanclares, Arellano, & DeFelipe, 2007). However, recent evidence suggests that pyramidal basket cells have decreased neurochemical expression of PV but do not undergo cell death (Sloviter et al., 2003). This finding indicates that pyramidal basket cells are resilient to cell death from seizures, but that the neurochemical expression of PV may be transiently or chronically affected by seizure activity.

1.7.3.4 Calretinin

CR represents the class of interneuron-selective interneurons. The majority of CR-expressing interneurons are GABAergic and display minimal overlap with other calcium-binding interneurons such as PV (Gulyas, Miettinen, Jacobowitz, & Freund, 1992; Liu, Fujise, & Kosaka, 1996; Miettinen, Gulyas, Baimbridge, Jacobowitz, & Freund, 1992). CR-expressing interneurons control the excitatory inputs onto principal cells through synchronization of dendritic inhibitory cells (Gulyas et al., 1996; Magloczky & Freund, 2005; Toth & Magloczky, 2014). CR appears particularly sensitive to excitotoxic damage associated with seizures, with several reports indicating a reduction of CR in experimental models of status epilepticus (Bouilleret, Loup, Kiener, Marescaux, & Fritschy, 2000; Huusko, Romer, Ndode-Ekane, Lukasiuk, & Pitkanen, 2013; Magloczky & Freund, 1995; van Vliet, Aronica, Tolner, Lopes da Silva, & Gorter, 2004). In addition, the number of CR interneurons are reduced in human patients with epilepsy and the surviving cells undergo significant neuronal reorganization (Toth et al., 2010).

1.8 Specific Aims and Goals

The specific aim of this dissertation is to investigate the relationship between aberrant plasticity and the behavioral and cognitive comorbidities associated with kindling. To address these issues, a few key questions were asked:

Question 1: What is the effect of amygdaloid kindling on operant fear learning and memory? Although there is considerable evidence that amygdaloid kindling produces significant alterations of unconditioned behaviors (Kalynchuk, 2000), the effect of kindled convulsions on operant fear learning and memory paradigms remains to be determined. To study this question, I conducted short- and long-term kindling of the BLA followed by amygdala- or hippocampal-dependent operant fear conditioning. To investigate the relationship between operant behavior and neuronal activity in discrete brain structures that mediate fear learning and memory, I analyzed post-mortem immunohistochemical labelling of the behaviorally relevant IEG Fos following fear memory retrieval.

Hypothesis: I expect that amygdaloid kindling will impair operant behaviors in a stimulation-dependent and task-specific manner. In line with past reports, I expect that long-term amygdala kindling will produce more robust behavioral impairments than short-term amygdala kindling (Kalynchuk et al., 1997). Furthermore, I expect that amygdaloid kindling will produce more broad impairments on amygdala-dependent fear conditioning compared to hippocampal-

dependent fear conditioning, as the amygdala is the site of seizure initiation in this study. However, I expect that long-term amygdala kindling will also impair hippocampal-dependent fear learning and memory, as the hippocampus undergoes substantial plasticity following long-term amygdaloid kindling (Fournier et al., 2009; Fournier et al., 2010). Lastly, I expect that conditioned freezing behavior will positively correlate with neuronal activity in structures that mediate amygdala- and hippocampal-dependent forms of fear learning and memory. (**Chapter 2**).

Question 2: Upon characterizing the operant fear learning and memory deficits produced by amygdaloid kindling, I was interested to determine whether fear memory impairments are due to convulsions in general or result from kindling of specific brain regions. It is well established that kindling of limbic brain regions impairs unconditioned behaviors (e.g., open field test, elevated plus maze) whereas kindling of non-limbic brain regions does not (Kalynchuk et al., 1998). However, there is no evidence whether this limbic vs non-limbic behavioral dichotomy also occurs in operant fear learning and memory paradigms. To study this issue, I conducted long-term kindling of limbic (e.g., BLA, dHip) or non-limbic (e.g., CN) brain regions followed by hippocampal-dependent trace fear conditioning. To evaluate the relationship between conditioned freezing behavior and hippocampal neuronal activity, I analyzed post-mortem immunohistochemistry of Fos following fear memory retrieval as in Chapter 2. Finally, to determine whether plasticity of hippocampal GABAergic interneurons may alter neural activity and contribute to memory impairments, I ran post-mortem analyses of NPY and its Y2 receptor.

Hypothesis: As my previous study (*Chapter 2*) indicated that amygdaloid kindling does not impair the acquisition of trace fear conditioning, I hypothesize that acquisition of hippocampal-dependent trace fear conditioning will be comparable for all groups. However, I expect that limbic, but non-limbic kindling, will impair memory retrieval of the fear association. In line with the results of *Chapter 2*, I expect that impaired conditioned freezing behavior will coincide with reduced immunoreactivity of the neural activity marker Fos. Because seizures promote robust plasticity of NPY and its Y2 receptors to inhibit neuronal activity (Vezzani et al., 1994; Vezzani et al., 1999; Vezzani & Sperk, 2004), I expect that immunoreactivity of NPY and its Y2 receptors will increase following kindling, with the greatest effect seen in limbic-kindled rats. An

upregulation of NPY and its Y2 receptor following limbic kindling may therefore explain the anticipated reductions of neural activity that parallel memory impairment. **(Chapter 3).**

Question 3: What is the effect of kindling different brain regions on measures of hippocampal neurogenesis? After discovering that limbic, but not non-limbic-kindled rats have hippocampal-dependent memory impairments, I sought to determine whether these behavioral differences are due to aberrant hippocampal plasticity associated exclusively with limbic convulsions. I focused my attention on adult hippocampal neurogenesis because adult generated neurons are strongly implicated in learning and memory, and seizures dramatically increase aberrant hippocampal neurogenesis (Parent et al., 2006). To study this question, I did post-mortem stereological analyses on two major immunohistochemical markers of neurogenesis, BrdU and DCX. Moreover, I investigated hippocampal protein expression of the pro-neurogenic growth factor BDNF. Finally, rats were subjected to hippocampal-dependent trace fear conditioning to evaluate the relationship between aberrant hippocampal neurogenesis and cognition.

Hypothesis: I expect that limbic kindling will increase hippocampal neurogenesis. This hypothesis is consistent with previous reports describing the neurogenic effects of amygdala (Parent et al., 1998) and hippocampal kindling (Bengzon et al., 1997). Since non-limbic kindling is not associated with behavioral or cognitive impairment, I expect that neurogenesis will remain at control levels in these subjects. However, seizure-induced neurogenesis is also characterized by aberrant features (HEGCs, HBDs, and dendritic hypertrophy) (Jessberger et al., 2007b; Parent & Murphy, 2008) that is hypothesized to contribute to cognitive impairment (Cho et al., 2015). Therefore, I expect that aberrant features of adult generated neurons will be most prevalent following limbic kindling, as kindling of these sites produces the largest behavioral and cognitive impairments. Because BDNF overexpression is associated with increased neural proliferation, aberrant neurogenesis (e.g., HBDs, HEGCs), and a long-lasting induction of NPY (*see **Chapter 3***) (Danzer, Crooks, Lo, & McNamara, 2002; Reibel et al., 2000; Scharfman et al., 2005), I hypothesize that hippocampal BDNF protein expression will dramatically increase following limbic, but not non-limbic kindling. Finally, I expect that aberrant hippocampal neurogenesis associated with limbic, but not non-limbic kindling will parallel impairments on hippocampal-dependent memory retrieval. **(Chapter 4).**

Question 4: Does kindling alter the number or morphology of distinct hippocampal GABAergic interneuron subpopulations? GABAergic interneurons broadly influence measures we investigated in *Chapters 2-4*, including learning, memory, and hippocampal neurogenesis (Andrews-Zwilling et al., 2012; Tozuka, Fukuda, Namba, Seki, & Hisatsune, 2005). However, the effect of kindling different brain regions on hippocampal GABAergic interneuron subpopulations remains largely unknown. This issue is important to address, as it may further explain the observed differences of limbic and non-limbic kindling on behavior and hippocampal plasticity. For example, do the memory deficits observed in limbic-kindled animals coincide with the loss of specific GABAergic interneuron subpopulations? Alternatively, is the enhancement of neurogenesis in limbic-kindled rats (*see Chapter 4*) a consequence of increased hippocampal GABA expression? To study these questions, I evaluated major immunohistochemical markers of hippocampal GABAergic interneurons: SOM, PV, CR and GAD67. Moreover, to determine whether kindling causes morphological plasticity of specific GABAergic interneuron subpopulations, I analyzed cell surface area measurements of immunoreactive interneuron subtypes. Lastly, to evaluate whether hippocampal protein expression of GAD67 is increased by kindling, a subset of rats were sacrificed for western blot analyses.

Hypothesis: A loss of hippocampal GABAergic interneurons following severe seizures is common in human TLE and rodent models of status epilepticus (Ben-Ari & Cossart, 2000; de Lanerolle et al., 1989; Houser & Esclapez, 1996; Sloviter, 1987). However, several reports have indicated that even after periods of extended amygdala kindling (e.g., 280-300 stimulations), there are few signs of gross tissue or neuronal damage (Brandt, Ebert, & Loscher, 2004; Michalakis et al., 1998). These findings are important, as they suggest that the behavioral and cognitive comorbidities associated with kindling do not require gross damage to the hippocampus. In line with this evidence, I hypothesize that kindling will not cause gross loss of hippocampal GABAergic interneuron subpopulations. However, it remains to be determined whether different interneuron subtypes show selective plasticity (e.g., increase or decrease in number) within the various subfields of the hippocampus. Moreover, I expect SOM-expressing interneurons will display signs of sprouting, which would be consistent with other models of epilepsy (Zhang et al., 2009) and previous reports that peptidergic interneurons (e.g., NPY and SOM) undergo robust plasticity following kindling (Schwarzer et al., 1996). Lastly, in line with

previous reports (Ramirez & Gutierrez, 2001), I hypothesize that hippocampal expression of GAD67 will increase following kindled seizures. (**Chapter 5**).

Question 5: Can novel therapeutic approaches ameliorate the interictal behavioral and cognitive comorbidities described in *Chapters 2-4*? As a preliminary investigation into this issue, I infused the extracellular matrix protein reelin into the dorsal hippocampus of rats that underwent 99 kindling stimulations of the amygdala, followed by 4 days of hippocampal-dependent auditory trace fear conditioning. To further understand the effects of reelin on the hippocampus during memory retrieval, I also did a preliminary analysis of the behaviorally relevant neural activity marker Fos.

Hypothesis: I hypothesize that reelin will partially restore the interictal behavioral and cognitive comorbidities associated with amygdala kindling. In the adult brain, reelin promotes numerous structure and functional changes, including dendritic spine development and maturation (Chameau et al., 2009; Niu, Yabut, & D'Arcangelo, 2008), synaptic plasticity (Beffert et al., 2005; Beffert et al., 2006; Pesold et al., 1998; Weeber et al., 2002), and postnatal neurogenesis (Pujadas et al., 2010; Won et al., 2006). My laboratory has previously shown that amygdala kindling causes a reduction in the number of reelin immunoreactive cells in the dentate gyrus (Fournier et al., 2010). Because of the diverse effects of reelin on measures of synaptic plasticity, a reduction in reelin in amygdaloid-kindled rats may contribute to the behavioral deficits my laboratory has previously reported. Interestingly, genetic overexpression or infusions of exogenous reelin into the brain normalizes deficits of behavior, cognition, and synaptic plasticity in a diverse array of animal models of neuropsychiatric illness, including, bipolar disorder, schizophrenia, Angelman syndrome, and Alzheimer's disease (Hethorn et al., 2015; Pujadas et al., 2014; Teixeira et al., 2011). Collectively, these findings led me to hypothesize that intrahippocampal infusion may also confer beneficial effects in the kindled brain. **(Chapter 6).**

CHAPTER 2

Amygdala kindling disrupts trace and delay fear conditioning with parallel changes in Fos protein expression throughout the limbic brain

J.J. Botterill [a], N.M. Fournier [b], A.J. Guskjolen [a], A.L. Lussier [a], W.N. Marks [a], L.E. Kalynchuk [c]

[a] Department of Psychology, University of Saskatchewan, Saskatoon, SK, S7N 5A5, Canada

[b] Department of Psychiatry, Yale University School of Medicine, Yale University, New Haven, CT, 06508, USA

[c] Department of Medicine, University of Saskatchewan, Saskatoon, SK, S7N 5A5, Canada

2.1 Introduction

Seizures are known to dramatically affect behavior and cognition. Patients with temporal lobe epilepsy often experience interictal (between seizure) behavioral and cognitive comorbidities that manifest as elevations of fear and anxiety, as well as memory problems (Dodrill & Batzel, 1986; Mula, 2013; Schwarcz & Witter, 2002; Strauss, Risser, & Jones, 1982). These comorbidities have a significant impact on quality of life and can be more debilitating for the patients than the seizures themselves (Cramer, 2002; Johnson et al., 2004; Perrine et al., 1995). Unfortunately, heterogeneity among patient populations is a major obstacle for understanding the neural mechanisms that underlie alterations in behavior and cognition in epileptic patients. To overcome many of these issues, researchers have adopted the use of animal models that can investigate these topics directly.

Kindling is an animal model that has frequently been used to study the pathophysiology of temporal lobe epilepsy. Kindling refers to the gradual development and intensification of motor seizures that result from daily electrical stimulation of a discrete brain site (Goddard et al., 1969). In addition to its epileptogenic effects, kindling is particularly useful for studying the aberrant neural plasticity that promotes interictal behavioral and cognitive comorbidities (Kalynchuk, 2000; Kalynchuk & Meaney, 2003; Kalynchuk, Pinel, & Meaney, 2006). In contrast to the well described effects of kindling on unconditioned fear and anxiety responses (Botterill, Guskjolen, Kalynchuk, & Caruncho, 2012), relatively little is known about the effects of kindling on learned fear responses. Fear conditioning is a form of Pavlovian conditioning that pairs a neutral conditioned stimulus (i.e., an auditory tone; CS) with an aversive unconditioned stimulus (i.e., a footshock; US) (LeDoux, 1995). Upon presentation(s) of the CS and US, the CS predicts an aversive outcome and comes to elicit a conditioned response (CR), such as defecation, piloerection, tachycardia, and freezing behavior (LeDoux, 1995). Lesion and pharmacological studies have revealed that cued (i.e., tone) and contextual fear learning are heavily reliant on the amygdala and hippocampus, respectively (Kim & Fanselow, 1992; Phillips & LeDoux, 1992; Selden, Everitt, Jarrard, & Robbins, 1991). Specifically, delay fear conditioning involves a co-terminating CS-US association, recruiting circuits that converge on the lateral amygdala and project to the central amygdala to elicit a CR (LeDoux, 2000). In contrast, trace fear conditioning involves a temporal gap between the CS-US presentations. Hippocampal projections containing contextual information converge on the basolateral

amygdala, which then project to the central amygdala to elicit a CR (LeDoux, 2000; O'Reilly & Rudy, 2001). The distinct neuroanatomical circuitry involved in trace and delay fear conditioning therefore provides an opportunity to investigate the functional consequences of amygdala kindling on fear memory and retrieval.

We have recently shown that long-term kindling to 99 stimulations impairs fear learning in rats subjected to trace fear conditioning (Fournier et al., 2013). However, we did not evaluate whether these deficits occur at an earlier time point (i.e., short-term kindling or 30 stimulations) or under different fear learning paradigms (i.e., delay fear conditioning). The issue of short-term vs long-term kindling is relevant because previous work has clearly shown that the magnitude of kindling-induced changes in fear and cognitive behaviors increases substantially with increasing numbers of stimulations (Kalynchuk, 2000). We therefore sought to characterize the effects of short- and long-term amygdaloid kindling on trace and delay fear conditioning. Rats were sacrificed following memory retrieval and cell counts were conducted on postmortem brain tissue immunostained for the presence of Fos protein. As Fos is a marker of behaviorally relevant neuronal activity (Guzowski et al., 2005; Morgan & Curran, 1991; Robertson, 1992), we hypothesized that the pattern of Fos immunoreactivity within the hippocampus and amygdala would parallel performance on these tasks.

2.2 Experimental Procedures

2.2.1 Animals

Male Long-Evans rats, weighing approximately 200-250g (7-8 weeks old) at the time of arrival (Charles River, Quebec, Canada) were used in this experiment. Rats were individually housed in rectangular polypropylene cages with standard laboratory bedding. Purina rat chow and water was provided ad libitum in a colony room maintained at an ambient temperature of 20 ± 1°C with a 12:12 h light-dark cycle (lights on at 8 a.m.). All experimental procedures were conducted during the light period of the light-dark cycle. Experimental manipulations were in accordance with the guidelines of the Canadian Council on Animal Care and a protocol approved by the University of Saskatchewan Committee on Animal Care and Supply. We made all possible efforts to minimize the number of animals used. A total of five rats were removed from the study due to incorrect electrode placement or head cap loss during kindling.

2.2.2 Surgery

All rats received daily handling and a minimum 1-week habituation to the colony room prior to surgery. To begin the surgery, each rat was individually anesthetized with isoflurane (5%) and injected with a preoperative analgesic (Anafen, Ketoprofen, 10mg/kg, s.c.) to reduce pain and inflammation. Once the rat was secured in a stereotaxic apparatus, a mixture of isoflurane and oxygen (5% initial, 2.5% maintenance) was provided through a mouth tube to maintain the anesthesia. A small incision was made down the scalp and surrounding connective tissue was excised. A single stainless steel bipolar stimulating electrode (MS-303-2-B-SPC, Plastics One, Roanoke, VA, USA) was chronically implanted into the left basolateral amygdala using the coordinates 2.8 mm posterior, 5.0 mm lateral, and 8.5 mm ventral to bregma in flat skull position (Paxinos & Watson, 1998). The electrode was secured to the skull with stainless steel screws (2 anterior, 2 posterior; 0-80 X 3/32, Plastics One) and dental acrylic. To minimize the risk of post-surgical infection, all rats received daily topical administration of Hibitane antibacterial-antifungal ointment Chlorhexidine acetate B.P. 1% (w/w) around the incision for a minimum of 1 week.

2.2.3 Kindling

The experimental outline of the study is shown in Fig. 2-1. All rats received a post-surgical recovery period of 10-14 days prior to the onset of kindling. The rats were then randomly divided into three separate groups such that the rats in each group began the experiment with approximately equal body weights. The three groups were long-term kindled (99 kindling stimulations, $n = 20$), short-term kindled (69 sham stimulations followed by 30 kindling stimulations, $n = 15$) and sham stimulated (99 sham stimulations, $n = 17$). All stimulations were delivered in a procedures room separate from the room in which the rats were housed. Rats received three stimulations per day, 5 days per week, with a minimum of 3 h between consecutive stimulations. The kindling stimulations were delivered using an isolated pulse stimulator (Model 2100, A-M Systems, Sequim, WA, USA) and comprised a 1 s, 60-Hz train of square-wave pulses, with each pulse lasting 1 ms with a biphasic amplitude of 800 μA (peak-to-peak). The sham stimulations were similar except that no electrical current was passed through the stimulation lead. Rats were returned to their home cage once all motor convulsions
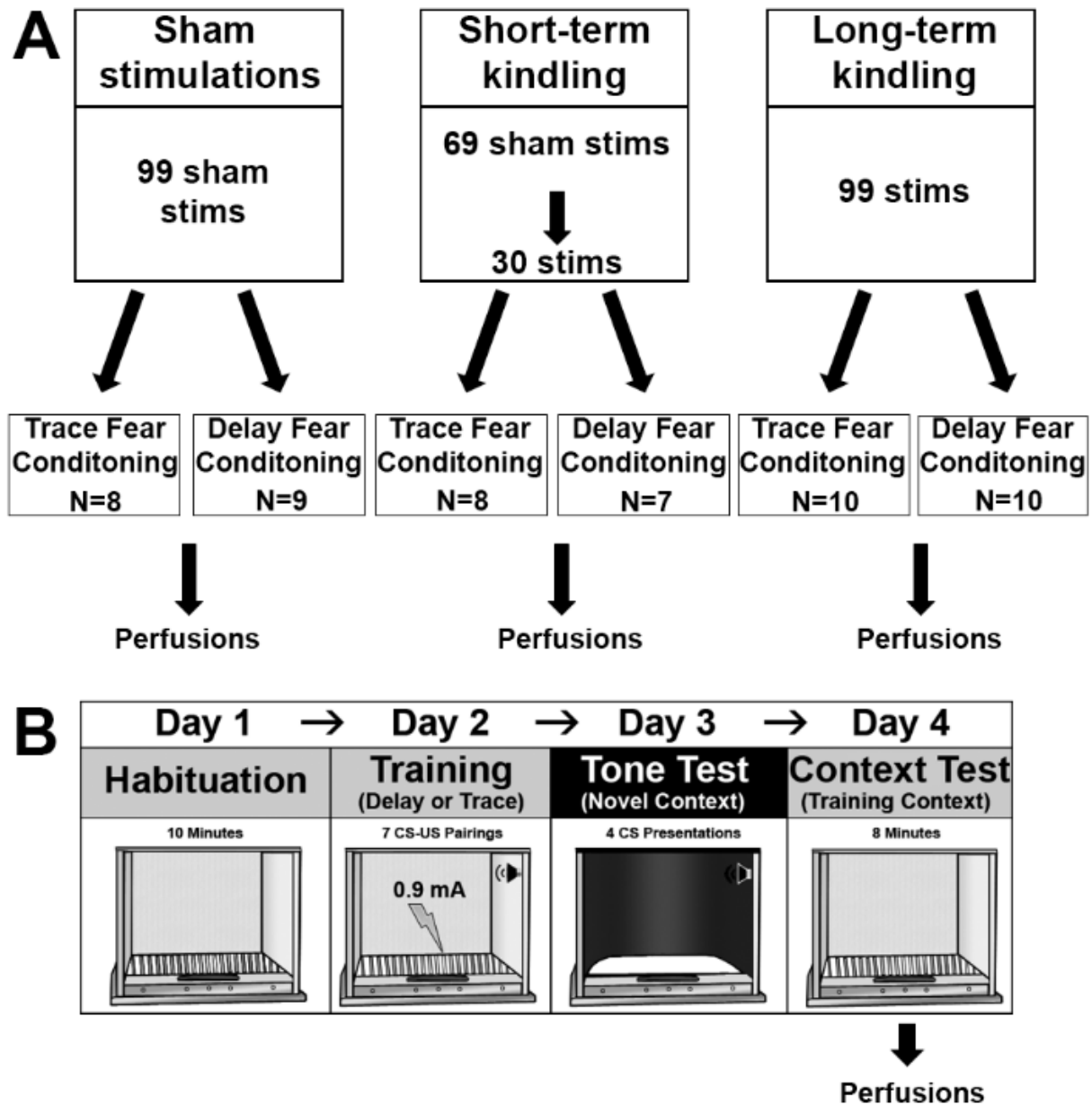
Figure 2-1. Experimental design. Panel A shows the three experimental groups that rats were randomly assigned to following surgery. Within 24 h of the final kindling or sham stimulation, approximately half of the rats in each group underwent trace conditioning while the other half underwent delay conditioning. Rats were sacrificed after completing the 4-day trace or delay conditioning paradigm. Panel B shows the fear conditioning paradigm used in the present study. On day 1, rats were habituated to the operant chamber for 10 min. On day 2, rats were returned to the chamber they were habituated in and subjected to seven CS-US pairings using a trace (30 s temporal gap between CS-US) or delay (co-terminating CS-US) protocol. On day 3, rats were placed in a novel operant chamber and presented with four CS's over 16 minutes. On day 4, rats were returned to the operant chamber they were trained in for 8 min. Rats were perfused 2 h after completing this final behavioral task.

had ceased or after 30 s for sham stimulations. To control for handling effects, all rats received a total of 99 kindling or sham sessions.

The convulsion elicited by each stimulation was scored using a revised eight class extension (Pinel & Rovner, 1978) of Racine's original five class scale (Racine, 1972). The classes were operationally defined as: Class 0: immobility, Class 1: orofacial automatisms, Class 2: orofacial automatisms with head nodding, Class 3: unilateral forelimb clonus, Class 4: rearing with bilateral forelimb clonus, Class 5: rearing with bilateral forelimb clonus followed by falling, Class 6: multiple class 5 convulsions and falling episodes, Class 7: previous classes with running fit, Class 8: previous classes with intermittent muscle tonus. Using this classification system, rats are considered to be "kindled" after three consecutive Class 5 convulsions (Pinel & Rovner, 1978).

2.2.4 Fear conditioning procedures

One day after the last sham or kindling stimulation, all rats underwent one of two fear conditioning procedures (trace or delay; Fig. 2-1B). Two identical sound attenuated operant chambers (VFC-008, Med Associates Inc, St. Albans, VT, USA) were used for all fear conditioning experiments. Each chamber was equipped with a miniature monochromatic video camera and VideoFreeze software (Med Associates Inc) that provided live observations and video recordings sampled at 30 frames per second for offline analyses. The chambers had aluminum walls and a clear Plexiglas front door and ceiling. Within the chamber, the grid floor had 19 aluminum rods (0.48 cm diameter, spaced 1.6 cm apart) that were connected to a shock generator (ENV-414S, Med Associates Inc). Auditory stimuli were delivered through a loudspeaker mounted in the chamber wall (ANL-926, Med Associates). An interface cabinet (DIG-700 F, Med Associates Inc) controlled tone-shock presentations. Operant chamber ventilation fans provided constant background noise (60 db) during the task. The chambers were cleaned thoroughly with 0.6% (v/v) acetic acid between subjects unless otherwise noted.

The rats in each group were randomly subdivided so that approximately half the rats were subjected to trace fear conditioning and the other half were subjected to delay fear conditioning. For the trace conditioning paradigm ($n = 26$), the breakdown of each group was as follows: sham stimulated ($n = 8$), short-term kindled ($n = 8$), and long-term kindled ($n = 10$). For the delay

conditioning paradigm ($n = 26$), the breakdown of each group was sham stimulated ($n = 9$), short-term kindled ($n = 7$), and long-term kindled ($n = 10$). The protocol for both trace and delay fear conditioning included habituation, training, and 2 testing days. The main variable of interest, freezing behavior, was measured during all sessions. We defined freezing as the termination of all motor movements except those movements necessary for respiration (Blanchard & Blanchard, 1972; Bolles, 1970). We scored freezing behavior using a modified version of a time sampling method that has been previously described (Quinn et al., 2005). Briefly, freezing behavior was scored every 2 s during tone presentations and every 4 s in the absence of tone. These observations were transformed into the percentage of time spent freezing by summing the total number of freezing observations together and dividing this value by the total number of observations for each period and then multiplying the resultant number by 100.

The fear conditioning tasks proceeded as follows. On day 1, the habituation day, all rats were placed in the operant chamber for 10 min. This was to ensure that rats were acclimatized to the operant chambers in the absence of aversive stimuli. During the habituation period, we assessed baseline ambulatory activity (midline crossing), operationally defined as all four limbs crossing the center of the chamber (Quinn et al., 2005). We collected these data to ensure that subsequent alterations in conditioned freezing could not be attributed to the novelty-induced hyperlocomotion that has been previously reported after long-term kindling (Fournier et al., 2009).

On day 2, the training day, rats were subjected to either a trace or delay training paradigm comprising seven CS (tone)–US (footshock) pairings (Quinn et al., 2005). For both paradigms, rats were returned to the same operant chamber they were habituated in and received a 180-s acclimation period prior to the first auditory CS presentation. In the trace conditioning paradigm, each trial comprised a 16-s auditory tone (85 dB, 2 kHz), followed by a 30-s trace interval, and then a 2-s footshock (.9 mA). In the delay conditioning paradigm, each trial comprised a 16-s auditory tone (85 dB, 2 kHz) that co-terminated with a 2-s footshock (.9 mA). The inter-trial interval was 180 s between tone onsets for both paradigms. After the seventh CS-US pairing, all rats remained in the operant chamber for an additional 180 s and were then returned to their home cages.

On day 3, the tone test day, rats received a 180-s acclimation period followed by four tone presentations (16 s, 85 dB, 2 kHz). The inter-trial interval was 198 s between tone onsets.

As on the training day, rats remained in the chamber for an additional 180 s after the final tone presentation before being returned to their home cages. The operant chambers and testing room were modified in a number of different ways to provide a novel context for the tone test. First, we changed the shape and lighting of the room by hanging blue curtains in a circular shape around the operant cubicles and disabling the overhead lights. Second, we placed Plexiglas inserts inside the operant chambers to modify the internal shape from a square to a semi-circle. Construction paper cutouts were placed on the Plexiglas inserts and on the inner walls of the operant cubicle to provide novel cues inside and outside the testing chamber. Third, we added black inserts to the operant chamber ceiling to alter house lighting within the operant chamber. Fourth, we replaced the grid bars on the chamber floor with a solid white Plexiglas sheet. Fifth, we scented the chamber with a dilute vanilla extract solution in a stainless steel pan placed below the operant chamber floor. And finally, we used a white noise generator (60 dB) to alter the sound within the chamber during the task. All rats were transported to the fear conditioning room with a dark cloth covering their home cage. Experimenters took additional paths and time to reach the room to prevent the rats from identifying the routes taken on the previous days and all rats were tested in the opposing operant chamber from the one they were trained in. The chambers were cleaned thoroughly with 0.64% NaOH between each rat.

On day 4, the context test day, all modifications made on day 3 were reverted back to the original habituation and training settings. All rats were placed into the operant chamber they had been trained in for a total of 480 s. No footshocks or tones were delivered during this time.

2.2.5 Perfusions and Immunohistochemistry

Two hours after the context test, each rat was deeply anesthetized with sodium pentobarbital (240 mg/ml, i.p.) and transcardially perfused using room temperature phosphate-buffered saline (PBS, 0.1 M, pH = 7.4) followed by ice-cold 4 % (w/v) formaldehyde fixative (pH = 7.4). The brains were extracted and immersed in the same formaldehyde fixative for up to 72 hours before coronal sectioning at 50 μm on a vibrating microtome (Vibratome 3000, Vibratome Company, St. Louis, MO, USA). All sections were collected and stored at -20 °C in a cryoprotectant solution comprised of 30 % (w/v) sucrose, 1 % (w/v) polyvinylpyrrolidone (PvP), and 30 % (v/v) ethylene glycol in PBS (0.1 M, pH = 7.4).

Immunostaining for Fos protein expression was conducted on free-floating sections using gentle agitation during all rinses and incubations as previously described (Fournier et al., 2013). All tissue from each fear conditioning procedure was processed in unison (i.e., delay fear conditioning tissue together and trace fear conditioning tissue together). All sections were treated with 0.3 % (v/v) H_2O_2 for 30 min at room temperature to block endogenous peroxidase activity, followed by 5 % (v/v) normal goat serum, 1 % (w/v) bovine serum albumin, and 0.3 % (v/v) Triton X-100 in 0.1 M PBS for 1 h to prevent non-specific antibody binding. The sections were then incubated for 72 h at 4 °C with c-Fos anti-rabbit polyclonal primary antibody (1:15,000, Calbiochem, La Jolla, CA, USA) diluted in blocking solution. The sections were then incubated in biotinylated goat anti-rabbit IgG secondary antibody (1:500, Vector Laboratories, Burlingame, CA, USA) diluted in 0.3 % (v/v) Triton X-100 in 0.1 M PBS for 2 h at room temperature, treated with avidin-biotin-peroxidase complex (1:500, Vectastain ABC Elite, Vector Laboratories) and visualized with nickel-intensified DAB comprised of 0.02 % (w/v) 3,3'-diaminobenzidine, 2.5 % (w/v) nickel ammonium sulfate and 0.000083 % (v/v) H_2O_2 in 0.175 M sodium acetate. The reaction was halted by rinsing the sections in 0.175 M sodium acetate. After these rinses, the sections were mounted onto glass slides using 0.2 M PB (pH = 7.4) and left to dry overnight. Within 24 h the slides were dehydrated using graded alcohols (2 min 70 %, 2 min 95 %, 4 min 100 %), a xylene series (1 min, 1 min, 5 min), and coverslipped with mounting medium (Entellan, EMD Chemicals Inc; Gibbstown, NJ, USA).

2.2.6 Quantification of Fos Immunoreactivity

A researcher blind to the treatment conditions conducted all quantitative analyses. Immunostained sections were examined using a Nikon Eclipse E800 microscope equipped with a motorized stage and digital camera (MicroFire, Optronics, Goleta, CA, USA) that was connected to a computerized stereology system (Stereo Investigator, v9, MicroBrightField Inc, Williston, VT, USA). Fos immunoreactivity was assessed bilaterally in a number of different brain regions: the CA1 and CA3 pyramidal cell layers, the granule cell layer and hilus of the dentate gyrus, the lateral, basolateral, central, and medial nuclei of the amygdala, the cingulate, perirhinal, and entorhinal cortices, and the periaqueductal grey. These brain regions were chosen for analysis because of their direct involvement in spatial memory, emotional responses, conditioned freezing and because they are thought to play distinct roles in trace vs. delay fear conditioning (Fanselow

& Poulos, 2005; Han et al., 2003; Kim & Jung, 2006; LeDoux, 2000; Maren, 2001; Orsini & Maren, 2012). Fos immunoreactivity was quantified on a minimum of five sections per brain with 300 µm between sections, ranging from -0.12 to -6.36 mm (relative to bregma). Fos+ cells were counted in similar anterior-posterior coronal planes across subjects to allow for more accurate cell count comparisons. We traced contours well within the boundaries of each region of interest at 4x magnification using a brain atlas as a guide (Paxinos & Watson, 1998). We used landmarks such as the size and shape of the lateral ventricles, hippocampus, and optic tract to assist with tracing the previously described areas. Fos+ cells were counted within these contours at 20x magnification using a meander scan profile counting method (Knapska & Maren, 2009). This technique uses an automated scanning procedure that allows for all cells within a traced region to be viewed and counted. For each subject, we divided the total number of Fos+ cells in each region of interest by the total area captured within the traced contours (in µm) to determine the density of Fos+ cells within each traced region. The density values were then re-expressed as the number of Fos+ cells per mm^2. Therefore, our reported values are not the absolute total number of Fos+ cells within each region. Instead, they reflect the average number of Fos+ cells within a defined contour within each region.

2.2.7 Statistical Analyses

All statistical analyses were conducted using IBM's Statistical Package for Social Sciences, v. 20. Group differences were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni adjusted for multiple pair wise comparisons as a post hoc test when appropriate. To determine the relationship between contextual freezing and Fos immunoreactivity, we conducted Pearson (*R*) product moment correlations. The criterion for statistical significance was P < 0.05 for all analyses. All graphs depict group means ± standard errors of the mean.

2.3. Results

2.3.1 Kindling Progression

Kindling progressed normally in all rats. The number of stimulations required to elicit the first class 5 convulsion was 13.7 ± 1.2 for the short-term kindled rats and 13.6 ± 1.4 for the long-

term kindled rats. The number of electrical stimulations required to achieve the fully kindled state (i.e., three consecutive class 5 convulsions) was 17.4 ± 2.1 for the short-term kindled rats and 20.9 ± 2.4 for the long-term kindled rats. The average total number of class 5 or higher convulsions was 10.1 ± 1.3 for the short-term kindled rats and 68.4 ± 3.7 for the long-term kindled rats. Consistent with earlier reports (Fournier et al., 2010; Kalynchuk et al., 1997; Pinel & Rovner, 1978), we did not observe spontaneous seizures at any point during the course of our experiments.

2.3.2 Effect of Kindling on Trace Fear Conditioning

There were no significant effects of kindling on acquisition of the trace fear conditioning task. As a baseline measure of motor activity, we analyzed the number of midline crossovers made during the 10-min exposure to the operant chambers (i.e., habituation day, Fig. 2-2A). There was no difference across groups in midline crosses ($F(2,25) = .922$, $p = .412$) indicating that amygdaloid kindling did not alter nonspecific motor activity in the conditioning chambers. On the training day, there was no difference in freezing ($F(2,25) = 1.982$, $p = .161$) prior to the first CS presentation (data not shown). In addition, all groups successfully learned the trace fear conditioning paradigm after seven CS-US pairings as indicated by increased levels of freezing with successive tone-shock pairings (Fig. 2-2B). Further analysis confirmed that all groups exhibited comparable levels of freezing during each presentation of the CS ($F(2,25) < 2.176$, $p > .136$). Finally, there were no significant differences in the average time spent freezing during the trace interval ($F(2,25) = .708$, $p = .503$) or post-shock periods ($F(2,25) = .901$, $p = .420$) suggesting that kindling did not impair or alter the display of appropriate fear-related behavior (Fig. 2-2C).

On the tone test day, rats were placed in a novel context and freezing behavior was assessed during both the presentation of the tones and the post-tone periods (Fig. 2-2D). There were significant group differences in the percentage of time spent freezing during the CS presentations (averaged across test tones, ($F(2,25) = 4.599$, $p = .021$) and the percentage of time spent freezing during the post-CS periods ($F(2,25) = 9.704$, $p = .001$). Subsequent post hoc analyses revealed that the long-term kindled rats froze significantly less than the sham-stimulated rats (all p values <.018) during both periods. However, the short-term kindled rats were not significantly different than the sham-stimulated rats on either measure (p values >.276).
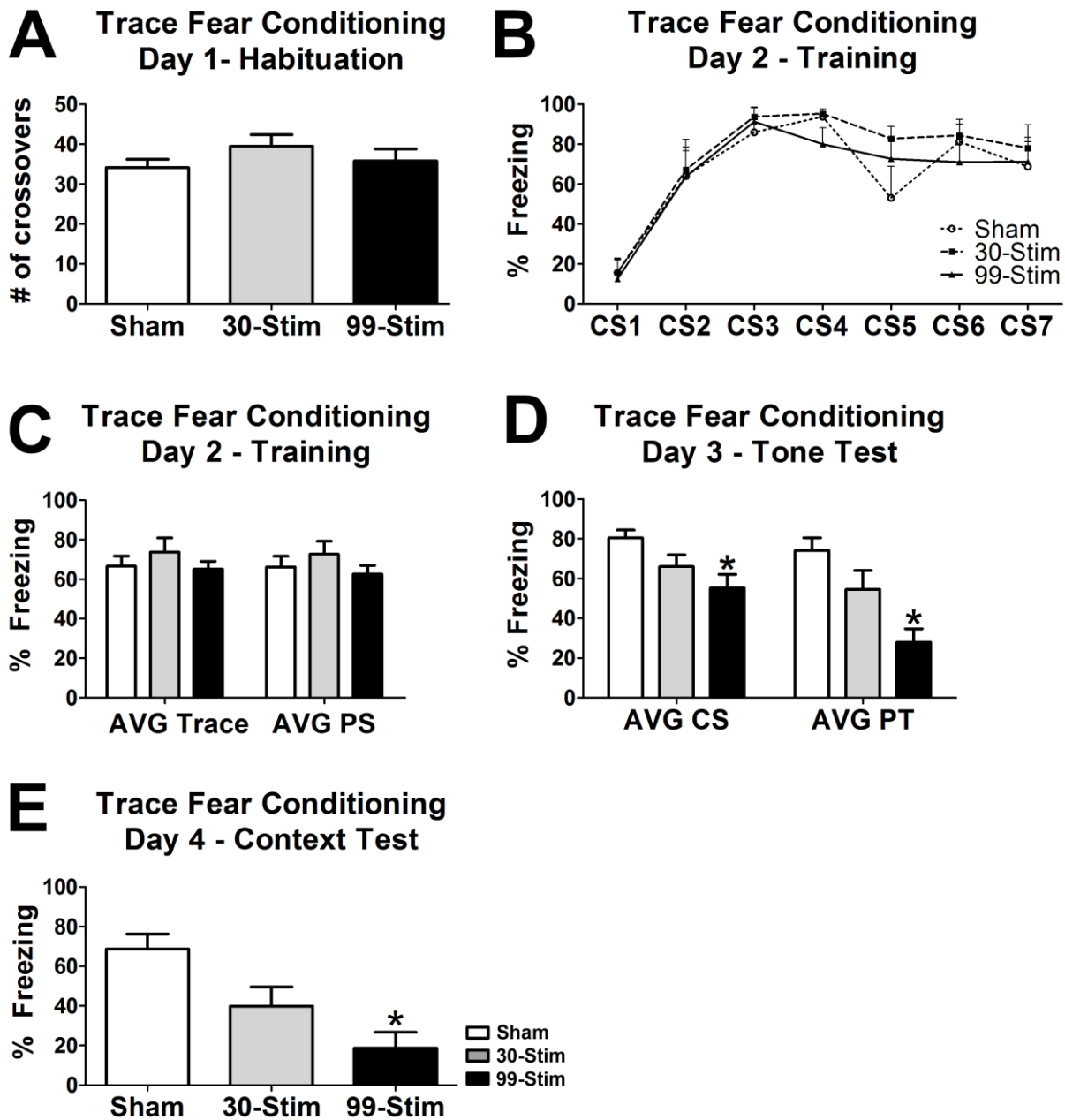
Figure 2-2. Effect of short and long-term amygdala kindling on trace fear conditioning. Panel A shows locomotor behavior during habituation and Panels B and C show the percent of time spent freezing during training. Kindling had no significant effect on any of these measures. Panel D shows the percent of time spent freezing during the tone test. Long-term kindling significantly reduced conditioned freezing during the presentation of discrete tones (CS) and the post-tone intervals (PT). Panel E shows the percent of time spent freezing during the context test. Long-term kindling significantly reduced conditioned freezing to the training context in absence of discrete CS cues. AVG Trace: average freezing during the 30 s trace interval. AVG PS: average freezing during the post-shock periods. AVG CS: average freezing during the tone presentations. AVG PT: average freezing during the post-tone periods. *Significantly different from sham-stimulated rats ($p < 0.05$)

On the context test day, the rats were returned to the original training environment to examine retrieval of context-dependent fear memories (Fig. 2-2E). During context testing, the long-term kindled rats froze significantly less than the sham-stimulated rats ($F(2,25) = 9.038$, $p = .001$), but the short-term kindled rats were not significantly different from the sham-stimulated rats on this measure ($p = .087$).

2.3.3 Effect of Kindling on Delay Fear Conditioning

In contrast to the trace fear conditioning results, kindling did appear to slow learning in the delay fear conditioning task. As expected, we did not find a significant difference in the number of midline crossovers on the habituation day ($F(2,25) = .143$, $p = .868$) (Fig. 2-3A). Further, as seen in the trace study, there were no significant group differences in freezing during the acclimation period of the training day ($F(2,25) = 2.185$, $p = .135$) (data not shown). Analyses of freezing behavior on the training day revealed no significant differences among the groups in freezing to the first CS presentation ($F(2,25) = .882$, $p = .427$), but we did find a significant group difference in freezing during the second CS presentation ($F(2,25) = 12.963$, $p < .001$; Fig. 2-3B). Post hoc analysis of this effect revealed that the short-term and long-term kindled rats froze significantly less than the sham-stimulated rats during this time (p values $< .030$). However, this effect was limited to the second CS presentation as freezing during subsequent tone presentations was not significantly different across groups ($F(2,25) < 2.946$, $p > .073$). Finally, we found a significant difference among the groups in freezing during the post-shock period ($F(2,25) = 8.27$, $p < .002$; Fig. 2-3C). Post hoc analysis revealed that the short-term and long-term kindled rats froze less during this time than did the sham-stimulated rats (all p values $< .008$).

On the tone test day, we examined freezing behavior in a novel context during tone presentations and the post-tone periods. We found significant group differences in freezing during both tone-on ($F(2,25) = 12.06$, $p < .001$) and post-tone ($F(2,25) = 48.165$, $p < .001$) periods (Fig. 2-3D). Post hoc analyses of these main effects revealed that both the short-term kindled and long-term kindled rats froze less than the sham-stimulated rats during these periods (p values $< .003$).

On the context test day, we examined freezing in the original training context. Our results were similar to those we observed for the tone test. That is, we found significant group differences in freezing to context ($F(2,25) = 40.916$, $p < .001$) and post hoc analyses revealed
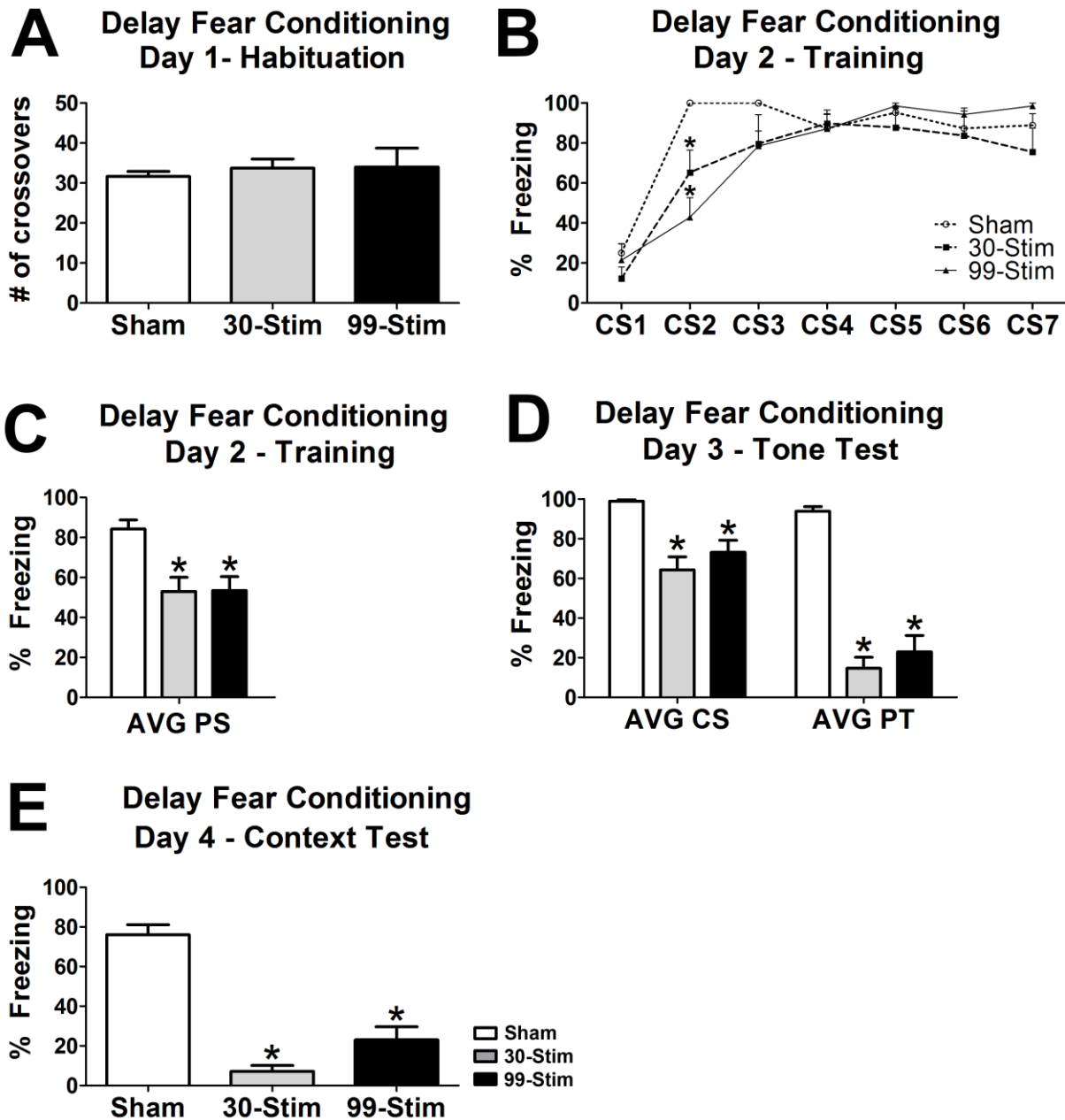
Figure 2-3. Effect of short and long-term amygdala kindling on delay fear conditioning. Panel A shows locomotor behavior during habituation and Panels B and C show the percent of time spent freezing during training. Short and long-term amygdala kindling significantly impaired freezing during the second tone presentation (CS2) and during the post-shock periods (AVG PS). Panel D shows the percent of time spent freezing during the tone test. Short- and long-term kindling significantly reduced conditioned freezing during the presentation of discrete tones (CS) and the post-tone intervals (PT). Panel E shows the percent of time spent freezing during the context test. Again both short- and long-term kindling significantly reduced conditioned freezing to the training context in absence of discrete CS cues. AVG PS: average freezing during the post-shock periods. AVG CS: average freezing during the tone presentations. AVG PT: average freezing during the post-tone periods. *Significantly different from sham-stimulated rats ($p < 0.05$)

that both the short-term and long-term kindled rats froze significantly less than did the sham-stimulated rats (p values $< .001$; Fig. 2-3E).

2.3.4 Fos Expression Following Trace or Delay Fear Conditioning

Following fear conditioning, we examined the effect of kindling on neuronal activation using the activity-dependent marker Fos. Fig. 2-4 provides a schematic of the brain regions we analyzed. Figs. 2-5 and 2-6 show examples of Fos immunoreactivity within these brain regions and provide histograms with our quantified Fos+ cell counts. In the sham-stimulated rats subjected to trace fear conditioning, we saw substantial Fos expression within multiple limbic, cortical, and subcortical regions. By comparison, Fos expression was substantially lower in the long-term kindled rats. These qualitative observations were supported by statistical analyses of quantitative cell counting. Within the hippocampus and parahippocampal regions, we found significant group differences in the number of Fos+ cells bilaterally in the granule cell layer [ipsilateral $F(2,25) = 5.224$, $p < .013$; contralateral $F(2,25) = 8.642$, $p < .002$], the CA3 pyramidal cell layer [ipsilateral $F(2,25) = 3.548$; $p < .045$; contralateral $F(2,25) = 5.169$, $p < .014$], the perirhinal cortex [ipsilateral $F(2,25) = 7.435$, $p < .003$; contralateral $F(2,25) = 10.205$, $p < .001$], and the entorhinal cortex [ipsilateral $F(2,25) = 9.830$; $p < .001$; contralateral $F(2,25) = 12.092$, $p < .001$]. For all of these main effects, post hoc analyses revealed that the long-term kindled rats had fewer Fos+ cells than did the sham-stimulated rats (all p values $< .042$). Within the amygdala, we found significant group differences in the number of Fos+ cells in the ipsilateral basolateral region ($F(2,25) = 9.438$, $p < .001$) and the contralateral lateral amygdala ($F(2,25) = 3.657$, $p < .042$). Again, post hoc analyses revealed that the long-term kindled rats had fewer Fos+ cells in these regions than did the sham-stimulated rats (all p values $< .044$). Our post hoc analyses also indicated that long-term kindled rats had fewer Fos+ cells than short-term kindled rats in the bilateral entorhinal cortex, the ipsilateral basolateral amygdala, and the contralateral granule cell layer and perirhinal cortex (all p values $< .034$). There were no significant differences in Fos+ cells in the hilus, CA1 subfield, periaqueductal gray, cingulate cortex, or the central and medial nuclei of the amygdala (all p values $> .085$). Finally, there were no significant differences between the short-term kindled rats and the sham-stimulated rats in any quantified region (all p values $> .215$).

Bregma -0.36 mm; Interaural 8.64 mm

Cingulate Cortex

Bregma -2.92 mm; Interaural 6.08 mm

CA1
CA3
Hilus
Granule Cell Layer

Lateral Amygdala
Central Amygdala
Basolateral Amygdala
Medial Amygdala

Bregma -4.44 mm; Interaural 4.56 mm

Perirhinal Cortex
Entorhinal Cortex

Bregma -6.12 mm; Interaural 2.88 mm

Periaqueductal Grey
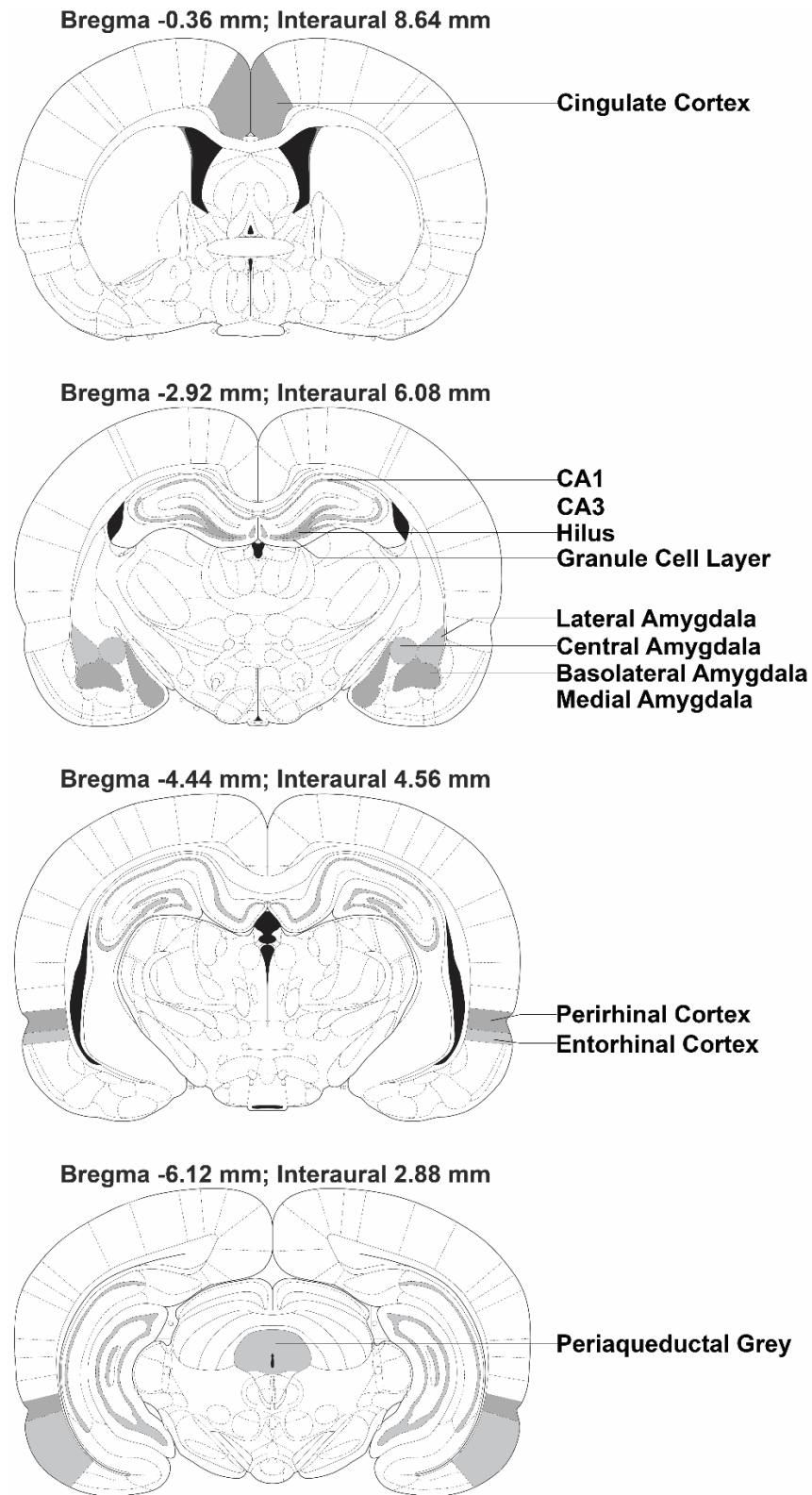
Figure 2-4. Schematic of the brain regions that were quantified in this experiment. Adapted from (Paxinos & Watson, 1998)
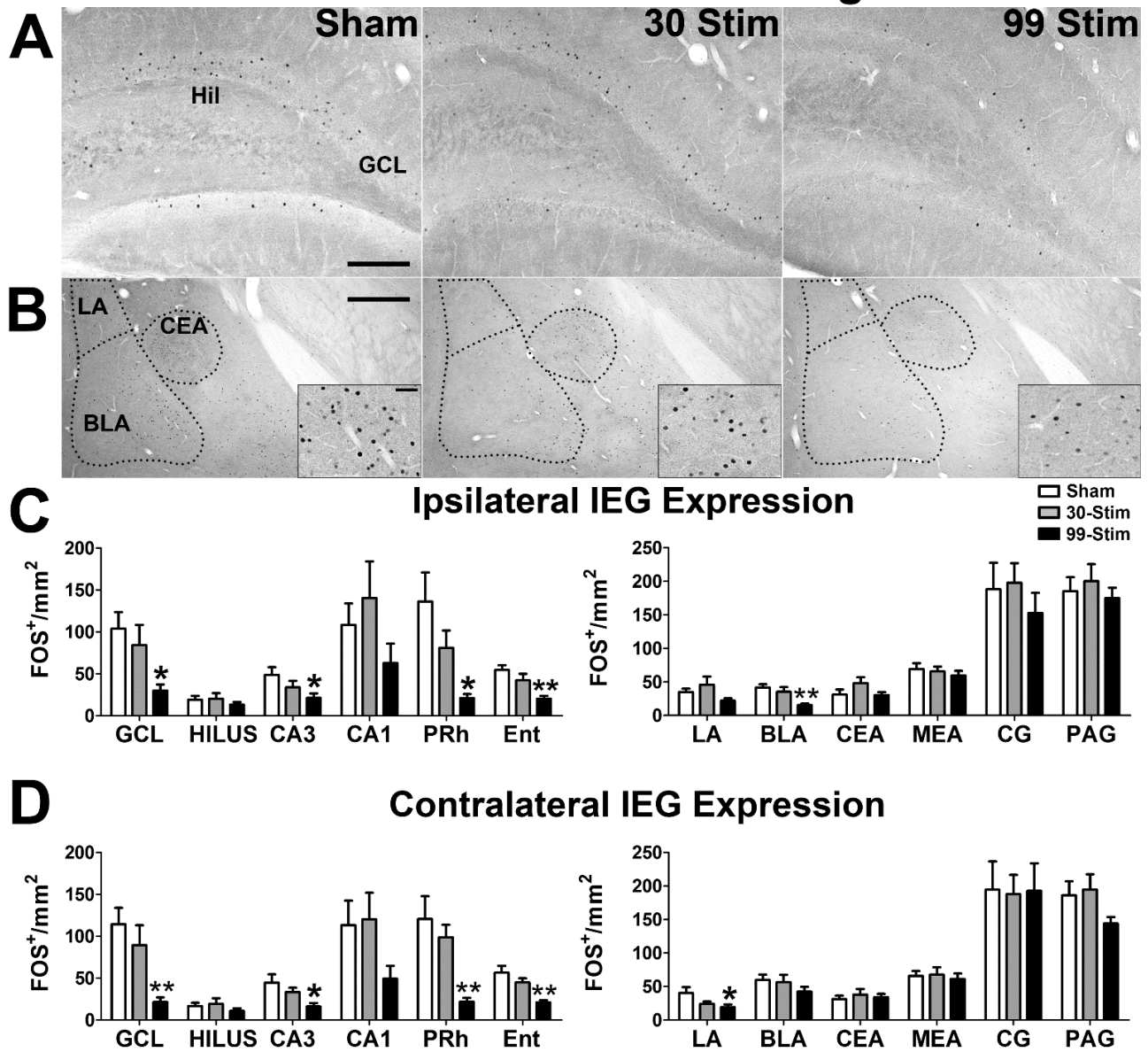
Figure 2-5. Effect of kindling on Fos expression following contextual memory retrieval in rats subjected to trace conditioning. Panels A and B show representative photomicrographs of Fos immunoreactivity in the dentate gyrus (Scale bar = 200μm) and amygdala (Scale bar = 500μm) respectively. Inserts are a magnification of the basolateral amygdala (Scale bar = 50 μm). Note the presence of Fos+ cells throughout these regions in the sham-stimulated rats. Panels C and D provide histograms of the quantified number of Fos+ cells across a number of brain regions. Only long-term kindling significantly decreased Fos expression. GCL, granule cell layer; PRh, perirhinal cortex; Ent, entorhinal cortex; LA, lateral amygdala; BLA, basolateral amygdala; CEA, central amygdala; MEA, medial amygdala; CG, cingulate gyrus; PAG, periaqueductal gray. *Significantly different from sham-stimulated rats ($p < 0.05$), **Significantly different from both sham-stimulated and short-term kindled rats ($p < 0.05$)
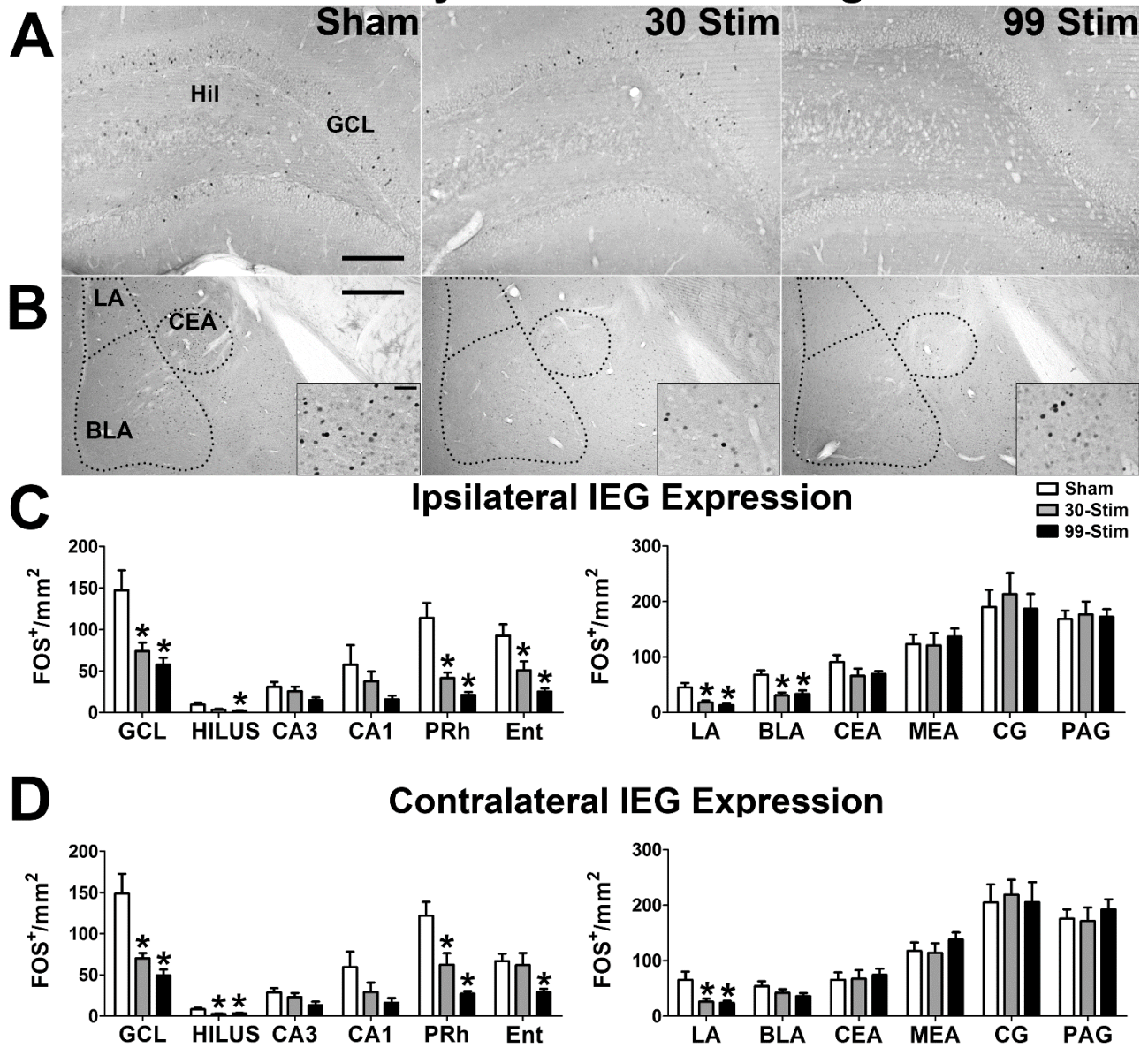
Figure 2-6. Effect of kindling on Fos expression following contextual memory retrieval in rats subjected to delay conditioning. Panels A and B show representative photomicrographs of Fos immunoreactivity in the dentate gyrus (Scale bar = 200μm) and amygdala (Scale bar = 500μm) respectively. Inserts are a magnification of the basolateral amygdala (Scale bar = 50 μm). Panels C and D provide histograms of the quantified number of Fos+ cells across a number of brain regions. In this case, both short- and long-term kindling significantly decreased Fos expression in several brain regions. GCL, granule cell layer; PRh, perirhinal cortex; Ent, entorhinal cortex; LA, lateral amygdala; BLA, basolateral amygdala; CEA, central amygdala; MEA, medial amygdala; CG, cingulate gyrus; PAG, periaqueductal gray. *Significantly different from sham-stimulated rats ($p < 0.05$).

In rats subjected to delay fear conditioning, we found significant group differences in the number of Fos+ cells bilaterally in the dentate granule cell layer [ipsilateral $F(2,25) = 8.843$, $p < .001$; contralateral $F(2,25) = 12.148$, $p < .001$], hilus [ipsilateral $F(2,25) = 6.136$, $p < .007$; contralateral $F(2,25) = 6.890$, $p < .005$], perirhinal cortex [ipsilateral $F(2,25) = 19.116$, $p < .001$; contralateral $F(2,25) = 16.787$, $p < .001$], and entorhinal cortex [ipsilateral $F(2,25) = 12.780$; $p < .001$, contralateral $F(2,25) = 5.610$, $p < .010$]. In each case, post hoc analyses revealed that the long-term kindled rats had fewer Fos+ cells compared to the sham-stimulated rats (all p values $< .016$). Interestingly, the short-term kindled rats also had fewer Fos+ cells than did the sham-stimulated rats bilaterally in the dentate granule cell layer and perirhinal cortex (all p values $< .019$), ipsilaterally in the entorhinal cortex ($p = .027$), and contralaterally in the hilus ($p = .011$). Within the amygdala, we found group differences in the number of Fos+ cells bilaterally in the lateral amygdala [ipsilateral $F(2,25) = 9.828$, $p < .001$; contralateral $F(2,25) = 6.091$, $p < .008$] and ipsilaterally in the basolateral amygdala ($F(2,25) = 8.380$, $p < .002$). Post hoc analyses revealed that both the short-term kindled rats and long-term kindled rats had fewer Fos+ cells in these regions compared to the sham-stimulated rats (all p values $< .031$). Finally, there were no group differences in the number of Fos+ cells in the CA3 and CA1 subfields, periaqueductal gray, cingulate cortices or the central and medial nuclei of the amygdala (all p values $> .063$).

2.3.5 Correlations between contextual freezing and Fos immunoreactivity

To better understand the relationship between fear learning and memory and neuronal activity in specific brain regions, we calculated correlations between freezing during the context test and Fos immunoreactivity across all quantified brain regions (i.e., all groups combined). To facilitate the interpretation of these analyses, we averaged the ipsilateral and contralateral Fos counts for each region. The results of these analyses are presented in Table 2-1. For the trace conditioning task, we found significant correlations between contextual freezing and Fos+ cells. In the granule cell layer ($p = .023$), perirhinal cortex ($p = .012$) and entorhinal cortex ($p = .049$). For the delay conditioning task, we found significant correlations between contextual freezing and Fos+ cells in the granule cell layer ($p = .005$), hilus ($p = .009$), perirhinal cortex ($p = .005$), and lateral amygdala ($p = .009$). No other significant correlations were found for either conditioning protocol (all p values $> .06$).

Table 2-1. Correlations between contextual freezing (day 4) and Fos immunoreactivity in brain regions.

Protocol	GCL	Hilus	CA3	CA1	PRh	Ent	LA	BLA	CEA	MEA	CG	PAG
Trace	**.444***	.262	.312	.135	**.485***	**.390***	.301	.319	-.229	-.280	-.283	.263
Delay	**.536***	**.501***	.301	.267	**.536***	.376	**.503***	.336	.095	-.214	-.289	-.115

GCL, granule cell layer; PRh, perirhinal cortex; Ent, entorhinal cortex; LA, lateral amygdala; BLA, basolateral amygdala; CEA, central amygdala; MEA, medial amygdala; CG, cingulate gyrus; PAG, periaqueductal gray.

* $p < 0.05$ (bolded values indicate statistically significant correlations).

2.4. Discussion

The results of this experiment make two important points. First, we found that amygdaloid kindling disrupts conditioned fear in a stimulation-dependent and task-specific manner. Specifically, all rats that underwent trace fear conditioning displayed normal freezing during the training period, but the long-term kindled rats showed significant reductions of freezing during subsequent tone and context tests. In contrast, in the delay fear conditioning task, both short- and long-term kindled rats displayed mild deficits during acquisition and significant reductions in conditioned freezing during the tone and context tests. These results suggest that long-term kindling produces greater impairments in fear learning and memory than short-term kindling and that amygdala-dependent tasks are more likely to be affected by short-term amygdala kindling than hippocampal-dependent tasks. Second, we showed that amygdaloid kindling markedly reduced Fos expression following fear memory retrieval in a manner that paralleled the decrease in freezing during the context test. That is, in rats subjected to trace conditioning, decreased Fos was apparent primarily in the long-term kindled rats but in rats subjected to delay conditioning, decreased Fos was seen in both short-term and long-term kindled rats. Interestingly, our correlational analyses revealed that conditioned freezing in rats subjected to trace conditioning positively correlated with Fos expression in hippocampal and parahippocampal regions, whereas conditioned freezing in rats subjected to delay conditioning was positively correlated with Fos expression in hippocampal, parahippocampal, and amygdala regions. These correlations are consistent with prior work that has implicated the hippocampus in in trace conditioning and the amygdala in delay conditioning (Fanselow & Poulos, 2005; Kim & Jung, 2006; LeDoux, 2000; Orsini & Maren, 2012).

What might account for the reduction of fear memory retrieval seen in the kindled rats in this experiment? It is probably not due to an inability of amygdala-kindled rats to engage in conditioned freezing, because all rats displayed an appropriate level of learning across successive CS-US pairings during training. Similarly, our habituation data revealed that kindling did not alter baseline ambulatory behavior and thus non-specific alterations of locomotor activity cannot explain our behavioral results. Furthermore, our results cannot be explained by amygdala lesions associated with implantation of the stimulating electrode, because the sham-stimulated rats showed normal performance on all tasks. Rather, we believe that the reduction of conditioned fear might be better explained by cognitive deficits instigated via stimulation-dependent changes

within neural circuits involved in fear learning and memory retrieval. Indeed, kindling is known to cause robust changes of gene expression within the amygdala and hippocampus that could affect learning and memory (Corcoran, Kroes, Burgdorf, & Moskal, 2011).

Another important question to ask is why short-term kindling affected delay conditioning but not trace conditioning in this experiment. The basolateral complex of the amygdala is a locus of sensory convergence (Maren, 2001) that directly facilitates associative learning between the CS and US. Kindling of the basolateral amygdala rapidly causes localized neuroplastic changes, such as alterations in long-term potentiation and gene expression (Adamec & Young, 2000). With continued stimulations, these neuroplastic changes begin to occur in other brain regions downstream from the amygdala, such as the periaqueductal gray and hippocampus. As delay conditioning is amygdala-dependent, the acquisition and retrieval deficits seen in the short-term kindled rats in this task likely reflect functional changes within the amygdala that occur during the early stages of kindling. This is reflected in the decreased Fos expression evident in amygdalar regions in the short-term kindled rats in the delay but not the trace conditioning task. This idea is also consistent with our previous observation that short-term amygdala kindling decreases Fos immunoreactivity with the amygdala after exploration of a novel open field (Fournier et al., 2009). Although it is difficult to interpret decreased Fos expression in terms of specific neuroplastic changes, one possibility is that a loss of synaptic terminals in the amygdala could contribute to impairments of associative LTP that would normally facilitate learning and consolidation of auditory delay fear conditioning. In contrast, trace fear conditioning requires the participation of additional circuits, including the hippocampus, entorhinal cortex, perirhinal cortex, and cingulate cortex, to facilitate learning of a CS-US association with a temporal gap. Abnormal plasticity within these hippocampal circuits is typically seen after extended kindling, such as the 99 stimulations used in this experiment (Fournier et al., 2010; Kalynchuk, 2000; Kalynchuk & Meaney, 2003). Therefore, it may be that more pervasive changes resulting from long-term kindling are required to impair the hippocampal and parahippocampal regions recruited by trace fear conditioning. In line with this suggestion, a recent study showed that inactivation of the amygdala with the GABA$_A$ agonist muscimol produced profound deficits in delay but not trace conditioning (Raybuck & Lattal, 2011).

Our correlational analyses revealed several significant correlations between Fos immunoreactivity in specific structures and contextual freezing behavior. For rats that were

subjected to trace fear conditioning, our cell counts showed that long-term kindled rats had significantly fewer Fos+ cells in the granule cell layer, CA3 subfield, the perirhinal and entorhinal cortices, and the amygdala. However, only Fos immunoreactivity in the dentate granule cell layer and perirhinal and entorhinal cortices was positively correlated with contextual freezing. These correlations likely reflect the strong contextual component of this task. Indeed, lesions of the perirhinal cortex dramatically impair auditory trace conditioning (Kholodar-Smith, Boguszewski, & Brown, 2008b) and contextual fear (Bucci, Phillips, & Burwell, 2000). In this experiment amygdaloid kindling may have altered perirhinal function, resulting in impaired consolidation or retrieval of visual information needed for appropriate conditioned fear behavior. As these hippocampal and parahippocampal regions normally provide sensory information to the basolateral amygdala, a reduction in Fos expression with long-term kindling likely reflects deficient information transfer from these regions into the amygdala, resulting in poor contextual information processing. Interestingly, we also found decreased Fos expression in the lateral amygdala on the contralateral side of long-term kindled rats. Although it is difficult to explain the laterality of this effect, our results may suggest a general impairment of incoming sensory information or amygdala function during memory retrieval.

Lesion studies have also revealed that the cingulate cortex is required for trace conditioning but not delay conditioning (Han et al., 2003). We did not observe any differences in Fos immunoreactivity within the cingulate cortex. It is possible that long-term kindling is not of sufficient magnitude to produce deficits in cingulate cortex function that are comparable to lesions. Alternatively, a recent study found that inhibition of NMDARs via the NR2 antagonist APV in the retrosplenial cortex, but not the anterior cingulate cortex, selectively impaired conditioned freezing to a trace-conditioned context (Corcoran et al., 2011). Future studies should determine whether the reduction of contextual freezing following amygdaloid kindling is related to impaired retrosplenial cortex activation.

As seizures are known to dramatically shift the balance of excitatory and inhibitory networks, we wonder whether elevated levels of ambient GABA within the hippocampus might explain the reduction of hippocampal Fos during memory retrieval in rats subjected to trace conditioning. This possibility needs to be considered carefully, as a decrease in Fos does not necessarily indicate enhanced inhibition. However, there is evidence in the literature to support this idea. For example, kindling is known to increase GABA-mediated

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inhibition in the dentate gyrus (Gutierrez & Heinemann, 2001). In addition, there is evidence that kindling promotes a GABAergic phenotype in normally glutamatergic dentate granule cells (Gomez-Lira, Lamas, Romo-Parra, & Gutierrez, 2005), evidenced by elevations of GAD67 expression (Ramirez & Gutierrez, 2001). These general reductions of hippocampal excitability may impede activation of neurons necessary for the recall of contextually-relevant information acquired during fear learning. Indeed, we have recently shown that long-term kindling impairs the recruitment of adult-generated granule neurons during contextual fear memory retrieval in rats subjected to the trace conditioning protocol used in the present study (Fournier et al., 2013). As adult-generated neurons are known to play an important role in hippocampal-dependent learning and memory and display activity-dependent immediate early gene expression as early as 2-4 weeks after birth (Snyder et al., 2009), one intriguing possibility is that the deficits in conditioned freezing seen in the present experiment reflect aberrant neurogenesis in rats subjected to long-term kindling, but not short-term kindling (Fournier et al., 2010).

  Our Fos analyses of rats that underwent delay conditioning revealed a different pattern of Fos activation related to amygdala-dependent fear learning and memory. Our cell counts indicated that short- and long-term kindled rats had significantly fewer Fos+ cells within the dentate granule cell layer, hilus, the perirhinal cortex, the entorhinal cortex, and the lateral and basolateral nuclei of the amygdala. We found positive correlations between conditioned freezing and Fos immunoreactivity in the granule cell layer, hilus, perirhinal cortex, and lateral amygdala. These results confirm the reductions of Fos expression within hippocampal and parahippocampal structures involved in contextual memory retrieval as discussed above, but also reinforce the critical role of the lateral amygdala as the main source of sensory convergence to facilitate learning and memory after amygdala-dependent delay conditioning. The fact that short- and long-term kindled rats had reduced Fos expression bilaterally within the lateral amygdala suggests that this region becomes compromised during the early phases of kindling and contributes to the deficits seen in the present study.

  To our knowledge, the few studies that have previously investigated fear conditioning in epileptic rats have reported similar impairments in conditioned fear (Cardoso et al., 2009; Kemppainen et al., 2006; Szyndler et al., 2005). The novelty of our study lies in the fact we examined the effect of two forms of amygdaloid kindling (i.e., short and long-term kindling) on two different conditioning paradigms (i.e., delay and trace) and their relationship to a marker of

neuronal activity. We found that both short- and long-term kindling impaired retrieval of fear memories after delay conditioning, whereas only long-term kindling impaired retrieval of fear memories after trace fear conditioning. These results are valuable because they suggest that emotional circuits anchored by the amygdala are affected more rapidly by amygdaloid kindling than cognitive circuits anchored by the hippocampus. These findings therefore promote the use of kindling as a paradigm to identify the relationship between functional changes in amygdalar and hippocampal circuits and fear learning and memory. Indeed, several features of the kindling model make it a versatile tool for investigating behavior and cognition. First, the greatest expression of fear-related behavior occurs after kindling of the amygdala (Kalynchuk et al., 1998; Kalynchuk et al., 1998) as shown using tests such as resistance to capture from an unfamiliar open field (Fournier et al., 2009; Hannesson et al., 2008; Kalynchuk et al., 1997; Nieminen et al., 1992; Wintink et al., 2003), escape-related behaviors on the elevated plus maze (Kalynchuk et al., 1997) and fear-potentiated startle (Rosen, Hamerman, Sitcoske, Glowa, & Schulkin, 1996). In addition to altering fear-related behaviors, some studies have shown that amygdaloid kindling can impair spatial learning (Cammisuli et al., 1997; Sherafat et al., 2013) and memory (Beldhuis, Everts, Van der Zee, Luiten, & Bohus, 1992). Future studies could evaluate the effects of kindling different brain sites (i.e., hippocampus, caudate) on conditioned fear behavior to determine whether similar patterns of cognitive deficits and Fos expression are present. Second, the comorbidities produced by kindling are known to occur in a stimulation-dependent manner. Long-term kindling (i.e., 99 stimulations) produces more robust and reliable behavioral changes than partial or short-term kindling (i.e., <30 stimulations) (Kalynchuk, 2000). The stimulation-dependent effects of kindling were well illustrated in the present study. Third, kindled fear is greatest within a few days of the last kindling session, yet it can persist for several weeks (Hannesson et al., 2005; Kalynchuk et al., 1998), suggesting that alterations in fear behavior represent relatively permanent changes in limbic system function.

A caveat of the present study is that we examined Fos expression at only one point in time after retrieval of fear memories (i.e., 2 h). We chose a 2-h time point because previous work has indicated that this would capture maximal levels of Fos protein expression (Armario, 2006). It is possible that we may have observed a different pattern of Fos expression at 1 hr or 4 h after memory retrieval, but we believe that this is unlikely given that long-term kindled rats show decreased hippocampal and perirhinal cortex Fos expression approximately 30 min after a period

of open field exploration (Kalynchuk et al., 2001). However, we suggest that future studies could investigate other markers of neural activity, such as Arc or *Zif268*, as these proteins have different induction times and can be recruited by different neuronal populations (Lonergan, Gafford, Jarome, & Helmstetter, 2010). Such studies could provide additional insight into the relationship between neural activity markers and fear memory retrieval following kindling stimulations.

2.5. Conclusions

Amygdala kindling significantly disrupted conditioned fear behavior. These disruptions were selective to kindling treatment and protocol. Specifically, short- and long-term amygdala kindling disrupted conditioned freezing in rats subjected to delay conditioning, whereas trace conditioning was only impacted following long-term kindling. Disruption of fear conditioning coincided with a general reduction of the neuronal activity marker Fos in hippocampal, parahippocampal, and amygdalar circuits related to the conditioning protocol. Amygdala kindling appears to promote functional changes within neural circuits involved in emotionally salient learning and memory.

Acknowledgements

CHAPTER 3

Limbic but not non-limbic kindling impairs conditioned fear and promotes plasticity of NPY and its Y2 receptor

J.J. Botterill [a], A.J. Guskjolen [a], W.N. Marks [a], H.J. Caruncho [b], L.E. Kalynchuk [c]

[a] Department of Psychology, University of Saskatchewan, Saskatoon, SK, S7N 5A5, Canada

[b] College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, S7N 5A5, Canada

[c] Department of Medicine, University of Saskatchewan, Saskatoon, SK, S7N 5E5, Canada

3.1 Introduction

Seizures have a profoundly negative impact on cognition. However, the neural mechanisms that underlie seizure-induced alterations in cognition are unclear. Interestingly, seizures are known to alter expression of a number of GABAergic interneuron subtypes within the hippocampus (Schwarzer et al., 1995; Sperk et al., 1992). In particular, neuropeptide Y (NPY), a peptide preferentially expressed in a subset of GABAergic interneurons, undergoes robust plasticity in the hippocampus after seizures (Vezzani et al., 1999). Under basal conditions, hippocampal NPY is localized to GABAergic interneurons that primarily inhibit granule and hilar neurons. However, after recurrent seizures, hippocampal NPY expression is dramatically increased in granule cell mossy fibers in both rodents and humans (Furtinger et al., 2001; Vezzani et al., 1999). Similarly, the NPY Y2 receptor (NPY2R), which presynaptically inhibits glutamate release, also undergoes *de novo* expression in mossy fibers and granule neurons (Furtinger et al., 2001; Gobbi et al., 1998; Schwarzer et al., 1998). These changes to the NPY system can be long lasting (i.e., > 6 months) and are thought to represent an anticonvulsant mechanism (Vezzani & Sperk, 2004). Indeed, NPY-deficient mice display increased susceptibility to seizure activity (Baraban et al., 1997), whereas NPY and the NPY2R attenuate epileptic after discharges (Colmers & El, 2003; Noe et al., 2010; Woldbye et al., 2010) and protect against excitotoxicity (Silva et al., 2003). Although NPY-mediated presynaptic blockade of glutamate release appears to be a compensatory mechanism to limit the future occurrence of seizures (Vezzani et al., 1999), relatively little is known about its effects on cognition (Redrobe, Dumont, St-Pierre, & Quirion, 1999). It is possible that interactions between NPY and the NPY2R in the epileptic brain could inadvertently limit glutamate-dependent information processing and cognition that is mediated by the same hippocampal circuits that are affected by seizures.

To investigate this issue, we used the kindling model of temporal lobe epilepsy (TLE). Kindling refers to the gradual development and intensification of elicited motor seizures resulting from daily electrical stimulation of a discrete brain site (Goddard et al., 1969). Kindling has been widely used to study the pathophysiology of epilepsy, but it has also been recognized as a valuable model for understanding the behavioral comorbidities and cognitive deficits associated with epilepsy (Kalynchuk, 2000). Previous research has shown that kindling of limbic brain sites (i.e., basolateral amygdala, BLA; dorsal hippocampus, dHip) impairs cognition and

enhances unconditioned fear behaviors in tasks such as the open field, elevated plus maze, and Morris water maze (Fournier et al., 2009; Fournier et al., 2013; Hannesson et al., 2001; Hannesson et al., 2008; Helfer, Deransart, Marescaux, & Depaulis, 1996; Kalynchuk et al., 1997; Kalynchuk et al., 1999; Nieminen et al., 1992). In contrast, kindling of non-limbic brain sites (i.e., caudate nucleus, CN) generally has little to no effect on unconditioned fear (Kalynchuk et al., 1998). These behavioral dissociations presumably reflect changes in limbic function that do not occur with non-limbic kindling.

In this experiment, we took advantage of the site-specific effects of kindling on behavior to determine whether seizure-induced alterations in NPY are related to seizure-induced deficits in cognition. We have recently shown that kindling of the BLA dramatically impairs the retrieval of conditioned fear memories (Botterill et al., 2014; Fournier et al., 2013). However, nothing is known about the effects of dHip or CN kindling on this form of learning and memory. Based on previous research that dissociates the effects of limbic and non-limbic kindling on unconditioned fear (e.g., (Kalynchuk et al., 1998), we hypothesized that limbic kindling would impair memory of conditioned fear associations, whereas non-limbic kindling would not. Further, if NPY plays a role in the cognitive deficits associated with recurrent seizures, we should also see enhanced hippocampal NPY in limbic-kindled rats but not non-limbic kindled rats. Within 24 h of the final kindling stimulation, rats kindled through the amygdala, hippocampus, or caudate nucleus progressed through a 4-day hippocampal-dependent trace fear-conditioning paradigm. To determine the relationship between contextual fear memory retrieval and neural activity within the hippocampus, we assessed the presence of Fos protein, which is a widely accepted marker of behaviorally-relevant neuronal activity (Guzowski et al., 2005). In addition, to determine the relationship between NPY plasticity and cognition, we assessed immunolabelling of NPY and the NPY2R in areas of the hippocampus known to play a role in cognition.

3.2 Materials and Methods

3.2.1 Animals

We used male Long-Evans rats that were purchased from Charles River (Quebec, Canada). They weighed approximately 250g at the time of arrival. Rats were individually housed and given access to food and water ad libitum. The housing room was maintained on a 12 h

light-dark cycle. All experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the University of Saskatchewan Committee on Animal Care and Supply. We made all possible efforts to minimize the number of animals used. A total of three rats were removed from the study due to incorrect electrode placement or head cap loss during kindling.

3.2.2 Surgery and Kindling

Surgery and kindling was conducted as previously described (Fournier et al., 2013). Briefly, we used bregma coordinates at a flat skull position to stereotaxically implant a single bipolar stimulating electrode (MS-303-2-B-SPC, Plastics One, Roanoke, VA, USA) into the CN (+0.2 mm anteroposterior, +3.2 mm mediolateral, -5.7 mm dorsoventral), BLA (-2.8 mm anteroposterior, +5.0 mm mediolateral, -8.5 mm dorsoventral) or dHip (-3.5 mm anteroposterior, +2.6 mm mediolateral, -3.1 mm dorsoventral) in the left hemisphere of each rat (Paxinos & Watson, 1998). The electrode assembly was secured to the skull with jeweller screws (0 - 80 X 3/32, Plastics One) and dental acrylic. Rats received a post-surgical recovery of 1 week and were then randomly assigned to one of four groups: CN kindling ($n = 8$), BLA kindling ($n = 6$), dHip kindling ($n = 7$) or control ($n = 7$). All rats from each treatment group were included in all measures, unless mentioned otherwise. For each kindling stimulation, a wire lead connected to an isolated pulse stimulator (Model 2100, A-M Systems, Sequim, WA, USA) was attached to the electrode assembly to deliver a 1s, 60 Hz train of square-wave pulses with a biphasic amplitude of 800 µA (peak-to-peak) and duration of 1 ms. A total of 99 electrical stimulations were delivered on a 3X per day, 5 day per week schedule, with a minimum of 3 h between consecutive stimulations. Control rats received sham stimulations, which were the same as the kindling stimulations except that no current was passed through the wire lead. The convulsion elicited by each stimulation was scored using a revised eight class extension (Pinel & Rovner, 1978) of Racine's original five class scale (Racine, 1972). The classes were operationally defined as: Class 0: immobility, Class 1: orofacial automatisms, Class 2: orofacial automatisms with head nodding, Class 3: unilateral forelimb clonus, Class 4: rearing with bilateral forelimb clonus, Class 5: rearing with bilateral forelimb clonus followed by falling, Class 6: multiple class 5 convulsions and falling episodes, Class 7: previous classes with running fit, and Class 8:

previous classes with intermittent muscle tonus. Using this classification system, rats are considered to be fully "kindled" after 3 consecutive class 5 convulsions (Pinel & Rovner, 1978).

3.2.3 Trace Fear Conditioning

One day after the final kindling stimulation, all rats underwent a trace fear conditioning protocol that has been described previously (Botterill et al., 2014). Two identical sound-attenuated operant chambers (VFC-008, Med Associates Inc, St. Albans, VT, USA) were used for all fear conditioning manipulations. Each chamber was equipped with a miniature monochromatic video camera and VideoFreeze software (Med Associates Inc), which provided live observations and video recordings sampled at 30 frames per second for offline analyses. The chambers had aluminum walls and a clear Plexiglas front door and ceiling. Within the chamber, the grid floor had 19 aluminum rods that were connected to a shock generator (ENV-414S, Med Associates Inc). Auditory stimuli were delivered through a loudspeaker mounted in the chamber wall (ANL-926, Med Associates). An interface cabinet (Dig-700 F, Med Associates Inc) controlled tone and shock presentations. Operant chamber ventilation fans provided constant background noise (60 db) during the task. The chambers were cleaned thoroughly with 0.6% (v/v) acetic acid between subjects unless mentioned otherwise.

Our primary behavioral measure was freezing, operationally defined as the absence of motor movements except those necessary for respiration. Freezing behavior was scored every 2s during tone presentations and every 4 s in the absence of tone. These observations were transformed into the percentage of time spent freezing by summing the total number of freezing observations together and dividing this value by the total number of observations for each period and then multiplying the resultant number by 100.

The fear conditioning task proceeded as follows. On day 1, the habituation day, each rat was placed in an operant chamber for 10 min. During this time we measured midline crosses to evaluate if kindling altered baseline ambulatory activity. On day 2, the training day, each rat was placed back into the same operant chamber. Rats received a 180 s acclimation period followed by 7 CS-US pairings. Each pairing comprised a 16 s auditory tone (85 dB, 2 kHz), followed by a 30 s trace interval that co-terminated with a 2 s footshock (0.9 mA). The inter-trial interval was 180 s between tone onsets. On day 3, the tone test, rats were transported to a contextually novel conditioning room and placed inside a novel operant chamber scented with dilute vanilla extract.

Freezing behavior was assessed across 4 tone presentations (16 s each). The inter-trial interval was 198 s between tone onsets. The chambers were thoroughly cleaned with 0.064 % NaOH between subjects. On day 4, the context test, rats were returned to the same operant chamber they were trained in for 480 s and freezing to contextual cues was assessed. No footshocks or tones were delivered during the context test.

3.2.4 Histology

3.2.4.1 Perfusions

Approximately 90 min after completion of the context test, each rat was deeply anesthetized with sodium pentobarbital (240 mg/kg, i.p.) and transcardially perfused with room temperature saline followed by ice cold 4 % (w/v) formaldehyde fixative (pH = 7.4). The brains were removed and stored in the same fixative for 48 h at 4 °C prior to coronal sectioning (50 µm) on a vibrating microtome (Vibratome 3000, Vibratome Company, St. Louis, MO, USA). Sections were stored at -20 °C in a cryoprotectant solution comprised of 30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, and 30% (v/v) ethylene glycol in 0.1 M PBS (pH = 7.4) until use.

3.2.4.2. Immunohistochemistry

We used a standard immunohistochemistry technique to assess the presence of Fos-immunoreactive (ir) neurons, the presence of NPY-ir interneurons, and the immunoreactivity of NPY. We used immunofluorescence to assess the expression of the NPYR2. We selected widely used and commercially available antibodies. The Fos antibody selectively recognizes the Fos protein, but not other Fos-family related proteins (Giorgi et al., 2008) and has been widely used in the literature (Botterill et al., 2014; Hale et al., 2008; Snyder et al., 2009). NPY is a synthetic peptide with 100 % cross reactivity with NPY (human, rat, porcine) and 0 % cross reactivity with PYY/VIP. This antibody has also been widely used in the literature (Cardoso, Freitas-da-Costa, Carvalho, & Lukoyanov, 2010; Huusko et al., 2013; Ramamoorthy & Whim, 2008; Tu, Timofeeva, Jiao, & Nadler, 2005). Specificity of the NPY2R antibody has been shown with Y2R knockout mice, hippocampal membrane fractions, and pre-absorption with cognate peptide (Stanic et al., 2006).

Immunostaining was conducted on free-floating sections in 6-well tissue culture plates. All tissue was processed in unison for each experiment (i.e., all sections for Fos at once) to keep the incubation periods and visualization steps constant for all animals. For each study, we also counterbalanced sections from each treatment group across all 6-well plates to ensure consistent immunohistochemical processing. Furthermore, to confirm the specificity of the antibodies, a well of free-floating sections that did not receive the primary antibody served as the immunoreaction control for each study. We did not observe any Fos, NPY, or NPY2R immunoreactivity in the absence of the primary antibody.

All rinses and incubations were conducted in 0.1 M Tris buffered saline (TBS, pH = 7.4). Sections were rinsed in 0.3 % H_20_2 for 30 min to block endogenous peroxidase activity (Fos, NPY), subjected to antigen retrieval in sodium citrate buffer (pH = 6.0) at 85 °C for 30 min (NPY, NPY2R), and then blocked in 5 % normal animal serum with 0.3 % Triton X-100. Sections were then incubated in anti-rabbit primary antibodies (1:15,000 Fos, #PC38, Calbiochem, La Jolla, CA, USA; 1:1,000 NPY, #T-4070, Peninsula Laboratories, San Carlos, CA, USA; 1:300 NPY2R, #RA-14112, Neuromics, Edina, MN, USA) in blocking solution for 48 (NPY) or 72 h (Fos, NPY2R) at 4 °C. Fos and NPY sections were then incubated in biotinylated goat anti-rabbit IgG secondary antibody (1:500,Vector Laboratories, Burlingame, CA, USA) and treated with avidin-biotin-peroxidase complex (1:500, Vector Laboratories). Immunoreactivity of Fos was visualized with 2.5% nickel-enhanced DAB (0.02%). Immunoreactivity of NPY was visualized using the glucose oxidase DAB (0.02%) method (Scharfman, Goodman, Sollas, & Croll, 2002). For the NPY2R, sections were incubated in Alexa-568 (1:300, Molecular Probes-Invitrogen, Eugene, OR, USA) secondary antibody. Sections were mounted onto glass slides and left to dry overnight. For bright field microscopy, sections were dehydrated in a graded alcohol series, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA). To assist with cell counting, a series of NPY sections were counterstained with 0.1% (w/v) cresyl violet acetate (Acros Organics, Fair Lawn, NJ, USA). For immunofluorescence, sections were coverslipped with Citifluor antifade mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA).

To determine whether 99 kindling stimulations promotes apoptosis and neuronal degeneration, we stained a subset of tissue with Fluoro-Jade B (Millipore, Bedford, MA, USA) as previously described (Schmued & Hopkins, 2000). Briefly, sections were mounted onto

microscope slides and heated for 30 min at 50 ° C. Sections were then placed in a slide rack and immersed in 100 % EtOH for 5 min, followed by 70 % EtOH for 2 min. Slides were then rinsed with distilled water and immersed in a solution containing 0.06 % potassium permanganate for 15 min under gentle agitation. Excess potassium permanganate solution was rinsed off in distilled water for 2 min. Slides were then immersed in a solution containing 0.001 % Fluoro-Jade B solution in 0.1 % glacial acetic acid for 30 min. Slides were rinsed three times with distilled water and allowed to dry overnight. The next day, slides were immersed in xylene three times for 2 min each. Slides were then coverslipped with Permount mounting medium (Fisher Scientific). For the Fluoro-Jade B study, we also included tissue from rats ($n = 5$) that received a standard dose of lithium chloride (3 mEq/kg, sc) and pilocarpine (30 mg/kg, sc). Pilocarpine treated rats were added to this experiment specifically to demonstrate and compare neuronal death-apoptosis in kindling versus status epilepticus models.

3.2.5 Quantification

3.2.5.1 Cell counting

A researcher blind to the treatment conditions conducted all quantitative analyses. Immunostained sections were examined using a Nikon Eclipse E800 microscope equipped with a motorized stage and digital camera (MicroFire, Optronics, Goleta, CA, USA) that was connected to a computerized stereology system (StereoInvestigator V 9.0, MicroBrightfield Inc, Williston, VT, USA). The number of Fos-ir and NPY-ir cells were analyzed bilaterally across 5 sections per brain with 300 µm between sections. All counts were done in similar anterior-posterior coronal planes (i.e., ranging between -2.52 to -5.60mm, relative to Bregma) across subjects to allow for more accurate cell count comparisons. Due to previous reports indicating stimulation-side specific effects of kindling (Fournier et al., 2010), we analyzed both the ipsilateral (i.e., left) and contralateral hemispheres for all experiments. Fos immunoreactivity was evaluated in the granule cell layer, hilus, CA3 and CA1 stratum pyramidale. To determine the effect of kindling on the number of NPY-ir cells, we analyzed several hippocampal subfields: the subgranular zone, hilus, stratum oriens, radiatum, and CA3 and CA1 pyramidal cell layer, and the CA3 stratum lucidum. The subgranular zone was defined as the area approximately two cell widths wide bordering the hilus and granule cell layer. NPY-ir cells in the granule cell layer were

mostly located in this area, and these cells were included in the subgranular zone counts. The granule cell layer was not counted separately because it contained very few NPY-ir cells. For each section, we traced contours within the boundaries of each region of interest at 4X magnification using the brain atlas as a guide (Paxinos & Watson, 1998). Cells were counted at 40X magnification using a meander scan profile counting method (Knapska & Maren, 2009). This procedure uses an automated scanning method that allows the user to view and count all cells within the traced contour. To determine the density of immunolabeled cells, we divided the total number of Fos-ir or NPY-ir cells in each region of interest by the total area measurement of traced contours (in μm). Cell counts are therefore represented as the average number of cells per mm^2.

3.2.5.2 Optical densitometry

Semiquantitative optical densitometry of NPY immunoreactivity was conducted on 3 sections per brain with 300 μm between sections using methods previously described (Fournier et al., 2009). With exposure and gain settings held constant for all images, the mean optical density of each region was calculated using ImageJ software (V1.46R, National Institutes of Health, Bethesda, MD, USA). Background staining was controlled by subtracting the mean optical density of the corpus callosum from each region of interest. Values were standardized between white (0) and black (255) and expressed as the percentage change from controls. For the NPY2R quantification, we evaluated fluorescence signal intensity on three randomly selected subjects per treatment condition. Fluorescent images were captured in monochrome with exposure and gain settings held constant. Optical densitometry of the monochrome fluorescent signal was measured bilaterally on two sections per rat. Due to the difficulty in discriminating fluorescent signal intensity between the borders of the subgranular zone–hilus and CA3 stratum pyramidale-lucidum regions, we combined our analyses within these regions. Values obtained in these analyses were also expressed as the percentage change from controls.

3.2.6 Statistics

All statistical analyses were conducted using IBM's Statistical Package for Social Sciences (SPSS v. 20). Group differences were analyzed using one-way analysis of variance

followed by Tukey HSD post hoc tests when appropriate. The criterion for statistical significance was set at $p < 0.05$ for all analyses. All graphs depict the mean ± the SEM. We initially conducted post-mortem analyses on the ipsilateral and contralateral hemispheres for all experiments to determine if there were stimulation-side specific effects of kindling. These initial analyses revealed minimal within-group differences between the ipsilateral and contralateral hemisphere and consistent between-group differences for each hemisphere. Furthermore, we also assessed whether any of our results varied along the dorsal or ventral segments of the hippocampus. These analyses also revealed minimal within-group dorsal-ventral differences and consistent between-group differences for both dorsal and ventral hippocampus. Therefore, we combined both the ipsilateral/contralateral and dorsal/ventral measurements throughout the hippocampus to facilitate the analysis and interpretation of our data.

3.3. Results

3.3.1 Kindling Progression

Kindling progressed normally in all rats. The first class 5 convulsion was elicited after 12.33 ± 2.37 stimulations for the BLA-kindled rats and 47.57 ± 5.60 stimulations for the dHip-kindled rats and the fully kindled state was achieved after 21.00 ± 6.49 stimulations for the BLA-kindled rats and 52.42 ± 6.32 stimulations for the dHip-kindled rats. The total number of class 5 or higher convulsions was 75.66 ± 7.65 for the BLA-kindled rats and 45.00 ± 6.22 for the dHip-kindled rats. In contrast to BLA and dHip kindling, electrical stimulation of the CN produced seizures characterized by rapid falling to one side and brief whole-body tonus (Kalynchuk et al., 1998). Near the completion of kindling, 2 of the 8 CN kindled rats displayed mild limbic convulsions (i.e., unilateral forelimb clonus) suggesting some generalization was occurring. Consistent with earlier reports (Fournier et al., 2010; Pinel & Rovner, 1978), we did not observe spontaneous seizures at any point during our experiment.

3.3.2 Limbic kindling impairs retrieval of fear memories despite normal acquisition of hippocampal-dependent fear conditioning

After the completion of kindling, we assessed acquisition and retrieval of fear memory in a hippocampal-dependent task. On the habituation day, we found a significant main effect of

kindling on baseline ambulatory activity $F(3,27) = 7.70$; $p < 0.001$, with post hoc analyses showing that the dHip-kindled rats engaged in more midline crosses than the control or CN-kindled rats (all p values < 0.005; Fig. 3-1A). On the training day we found no differences in baseline freezing during the 3 min acclimation period prior to training $F(3,27) = 0.094$; $p = 0.963$ (Fig. 3-1B). All groups successfully learned the fear association, indicated by increased levels of freezing with successive CS-US pairings. We found no group differences in freezing during the CS presentations $F(3,27) = 0.797$; $p = 0.507$, the trace intervals $F(3,27) = 2.467$; $p = 0.09$ or the post-shock periods $F(3,27) = 1.916$; $p = 0.154$. In contrast, we found significant group differences on the tone test day in the percent time spent freezing during the CS presentations $F(3,27) = 12.654$; $p < 0.001$ and during the post-CS periods $F(3,27) = 15.968$; $p < 0.001$. Post hoc analyses revealed that the BLA and dHip-kindled rats froze significantly less than the control and CN-kindled rats in both cases (all p values < 0.015; Fig. 3-1C). On the context test day, we also found significant group differences in freezing $F(3,27) = 7.895$; $p = 0.001$, with post hoc analyses showing that the BLA and dHip-kindled rats froze less than the control rats (all p values < 0.031; Fig. 3-1D). Further, the BLA-kindled rats also froze less than the CN-kindled rats ($p = 0.013$). The CN-kindled rats did not differ from the control rats on any behavioral measure (all p values > 0.08).

3.3.3 Limbic-kindled rats have significantly fewer Fos-ir cells after memory retrieval

We next determined the effect of kindling on the activity-dependent marker Fos following fear memory retrieval. We found significant group differences in Fos expression in the granule cell layer $F(3,27) = 32.559$; $p < 0.001$, hilus $F(3,27) = 8.995$; $p < 0.001$, CA3 pyramidal cell layer $F(3,27) = 9.506$; $p < 0.001$, and CA1 pyramidal cell layer $F(3,27) = 9.649$; $p < 0.001$ (Fig. 3-2). Post hoc analyses revealed that the dHip-kindled rats had significantly fewer Fos-ir cells than the control and CN-kindled rats in all of these regions (all p values < 0.005). Similarly, the BLA-kindled rats had significantly fewer Fos-ir cells than the control and CN-kindled rats in the granule cell layer and hilus (all p values < 0.015). Further, the BLA-kindled rats had significantly fewer Fos-ir cells than the CN-kindled rats in the CA3 and CA1 regions (all p values < 0.031). There were no significant differences between the CN-kindled and control rats in any region (all p values > 0.68).
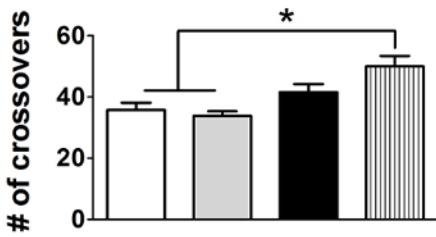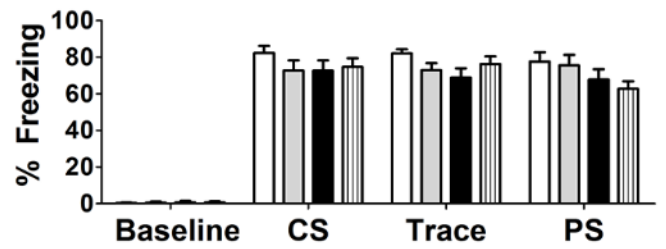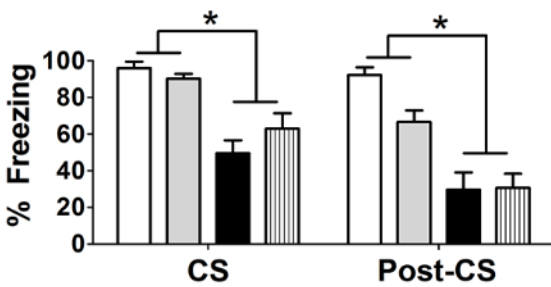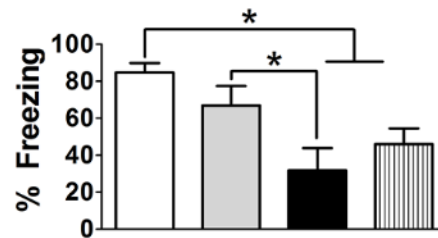
Figure 3-1. Limbic-kindled rats display impaired conditioned fear following trace fear conditioning. **(A)** dHip-kindled rats engaged in significantly greater midline crosses than control and CN-kindled rats during habituation to the operant chambers. **(B-D)** During the training period, we found no significant group differences in baseline or conditioned freezing during tone presentations (CS), the trace period, or post-shock intervals (PS), indicating acquisition was normal for all groups. However, the BLA and dHip-kindled rats displayed significantly less freezing than the control rats during subsequent **(C)** tone and **(D)** context memory tests. They also froze less than the CN-kindled rats during the tone test and the BLA-kindled rats froze less than the CN-kindled rats during the context test. *$p < 0.05$
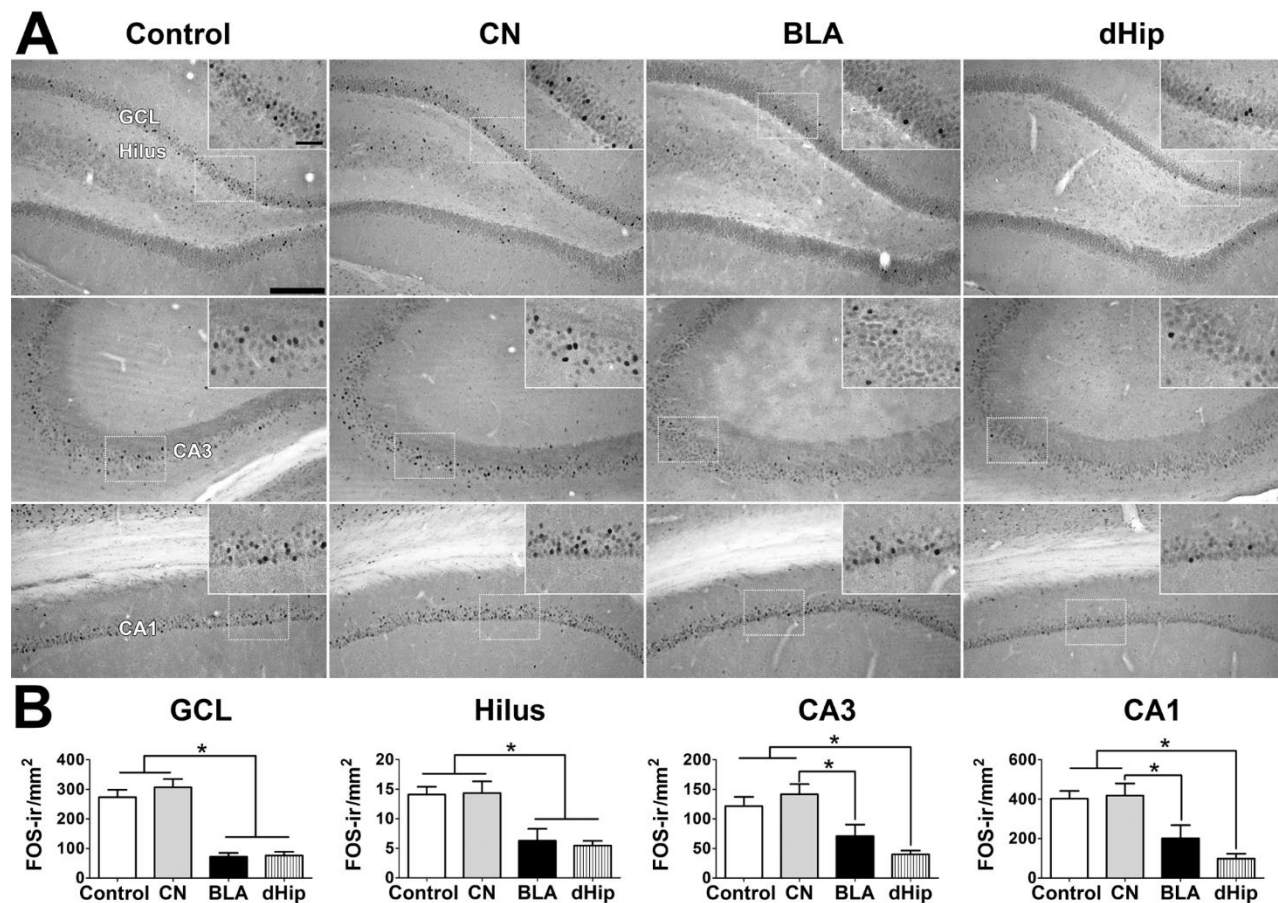
Figure 3-2. Limbic-kindled rats have fewer hippocampal Fos-ir cells following contextual fear memory retrieval. **(A)** Representative photomicrographs of Fos expression in the dentate gyrus, CA3, and CA1 from rats in each group (Scale bar = 200μm). Insets show high power magnification (Scale bar = 50μm). **(B)** Quantitative cell counts. The BLA and dHip-kindled rats had significantly fewer Fos-ir cells in the granule cell layer and hilus compared to controls. Further, the dHip-kindled rats had significantly fewer Fos-ir cells in the CA3 and CA1 than controls. The BLA and dHip-kindled rats had significantly fewer Fos-ir cells in the granule cell layer, hilus, CA3, and CA1 compared to CN-kindled rats. Importantly, the CN-kindled rats did not differ from controls in any region. $*p < 0.05$

3.3.4 Limbic kindling promotes plasticity of NPY following chronic seizures

Next, we determined whether kindling altered the number of detectable NPY-ir cells in hippocampal subfields. We found significant group differences in the number of NPY-ir cells in the subgranular zone $F(3,27) = 67.880$; $p < 0.001$, hilus $F(3,27) = 38.970$; $p < 0.001$, CA3 stratum oriens $F(3,27) = 32.047$; $p < 0.001$, CA3 stratum radiatum $F(3,27) = 12.536$; $p < 0.001$, CA3 stratum lucidum $F(3,27) = 48.388$; $p < 0.001$, CA3 stratum pyramidale $F(3,27) = 44.162$; $p < 0.001$, CA1 stratum oriens $F(3,27) = 3.743$; $p = 0.024$ and CA1 stratum radiatum $F(3,27) = 4.129$; $p = 0.017$ (Fig. 3-3). Post hoc analyses revealed that the BLA and dHip-kindled rats had significantly fewer NPY-ir cells than the control and CN-kindled rats within the subgranular zone, hilus, and CA3 stratum oriens, radiatum, lucidum, and pyramidale (all p values < 0.01). Further, the dHip-kindled rats had significantly fewer NPY-ir cells than the BLA-kindled rats in the subgranular zone and hilus (all p values < 0.04). Within the CA1 stratum oriens, post hoc analyses revealed that the BLA-kindled rats had significantly more NPY-ir cells than the CN-kindled rats ($p = 0.023$). Within CA1 stratum radiatum, the dHip-kindled rats had significantly fewer NPY-ir cells than the control rats ($p = 0.012$). Finally, the CN-kindled rats had significantly fewer NPY-ir cells than the control rats within the subgranular zone and CA3 stratum pyramidale (all p values < 0.012). No other group differences were statistically significant (all p values > 0.07). While counting NPY-ir cells, we included a small percentage of cells with perinuclear cytoplasmic staining in the analyses.

3.3.5 Limbic kindling increases hippocampal NPY immunoreactivity

While counting NPY-ir cells, we noticed qualitative differences in the level of NPY immunoreactivity in the neuropil, including the mossy fiber terminals. To investigate these qualitative observations, we ran a second series of NPY immunohistochemistry without cresyl violet counterstain and measured optical density of the NPY-immunoreaction product within these sections (Fig. 3-4). Our analysis revealed significant group differences in NPY-ir in the subgranular zone $F(3,27) = 68.818$; $p < 0.001$, hilus $F(3,27) = 23.776$; $p < 0.001$, molecular layer $F(3,27) = 4.400$; $p < 0.013$, stratum-lacunosum molecular $F(3,27) = 8.274$; $p < 0.001$, CA3 stratum lucidum $F(3,27) = 35.391$; $p < 0.001$ and stratum pyramidale $F(3,27) = 5.369$; $p < 0.006$, and CA1 stratum oriens $F(3,27) = 3.088$; $p < 0.046$ and stratum radiatum $F(3,27) = 4.116$; $p <$
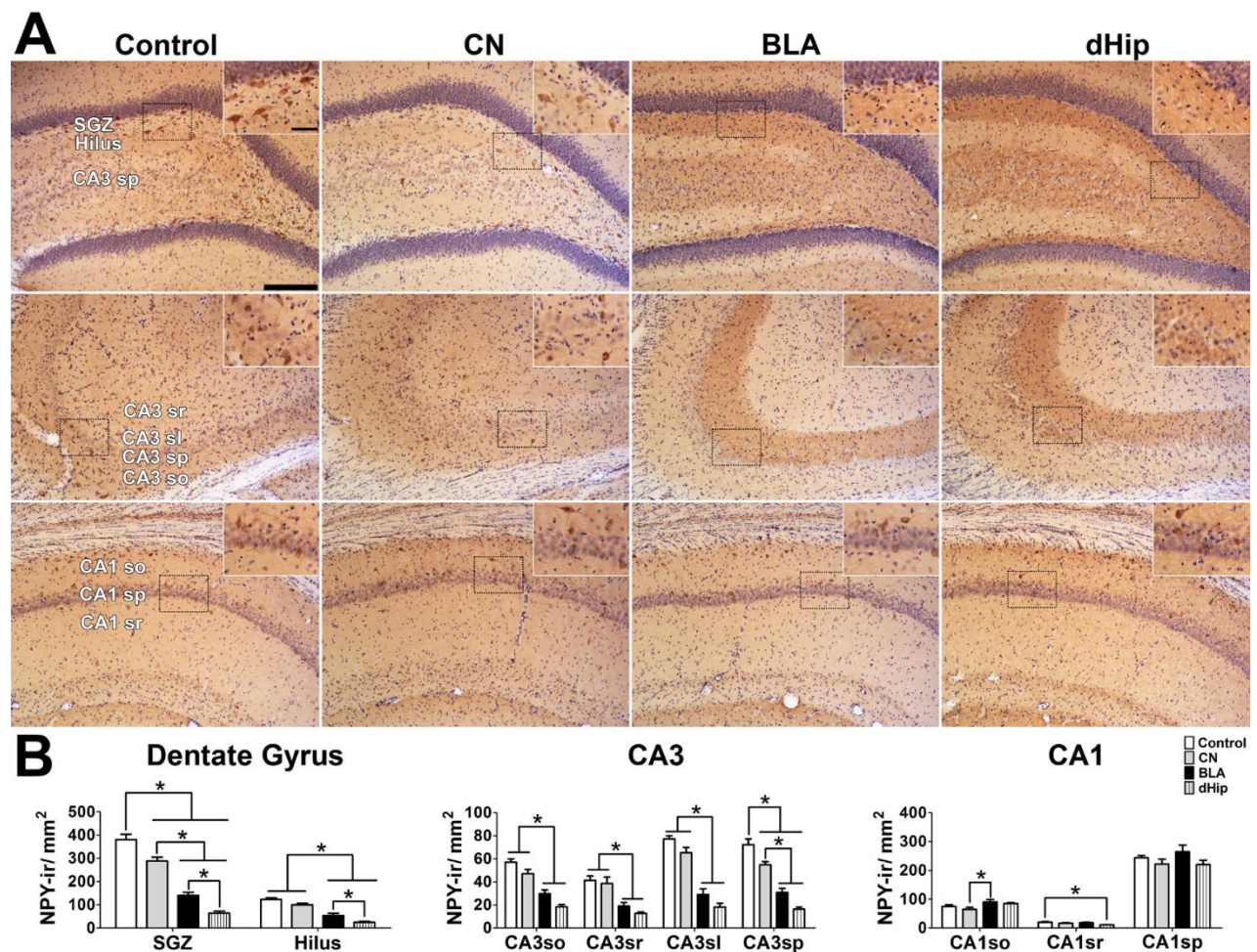
Figure 3-3. Limbic-kindled rats have significantly fewer detectable NPY-ir cells in several hippocampal subfields. **(A)** Representative photomicrographs of the dentate gyrus, CA3, and CA1 regions from each group (Scale bar = 200µm). Insets are high power magnification (Scale bar = 50µm). **(B)** Quantitative cell counts. The BLA and dHip-kindled rats had significantly fewer NPY-ir cells in the dentate gyrus and all CA3 subfields, relative to control and CN-kindled rats. Further, the dHip-kindled rats had significantly fewer NPY-ir cells than controls within the CA1sr and the BLA-kindled rats had significantly more NPY-ir cells than CN-kindled rats in the CA1so. Finally, the CN-kindled rats had significantly fewer NPY-ir cells than controls within the SGZ and CA3sp. SGZ, subgranular zone; SO, stratum oriens; SR, stratum radiatum; SL, stratum lucidum; SP, stratum pyramidale. *$p < 0.05$

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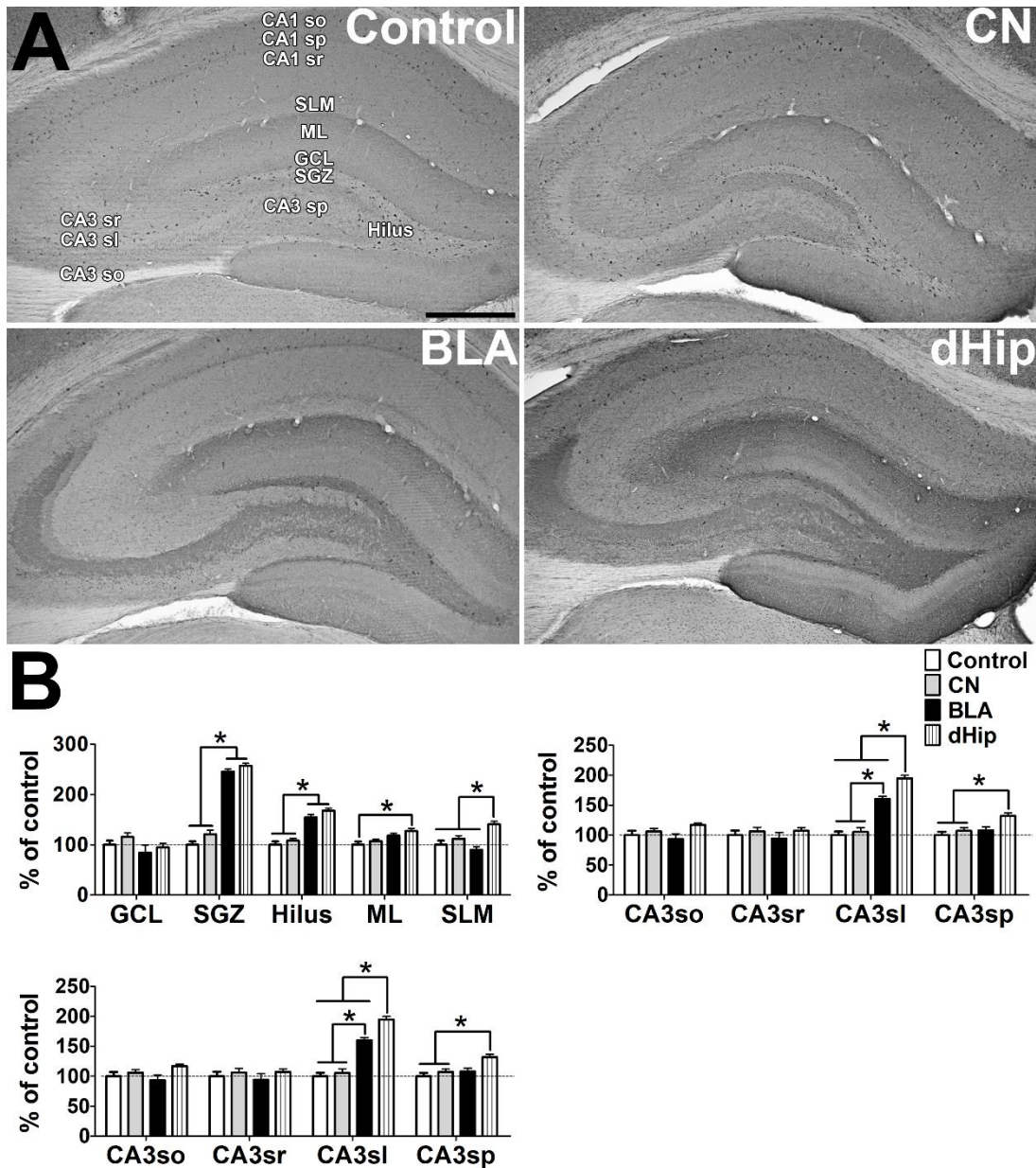
Figure 3-4. Limbic kindling promotes robust hippocampal NPY immunoreactivity. **(A)** Representative photomicrographs of hippocampal NPY immunoreactivity across the groups (Scale bar = 500μm). **(B)** Semiquantitative densitometry values normalized to percentage of controls. The BLA and dHip-kindled rats had significantly more NPY immunoreactivity than the CN-kindled and control rats within the SGZ, hilus, and CA3sl. Further, the dHip-kindled rats had more NPY immunoreactivity than the control rats in the ML and CA1so, than the rats in all other groups in the SLM, than the BLA-kindled rats in the CA3sl, than the control and CN-kindled rats in the CA3sp, and than the control and BLA-kindled rats in the CA1so. The CN-kindled rats were not significantly different from controls in any region. GCL, granule cell layer; SGZ, subgranular zone; ML, molecular layer; SLM, stratum-lacunosum molecular; SO, stratum oriens; SR, stratum radiatum; SL, stratum lucidum; SP, stratum pyramidale. *$p < 0.05$

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0.017. Post hoc analyses revealed that the BLA and dHip-kindled rats had significantly greater NPY immunoreactivity than the control and CN-kindled rats within the subgranular zone, hilus, and CA3 stratum lucidum (all p values < 0.001). Further, the dHip-kindled rats had significantly greater NPY immunoreactivity than the control rats in the molecular layer and CA1 stratum oriens (all p values < 0.05), than all groups in the stratum-lacunosum molecular (all p values < 0.043), than the control and CN-kindled rats in the CA3 stratum pyramidale (all p values < 0.029), than the control and BLA-kindled rats in the CA1 stratum radiatum (all p values < 0.034), and than the BLA-kindled rats in the CA3 stratum lucidum ($p = 0.026$). No other group differences were found (all p values > 0.09).

3.3.6 *De novo* expression of NPY2R following limbic kindling

Finally, we assessed the effect of kindling on hippocampal NPY2R expression (Fig. 3-5). Our photomicrographs clearly show an increase of NPY2R immunolabelling in the dentate gyrus and CA3 of limbic-kindled rats compared to control and CN-kindled rats (i.e., Fig. 3-5D-G). Although we saw some NPY2R immunolabelling in the granule cell layer and pyramidal cell layers for all subjects, we also found significant NPY2R immunolabelling in the neuropil and mossy fiber terminals of limbic-kindled rats. In contrast, the control and CN-kindled rats had minimal immunolabelling in the neuropil and mossy fiber terminals. Our analysis confirmed these qualitative observations. Indeed, we found significant group differences in NPY2R immunofluorescence labelling within the granule cell layer $F(3,11) = 21.077$; $p < 0.001$, subgranular zone/hilus $F(3,11) = 14.293$; $p < 0.001$ and CA3 stratum pyramidale-lucidum $F(3,11) = 10.866$; $p = 0.003$, but not the CA1 stratum pyramidale $F(3,11) = 0.040$; $p = 0.989$. Post hoc analyses revealed that the BLA and dHip-kindled rats had significantly greater NPY2R immunofluorescence than the control or CN-kindled rats in the granule cell layer, subgranular zone/hilus and CA3 stratum pyramidale-lucidum (all p values < 0.037).

3.3.7 Kindled rats show little Fluoro-Jade B staining

Given the decrease on the number of detectable NPY-ir neurons in the limbic-kindled rats, we assessed whether kindling enhances neuronal degeneration or apoptosis within the hippocampus. To our knowledge, no studies have investigated Fluoro-Jade B staining after long-

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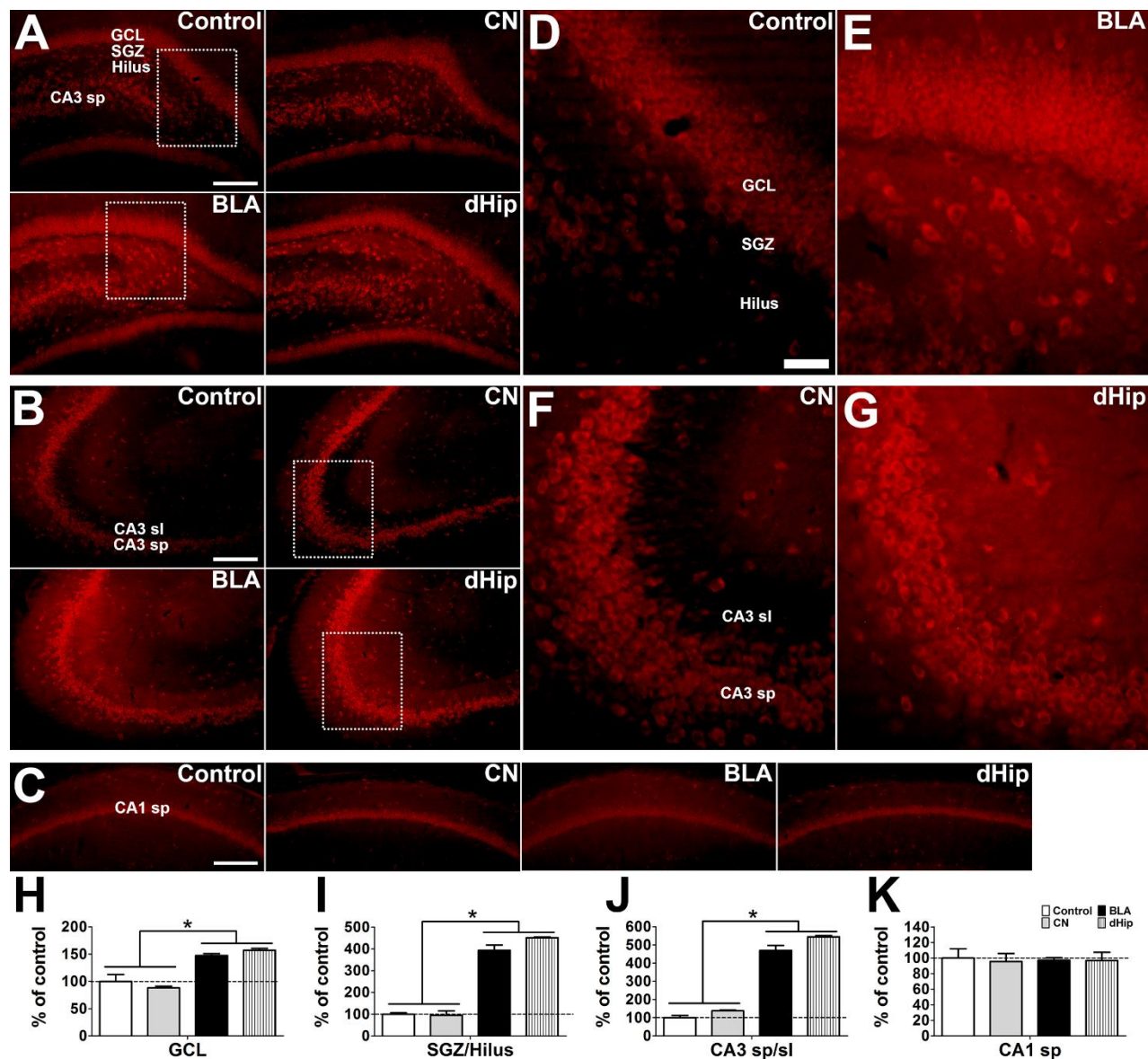
Figure 3-5. Limbic kindling increases NPY2R expression. Representative photomicrographs of the **(A)** dentate gyrus **(B)** CA3, and **(C)** CA1 (Scale bar = 200μm). **(D-G)** High power magnification (Scale bar = 50 μm) of the dentate gyrus in **(D)** control and **(E)** BLA-kindled rats and the CA3 in **(F)** CN-kindled and **(G)** dHip-kindled rats. Note the minimal NPY2R immunolabelling in the neuropil of control and CN-kindled rats. Quantification of NPY2R. Limbic-kindled rats had significantly more NPY2R fluorescence immunolabelling in the **(H)** GCL **(I)** SGZ-hilus and **(J)** CA3sp-sl, but not the **(K)** CA1sp, compared to control and CN-kindled rats. GCL, granule cell layer; SGZ, subgranular zone; SP, stratum pyramidale; SL, stratum lucidum. $*p < 0.05$

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term kindling of different brain sites. Our photomicrographs (Fig. 3-6) clearly show that Fluoro-Jade B staining is largely absent in kindled rats and controls, suggesting that neuronal degeneration or apoptosis is minimal in these rats at this time point. In contrast, rats treated with lithium-pilocarpine, which produces status epilepticus and is known to promote neurodegeneration (Turski et al., 1983), displayed robust Fluoro-Jade B staining.

3.4. Discussion

3.4.1 Limbic-kindled rats show impaired memory retrieval

Kindling had site-specific effects on behavior in this experiment. Our habituation period revealed that dHip kindling significantly increased locomotor activity in a novel environment. Increased activity and exploration in a novel environment is characteristic of hippocampal lesions and could represent hippocampal dysfunction following dHip kindling (Cassel et al., 1998; Hannesson et al., 2001). On the training day, kindling had no effect on the acquisition of hippocampal-dependent trace fear conditioning—all groups showed normal acquisition of the tone-shock fear association. This result is clearly demonstrated by all groups displaying equivalent increases of freezing to the tone presentations, trace intervals, and post-shock periods compared to baseline levels. This indicates that short-term memory was unaffected by kindling and that the locomotor effect observed during the habituation period did not impair learning or the ability to engage in conditioned freezing during training. In contrast, we found that limbic, but not non-limbic, kindling impaired the retrieval of fear memories. The BLA and dHip-kindled rats showed decreased freezing relative to the control rats during the tone test and the context test but the CN-kindled rats did not. These findings are particularly interesting given that kindling of the amygdala or hippocampus enhances unconditioned fear whereas non-limbic kindling does not (Kalynchuk et al., 1998). The site-specific effects of kindling on behavior are important, because they suggest that seizure activity per se is not sufficient to induce epilepsy-related behavioral comorbidities. Instead, these behavioral alterations are more likely to result from neurobiological changes that result from seizure activity within specific brain circuits.

3.4.2 Limbic-kindled rats have fewer hippocampal Fos-ir cells following fear memory retrieval

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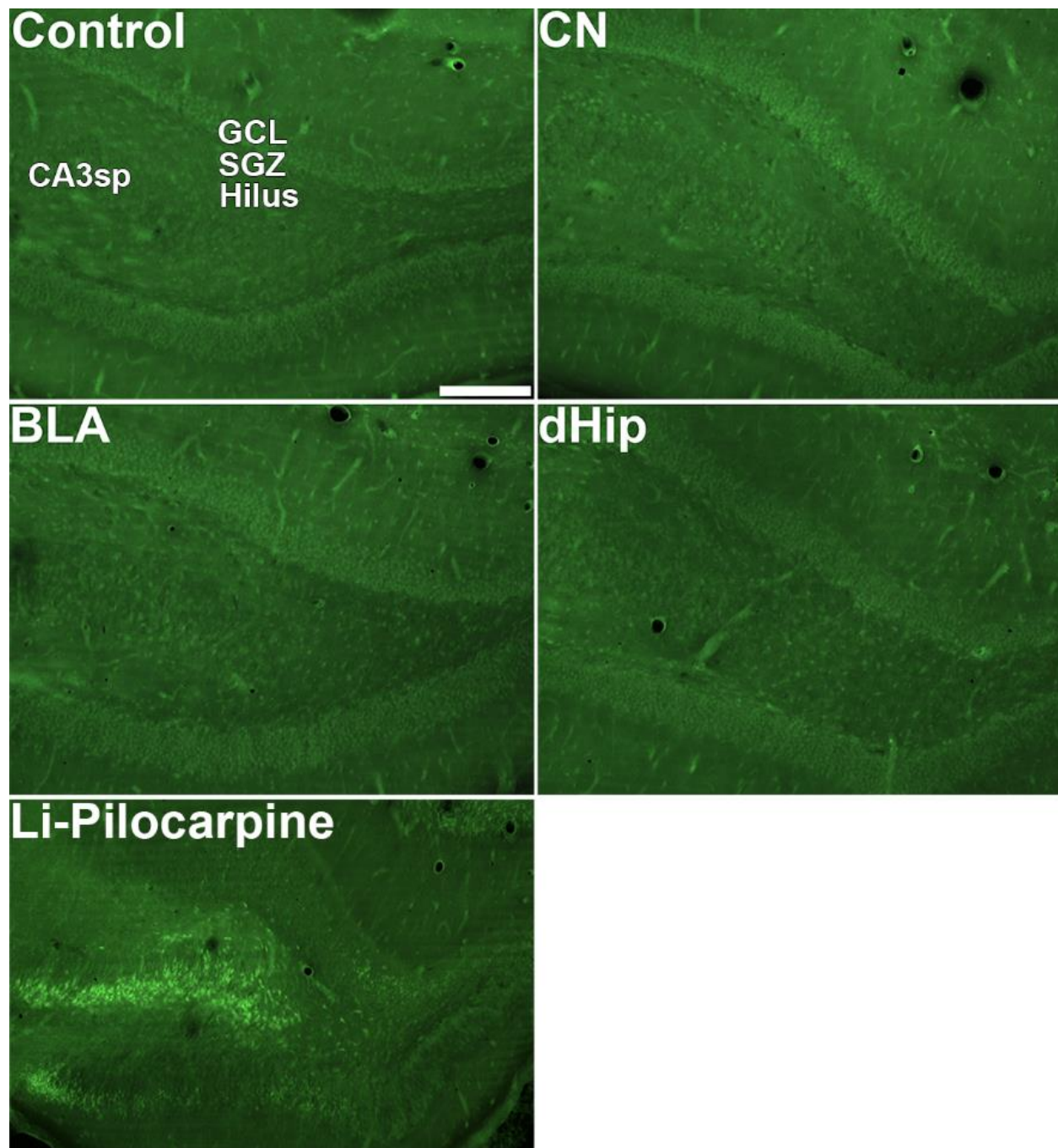
Figure 3-6. Fluoro-Jade B staining in control, kindled, and pilocarpine treated-rats. In contrast to the robust Fluoro-Jade B staining seen in lithium pilocarpine-treated rats, the control and kindled rats displayed no staining, indicating a lack of neurodegeneration after kindled seizures. Representative photomicrographs of the dentate gyrus from each group are shown (Scale bar = 200 μm).

We used Fos immunohistochemistry to begin to examine the role of hippocampal circuitry in the cognitive deficits produced by limbic seizures. Our results paralleled the behavioral findings in that we saw general reductions of hippocampal Fos expression after limbic kindling but not non-limbic kindling. Specifically, the dHip-kindled rats had fewer Fos-ir cells than the control rats in all hippocampal subfields measured. Further, the BLA-kindled rats had fewer Fos-ir cells than the control rats in the granule cell layer and hilus. In contrast, the CN-kindled rats did not differ from the control rats in the number of Fos-ir cells across the hippocampus. These results suggest that non-limbic kindling does not impair neuronal activity associated with memory retrieval. Importantly, our finding of reduced hippocampal Fos expression after memory retrieval in limbic-kindled rats is consistent with recent studies published by our laboratory using BLA-kindled rats (Botterill et al., 2014; Fournier et al., 2013) and provides a general indication of hippocampal dysfunction after recurrent limbic seizures.

What mechanism might account for the cognitive deficits and decreased hippocampal Fos expression seen in the present study? Although many possibilities exist, one explanation could be that seizures dramatically alter inhibitory neurotransmission. Seizures are known to increase NPY mRNA in granule cell layer and hilar neurons, presumably from increased synthesis of the peptide (Schwarzer et al., 1995; Sperk et al., 1992). After chronic seizures, NPY is transported through the mossy fibers to their nerve terminals where it is made available for release. This is a particularly interesting phenomenon, as this is the only known occurrence where NPY is released from a glutamatergic pathway (Nadler, Tu, Timofeeva, Jiao, & Herzog, 2007). Furthermore, several changes to the NPY2R also occur. Specifically, NPY2R mRNA in dentate granule cells is increased, the number of NPY2R receptor sites in the hilus increases as much as 800%, and receptor binding affinity increases (Gobbi et al., 1998; Schwarzer et al., 1998). Interestingly, these findings from rodent studies also apply to patient populations. Tissue samples from patients with intractable mesial TLE contain an increased number and length of NPY immunoreactive fibers and more NPY2R binding (Furtinger et al., 2001).

3.4.3 Limbic kindling promotes plasticity of NPY and its Y2 receptor

The results of this experiment point to the brain site-specific effect of seizures on hippocampal NPY plasticity and how this plasticity may affect behavior. We found that kindling significantly decreased the detectable number of NPY-ir cells in several hippocampal subfields.

The most pronounced decrease was observed within the dentate gyrus and CA3 subfields in limbic-kindled rats. In contrast, the effect of kindling on NPY-ir cell distribution in the CA1 was quite modest, with the only significant reduction seen in the CA1 stratum radiatum of dHip-kindled rats, compared to controls. The CN-kindled rats also had fewer NPY-ir cells than controls within the subgranular zone and CA3 stratum pyramidale. This result was particularly thought-provoking, as it could suggest that alterations in NPY distribution occur in these hippocampal subfields first and that additional subfields become affected once limbic seizures generalize. Or, given that the CN-kindled rats did not show cognitive impairments, it could indicate that the absolute number of NPY-ir cells is not important for the processing of cognitive information. The induction of status epilepticus using kainic acid or pilocarpine produces a significant reduction in the number of NPY-ir cells (Cardoso et al., 2010; Kuruba, Hattiangady, Parihar, Shuai, & Shetty, 2011; Long et al., 2011; Lurton & Cavalheiro, 1997; Nadler et al., 2007). In addition, hippocampal tissue taken after perforant path stimulation (Sloviter et al., 2003), rapid kindling (Sun et al., 2007), and autopsies from human patients with TLE (de Lanerolle et al., 1989) have all revealed reductions in the number of NPY-ir cells. The general consensus from these studies is that the decrease in the number of NPY-ir cells is likely due to neuronal degeneration resulting from prolonged seizure activity. This hypothesis has been supported by intense labelling of degenerative neuronal markers, such as Fluoro-Jade B after pilocarpine or kainic-acid induced status epilepticus (Long et al., 2011; Sloviter et al., 2003; Sun et al., 2007). The reduction of NPY-ir cells has therefore been suggested to represent a reduction of inhibition that increases susceptibility to subsequent seizures (Sloviter et al., 2003). However, there is evidence that kindling does not cause robust degeneration of peptidergic interneurons (Schwarzer et al., 1996). As we found that kindling had a minimal effect on Fluoro-Jade B staining at 4 days following the last kindled seizure, our interpretation of these results is that under basal conditions, NPY immunoreactivity tends to accumulate in the cell body. However, under conditions of repeated limbic seizures, NPY immunoreactive product is mostly observed as diffuse staining in the neuropil and mossy fiber terminals. Accumulation of NPY into the neuropil and mossy fiber terminals under conditions of repeated seizures could increase the speed and efficiency of NPY neurotransmission. Therefore, one possibility of why limbic-kindled rats had fewer detectable NPY-ir cells is because NPY may have been transported from the cell bodies of these neurons to the nerve terminals. As our NPY cell counting technique relies

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on the presence of NPY in cell bodies, this would have created the appearance of a loss of NPY-ir neurons.

Our densitometry analyses of NPY and the NPY2R support this idea. We found that limbic-kindled rats had dramatically increased immunolabelling of NPY and NPY2R in the dentate gyrus and CA3 subfield, which are the regions where significant mossy fiber sprouting occurs with seizures. Further, close examination (Fig. 3-5D-G), shows quite clearly that the NPY2R is prominently expressed in the neuropil of hilar and CA3 neurons in BLA and dHip-kindled rats, but it is virtually absent outside the soma of these neurons in the CN-kindled and control rats. This re-distribution of the NPY2R would change the way in which extracellular NPY affects neuronal activity, as discussed below. With the exception of previously discussed decreases in the number of NPY-ir cells in the subgranular zone and hilus, the CN-kindled rats were not significantly different from the control rats on any other measure of NPY plasticity. Our results therefore provide novel evidence that hippocampal NPY plasticity is not always a consequence of generic recurrent seizures. Instead, they suggest that limbic seizures *per se* are required to promote plasticity of NPY and the NPY2R within specific subfields of the hippocampus.

3.4.4 The link between NPY and cognition

Under normal experimental conditions, NPY is preferentially released by large dense core vesicles in response to high frequency stimulation (Hokfelt, 1991). However, there is evidence that the pattern of NPY release is altered after recurrent seizures. That is, the degree of NPY release after seizures appears to be specific to the severity of seizures and experimental timeline. Indeed, during the epileptogenesis phase, basal release of NPY is moderately increased but not statistically different from control levels (Rizzi, Monno, Samanin, Sperk, & Vezzani, 1993). In contrast, basal release of NPY is approximately doubled following chronic recurrent seizures (Vezzani et al., 1994) and significantly greater amounts of NPY are released in response to depolarizing conditions (Rizzi et al., 1993). Further, *in vitro* application of the selective NPY2R antagonist BIIE0246 alone was shown to enhance recurrent mossy fiber synaptic transmission, the frequency of miniature excitatory postsynaptic currents, and the magnitude of granule cell epileptiform activity evoked by recurrent mossy fibers in slices from pilocarpine treated rats (Tu et al., 2005). Therefore, enhanced tonic release of NPY after limbic seizures may

augment presynaptic inhibition of glutamate release (Tu et al., 2005), possibly due to the increased number and affinity of presynaptic NPY2R binding sites (Vezzani et al., 1999). This may be an effective compensatory mechanism for increasing seizure thresholds, but it could have detrimental functional consequences on behavior. There is evidence that enhancing NPY in otherwise experimentally naïve subjects can impair learning and memory. Specifically, acute systemic administration of the selective NPY2R agonist PYY3-36 impairs spatial memory performance (Stadlbauer, Langhans, & Meyer, 2013) and intracerebroventricular injection or adeno-associated viral overexpression of NPY attenuates hippocampal long-term potentiation (LTP) by approximately 50 % and delays hippocampal memory consolidation (Sorensen et al., 2008; Whittaker, Vereker, & Lynch, 1999). Importantly, selective antagonism of NPY2R with BIIE0246 restores LTP to control levels, suggesting that LTP can be disrupted through NPY actions on the NPY2R (Sorensen et al., 2008). These findings are important from our perspective, as kindling is also known to disrupt LTP and performance on hippocampal-dependent memory tasks (Sherafat et al., 2013), such as the trace fear conditioning task used in this experiment. This evidence is particularly interesting in relation to cognition, as glutamate is critical for LTP and contextual memory (Levenson et al., 2002; Richter-Levin, Canevari, & Bliss, 1995) and the presence of NPY in mossy fibers can directly modulate glutamate release through interactions with NPY2R. Our data therefore raise the intriguing possibility that limbic seizures produce an exaggerated basal inhibitory tone that persists during interictal periods and subsequently dampens the neuronal activity that is required for consolidation and/or retrieval of hippocampal-dependent memories.

It is worth considering our results in the context of findings that limbic kindling can enhance adult hippocampal neurogenesis (Fournier et al., 2010). There is considerable debate in the literature about why seizures enhance neurogenesis (Scharfman & McCloskey, 2009), given that hippocampal neurogenesis is generally thought to be beneficial, whereas epilepsy is a pathological state. We recently reported that 4 week old adult-generated dentate granule neurons labelled with bromodeoxyuridine and Fos were relatively quiescent after memory retrieval in BLA-kindled rats (~ 1 % co-localization) but not in control rats (~ 10 % co-localization) (Fournier et al., 2013). In this case, memory was impaired in the BLA-kindled rats relative to the control rats. This result was quite interesting, as adult-generated neurons are thought to display lower thresholds for depolarization and to be preferentially recruited into hippocampal-

dependent learning circuits (Kee et al., 2007), but we observed memory impairments and little functional integration of new neurons. Given the results reported here, it is possible that interactions between NPY and the NPY2R after limbic seizures may impair functional recruitment of these "plastic" adult generated neurons and negatively impact performance on hippocampal-dependent tasks, such as spatial learning and certain forms of fear memory. In line with this, it has been suggested that tonic release of NPY impairs the ability of recurrent mossy fibers to synchronize granule cell discharge (Nadler et al., 2007). It may be fruitful in future studies to further examine the role of NPY in the regulation of adult neurogenesis.

Our results indicate that limbic kindling significantly impairs memory retrieval following hippocampal-dependent trace fear conditioning. These impairments coincide with a general reduction of hippocampal Fos expression and concurrent hippocampal subfield-specific decreases in the number of NPY-ir cells but a dramatic increase in NPY and NPY2R immunolabelling. In contrast, non-limbic kindling had no effect on conditioned behavior or Fos-ir, and few effects on NPY plasticity. Overall, these results suggest that limbic kindling promotes plasticity of NPY and its Y2 receptor, resulting in an augmented inhibitory tone that could negatively impact cognition. Future studies should further investigate the role NPY and its receptors play in the regulation of interictal cognition by conducting *in vivo* manipulations of NPY following limbic and non-limbic kindling. For example, it would be interesting to directly infuse the selective NPY2R antagonists BIIE0246 or JNJ-5207787 into the hippocampus and determine whether these drugs can ameliorate the cognitive impairments seen in the present study.

Acknowledgements

CHAPTER 4

Aberrant hippocampal neurogenesis after limbic kindling: Relationship to BDNF and hippocampal-dependent memory

J.J. Botterill [a], K.J. Brymer [a], H.J. Caruncho [b], L.E. Kalynchuk [c]

[a] Department of Psychology, University of Saskatchewan, Saskatoon, SK, S7N 5A5, Canada

[b] College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, S7N 5E5, Canada

[c] Department of Medicine, University of Saskatchewan, Saskatoon, SK, S7N 5E5, Canada

4.1 Introduction

Neurogenesis, the birth of new neurons, was long thought to be exclusive to the prenatal brain. However, a plethora of findings has revealed that in the mammalian brain, neurogenesis occurs throughout adulthood (Altman, 1962; Eriksson et al., 1998) and is regulated by a variety of physiological, pharmacological, or pathological stimuli (Gould et al., 1999; Malberg, Eisch, Nestler, & Duman, 2000; Parent et al., 1997; van Praag et al., 1999). One of the most studied neurogenic brain regions is the dentate gyrus of the hippocampus, where newborn neurons of the subgranular zone (SGZ) migrate to the granule cell layer (GCL) and integrate into local hippocampal circuitry. Adult generated neurons in the hippocampus are functionally important, with numerous reports indicating that these new neurons play crucial roles in learning and spatial memory (Deng et al., 2010; Jessberger et al., 2009; Kee et al., 2007; Suarez-Pereira, Canals, & Carrion, 2014), associative fear learning (Kirby et al., 2012; Kitamura et al., 2009) and anxiety-related behaviors (Revest et al., 2009).

Although the integration of adult generated neurons into hippocampal circuitry is generally considered to be beneficial for cognition and emotion, pathological states such as temporal lobe epilepsy (TLE) seem to promote an aberrant form of neurogenesis that could contribute to the cognitive impairment and emotional dysfunction often seen in patients with this disorder (Fournier et al., 2013; Scharfman & Hen, 2007). For example, seizures are known to increase hippocampal cell proliferation and survival, and many of these newborn neurons migrate ectopically into the hilus and develop basal dendrites (Fournier et al., 2010; Fournier et al., 2013; Jessberger et al., 2007b; Parent et al., 1998; Parent et al., 2006). Many other newborn neurons do migrate normally into the GCL, but they mature more rapidly than normal. However, colocalization of adult generated neurons with the behaviorally relevant immediate early gene c-Fos (Guzowski et al., 2005) has indicated that mature adult generated neurons born under conditions of kindling may not be functionally active during hippocampal-dependent memory tests (Fournier et al., 2013). One issue that remains unresolved is whether the effect of seizures on adult hippocampal neurogenesis depends on the brain site where the seizures originate or whether epileptic activity anywhere in the brain can promote aberrant neurogenesis. This is an important question because there is evidence that the behavioral co-morbidities associated with TLE vary depending on the location of the epileptic focus. This is seen quite clearly in the preclinical kindling model of TLE, where kindling of the basolateral amygdala (BLA) or dorsal

hippocampus (dHip) impairs hippocampal-dependent fear conditioning (Botterill et al., 2014; Botterill, Guskjolen, Marks, Caruncho, & Kalynchuk, 2015; Fournier et al., 2013) and spatial learning in the Morris water maze and radial arm maze (Hannesson et al., 2001; Leung et al., 1996), and enhances unconditioned fear behaviors in the open-field test and elevated plus maze (Fournier et al., 2009; Kalynchuk et al., 1997; Kalynchuk et al., 2006). In contrast, kindling of nonlimbic brain sites, such as the caudate nucleus (CN), has minimal effects on any of these behavioral and cognitive comorbidities (Botterill et al., 2015; Kalynchuk et al., 1998). As adult hippocampal neurogenesis is involved in anxiety-related behaviors and spatial learning and memory, these behavioral dissociations raise the possibility that aberrant neurogenesis may be present following seizures that originate in limbic, but not nonlimbic, regions of the brain.

To test this idea, we examined the effect of different site kindling on aberrant adult hippocampal neurogenesis and cognition. We also investigated the effects of kindling on brain-derived neurotrophic factor (BDNF) expression across the hippocampus. Brain-derived neurotrophic factor is a modulator of many physiological functions, including synaptogenesis, neurogenesis, and activity-dependent plasticity (Binder & Scharfman, 2004; Chao, 2003; Lee & Son, 2009). Brain-derived neurotrophic factor belongs to the neurotrophin family of growth factors and is synthesized as a precursor protein (pre-pro BDNF) that is cleaved into proBDNF and mature BDNF (mBDNF), which in turn activates TrkB receptors. Previous studies have shown that seizures significantly increase BDNF expression, regardless of the method of seizure induction (Ernfors, Bengzon, Kokaia, Persson, & Lindvall, 1991; Humpel, Wetmore, & Olson, 1993; Isackson, Huntsman, Murray, & Gall, 1991; Nanda & Mack, 2000; Vezzani et al., 1999). Brain-derived neurotrophic factor is known to increase hippocampal neurogenesis (Lee & Son, 2009; Rossi et al., 2006), promote hilar-CA3 ectopic granule cells (Scharfman et al., 2005), influence the morphological features of dendritic spines (Murphy, Cole, & Segal, 1998; Tyler & Pozzo-Miller, 2003) and stimulate axonal growth and the development of hilar basal dendrites in dentate granule cells (Danzer et al., 2002). Elevated levels of BDNF following seizures could therefore stimulate aberrant forms of neurogenesis. We expected to see kindling-induced changes in hippocampal BDNF expression that parallel changes in hippocampal neurogenesis and cognition. That is, if limbic kindling promotes aberrant neurogenesis and impairs hippocampal-dependent cognition, it should also enhance hippocampal BDNF expression. Further, if

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nonlimbic kindling does not influence hippocampal neurogenesis or cognition, it should have no effect on BDNF levels.

4.2 Materials and Methods

4.2.1 Animals

We used 88 male Long-Evans rats (n = 40 for post-mortem analyses; n = 48 for behavioral analyses) that were purchased from Charles River (QC, Canada). The rats weighed 200-250g at the time of arrival from the breeder. Rats were individually housed in rectangular polypropylene cages containing standard laboratory bedding with free access to food and water. The colony room was maintained at a temperature of $20 \pm 1 \, ^{\circ}C$ on a 12:12h light-dark cycle (lights on at 8 a.m.). All experimental procedures were in accordance with the guidelines of the Canadian Council on Animal Care and an animal care protocol approved by the University of Saskatchewan Committee on Animal Care and Supply. We made all possible efforts to minimize the number of rats used in the present study.

4.2.2 Surgery

Surgery was conducted as previously described (Botterill et al., 2014). Briefly, rats were deeply anesthetized with isoflurane (5% initial, 2-2.5% maintenance) and secured into a stereotaxic apparatus using ear bars. At a flat skull position, a single bipolar stimulating electrode (MS-303-2-B-SPC, Plastics One, Roanoke, VA, USA) was chronically implanted into the left hemisphere of one of three discrete brain sites, relative to bregma: CN (+ 0.2 mm anteroposterior, + 3.2 mm mediolateral, - 5.7 mm dorsoventral), BLA (- 2.8 mm anteroposterior, + 5.0 mm mediolateral, - 8.5 mm dorsoventral) or dHip (- 3.5 mm anteroposterior, + 2.6 mm mediolateral, - 3.1 mm dorsoventral) (Paxinos & Watson, 1998). The electrode assembly was secured to the skull with 4 jeweler screws and dental acrylic.

4.2.3 Kindling

Rats received a post-surgical recovery period of 1 week and were then randomly assigned to CN (n = 22), BLA (n = 22), dHip (n = 22) kindling or control (n = 22) groups. Control rats included a set of CN, BLA, and dHip electrode-implanted rats. Kindling was conducted as

previously described (Botterill et al., 2014). Briefly, a wire lead connected to an isolated pulse stimulator (Model 2100, A-M Systems, Sequim, WA, USA) was attached to the electrode assembly to deliver a 1 s, 60 Hz train of square-wave pulses with a biphasic amplitude of 800μA (peak-to-peak) and duration of 1 ms. A total of 99 electrical stimulations were delivered on a 3-times-per-day, 5-days-per-week schedule (i.e., 15 stimulations per week), with a minimum of 3 h between consecutive stimulations. Control rats were connected to the electrode lead on the same schedule, but they did not receive any electrical stimulation. The convulsion elicited by each stimulation was scored as previously described (Pinel & Rovner, 1978; Racine, 1972). The convulsion classes were operationally defined as: class 0: immobility; class 1: orofacial automatisms; class 2: orofacial automatisms with head nodding; class 3: unilateral forelimb clonus; class 4: rearing with bilateral forelimb clonus; class 5: rearing with bilateral forelimb clonus and loss of equilibrium; class 6: multiple class 5 convulsions and falling episodes; class 7: previous classes with running fit; and class 8: previous classes with intermittent muscle tonus. Under this classification criteria, rats are considered to be fully "kindled" following 3 consecutive class 5 convulsions (Pinel & Rovner, 1978).

4.2.4 BrdU Injections

Between kindling stimulations 70-72, a subset of rats that was to be used for post-mortem immunohistochemistry analyses (n = 24; 6 per group) received two BrdU injections (100 mg/kg, 20 mg/ml; i.p. each injection; B-5002, Sigma; St. Louis, MO, USA). The injections were spaced 12 h apart (8 am and 8 pm). Bromodeoxyuridine was dissolved in warm physiological saline and sterile-filtered. The timing of these BrdU injections allowed us to birth-date proliferating cells after a period of extended kindling and to follow the survival and maturation of these cells under conditions of repeated seizures. Kindling continued for an additional two weeks following the BrdU injections.

4.2.5 Perfusions and Immunohistochemistry

The day after the final kindling stimulation, the subset of rats that received BrdU was deeply anesthetized with sodium pentobarbital (240 mg/kg; i.p.). Each rat was then transcardially perfused using room-temperature physiological saline, followed by ice-cold 4% (w/v)

formaldehyde fixative (pH = 7.4). The brains were extracted and immersed in the same fixative for 48 h at 4 °C. Brains were then sectioned in the coronal plane at 50 μm on a vibrating microtome (Vibratome 3000; Vibratome Company, St. Louis, MO, USA). Sections were collected and stored at – 20 °C until use in a cryoprotectant solution containing 30 % (w/v) sucrose, 1 % (w/v) polyvinylpyrrolidone, and 30 % (v/v) ethylene glycol in 0.1 M PBS (pH = 7.4).

We used a standard immunohistochemistry technique to assess the presence of BrdU-ir and DCX-ir neurons with widely used and commercially available antibodies. Immunostaining was conducted on free-floating sections in 6-well tissue culture plates. All tissues were processed in unison with treatment groups counterbalanced across all tissue plates to ensure consistent immunohistochemical processing. To confirm specificity of the BrdU and DCX antibodies, we omitted the primary antibody from an additional well of free-floating sections. In absence of the primary antibody, we did not detect any BrdU-ir or DCX-ir cells.

We conducted BrdU immunohistochemistry as previously described (Fournier et al., 2013). Briefly, endogenous peroxidase activity was blocked with 0.3 % (v/v) H_2O_2 in 0.1 M PBS for 30 min. Next, sections were treated with 2 N HCL at 37 °C for 1 h to denature the DNA and expose the BrdU antigen. Sections were then blocked for 1 h in 0.1 M PBS containing 5 % (v/v) normal horse serum, 1 % (w/v) bovine serum albumin (BSA), and 0.3 % (v/v) Triton X-100 and incubated for 48 h at 4 °C in mouse anti-BrdU primary monoclonal antibody (1:500, Roche Diagnostics GmbH, Mannheim Germany) diluted in blocking solution. Sections were then incubated for 2 h in biotinylated horse anti-mouse secondary antibody (1:500, Vector Laboratories, Burlingame, CA, USA), followed by incubation for 1 h in avidin-biotin peroxidase complex (1:200, Vectastain ABC Elite, Vector Laboratories). Immunolabelling was visualized using 0.033 % (w/v) 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.00786 % (v/v) H_2O_2 diluted in 0.1 M PBS. Sections were counterstained with 0.1 % cresyl violet to assist with cell counting.

DCX immunohistochemistry was also conducted as previously described (Lussier et al., 2013). Sections underwent heat-induced epitope retrieval in sodium citrate buffer (pH = 6.0) at 95 °C for 30 min. Sections were then blocked in 5 % (v/v) normal goat serum (NGS), 1 % (w/v) BSA, and 0.5 % (v/v) Triton X-100 in 0.1 M TBS, followed by rabbit anti-DCX polyclonal primary antibody (1:1000, Cell Signaling Technology, Danvers MA, USA) diluted in blocking

solution for 24 h at room temperature. Sections were then treated with 5 % (v/v) H_2O_2 in 0.1 M TBS for 30 min to block endogenous peroxidase activity. Next, the sections were incubated for 1 h in biotinylated goat anti-rabbit secondary antibody (1:500, Vector Laboratories) diluted in 5% (v/v) NGS, 1% (w/v) BSA, and 0.5% Triton X-100 in 0.1 M TBS, followed by avidin-biotin peroxidase complex (1:500, Vector Laboratories) for 1 h. Sections were rinsed with 0.175 M sodium acetate (pH = 6.8) and visualized with 0.025 % (w/v) DAB, 4.167 % (w/v) $NiSO_4$ and 0.002 % (v/v) H_2O_2. Sections were then mounted onto glass slides using 0.2 M PB (pH = 7.4), air dried overnight, dehydrated using graded alcohols, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA).

4.2.6 Quantification of Immunohistochemistry

All analyses were conducted by researchers blind to the treatment conditions. Immunostained sections were examined using a Nikon Eclipse E800 microscope with a motorized stage and digital camera (MicroFire, Optronics, Goleta, CA, USA) connected to a computer. We counted BrdU-ir and DCX-ir cells at 40X magnification. The dentate SGZ (defined as a two-cell width zone in between the inner granule cell layer and the hilus) and GCL were traced using a computerized stereology system program (StereoInvestigator, MicroBrightfield, Williston, VT, USA). Bromodeoxyuridine and DCX-ir cells ipsilateral and contralateral to the site of stimulation were counted in both of these regions. In order to assess ectopic migration of newborn cells, BrdU-ir cells located in the hilar-CA3 border were counted separately. For all counts, we employed unbiased stereology using a modified optical fractionator method that excludes cells in focus at the uppermost focal plane to reduce oversampling (Kuhn, Winkler, Kempermann, Thal, & Gage, 1997). The total number of BrdU-ir and DCX-ir cells was estimated using the following formula: $N_{total} = \sum Q^- \times 1 / ssf \times A(x, y \text{ step}) / a(\text{frame}) \times t / h$. $\sum Q^-$ represents the number of counted cells, ssf is the section sampling fraction (1 in 12), $A(x, y \text{ step})$ is the area associated with each x,y movement ($10,000\mu m^2$), $a(\text{frame})$ is the area of the counting frame ($3,600 \ \mu m^2$), t is the weighted average section thickness, and h is the height of the dissector ($12 \ \mu m$) (Fournier et al., 2010; Lussier et al., 2013). To avoid counting sectioning artifacts, we used a guard zone of 2 μm.

We used a previously established dendritic categorization method for DCX-ir cells

(Lussier et al., 2013; Plumpe et al., 2006) to determine whether kindling has site-specific effects on the dendritic morphology of immature neurons. A meander scan method was used to randomly select 100 DCX-ir cells from each rat and assign them to one of six categories based on the presence and extent of apical dendrites (shown in Fig. 4-3C). Category one and two cells represented proliferative cells with either no process (category one) or one small process (category two). Category three and four cells represented intermediate stages of development, with a medium process reaching the granule cell layer (category three) or a process reaching the molecular layer (category four). Category five and six cells represented the postmitotic stage, and included cells with one major process extending into the molecular layer (category five), or a defined dendritic tree with delicate branching in the granule cell layer (category six). As seizures can increase the number of adult generated neurons containing hilar basal dendrites (Fournier et al., 2010; Jessberger et al., 2007b), we also recorded the number of randomly sampled cells containing processes extending into the hilus. The data are presented as the percentage of DCX-ir cells in each of the six categories and the percentage of sampled cells containing hilar basal dendrites.

4.2.7 BDNF Protein ELISA

The day after the final kindling stimulation, a subset of rats (n = 16; 4 per group) was deeply anesthetized with sodium pentobarbital (240 mg/kg; i.p.) and decapitated with a standard rodent guillotine. The entire hippocampus was rapidly dissected, snap frozen in liquid nitrogen and stored at -80 °C. Hippocampi were mechanically homogenized in a 0.3 M sucrose Tris-EDTA solution containing a protease inhibitor cocktail (cOmplete Mini Protease Inhibitor Cocktail, Roche Diagnostics GmbH) and centrifuged. The resulting supernatant was used to measure BDNF levels in the entire ipsilateral hippocampus (i.e., the stimulated side) with a commercially available and widely used ChemiKine BDNF sandwich ELISA kit (CYT306, Millipore, Billerica, MA, USA). Assays were conducted according to the manufacturer's instructions in a 96-well microtiter plate that was precoated with anti-human BDNF monoclonal antibody. A series of 7 serially diluted standards (15.63 - 1000 pg/ml) and samples (100 µl each; diluted 1:2 in sample diluent) were probed in duplicate and incubated overnight at 4 °C. On the following day, the plate was washed 4 times and incubated in 100 µl of biotinylated mouse anti-BDNF monoclonal antibody (1:1000) for 3 h at room temperature. The samples then received 4

additional washes, followed by incubation in 100 µl of streptavidin-HRP conjugate (1:1000) for 1 h at room temperature. After a final series of washes, each sample received 100 µl of room temperature TMB/E solution for 15 min followed by 100 µl of stop solution. The plate was immediately read at 450 nm using a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) connected to a computer running SoftMax Pro software v6.4 (Molecular Devices). The standard curve ($R^2 = 0.994$) was then used to convert the optical density units of each sample to pg of BDNF per ml of hippocampal homogenate.

4.2.8 Trace Fear Conditioning

To determine the effects of limbic and non-limbic kindling on hippocampal-dependent learning and memory, a subset of rats (n = 48; 12 per group) underwent trace fear conditioning 24 h after the final kindling stimulation. None of these rats were used for any of the previously described post-mortem analyses. Two identical sound-attenuated operant chambers (VFC-008, Med Associaties Inc, St. Albans, VT, USA) equipped with miniature monochromatic video cameras were used for all fear conditioning procedures. The chambers contained a grid floor with 19 aluminum rods connected to a shock generator (ENV-414S, Med Associates Inc). Auditory stimuli were presented through a loudspeaker mounted in the chamber wall (ANL-926, Med Associates Inc). Tone and shock pairings were controlled through an interface cabinet (Dig-700 F, Med Associates Inc) connected to a computer running Video Freeze Software (Med Associates Inc). The chambers were cleaned thoroughly with 0.6 % (v/v) acetic acid between subjects. Our primary behavioral measure was conditioned freezing, operationally defined as the absence of motor movements except those necessary for respiration. Freezing behavior was scored every 2 s during tone presentations and every 4 s during contextual freezing observations. The freezing observations were transformed into a percentage of time spent freezing by summing the total number of freezing observations and dividing by the total number of observations for each measure, and multiplying the resultant number by 100.

On the training day, each rat was individually placed into one of the two identical sound attenuated operant chambers. Following a 180-s acclimation period, each rat received 7 CS-US pairings. Each pairing consisted of an auditory tone (16 s, 85 db, 2 kHz) followed by a 30-s trace interval that coterminated with a 2-s footshock (0.9 mA). The intertrial interval was set at 180 s between tone-onsets. During the training day, we measured conditioned freezing to both the

auditory tone (CS) and training context (trace and postshock intervals). Each rat was returned to the same conditioning chamber that it was trained in 48 h later for the contextual memory retrieval test. We measured conditioned freezing to the training context over a period of 8 min. No footshocks or tones were delivered during the context memory retrieval test.

4.2.9 Statistical Analyses

All statistical analyses were done using IBM's Statistical Package for Social Sciences v20. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc tests when appropriate. To determine the relationship between the number and severity of seizures and hippocampal neurogenesis, we conducted Pearson (R) product moment correlations. The criterion for statistical significance was set at $P < 0.05$. All graphs depict the mean ± standard error.

4.3 Results

4.3.1 Kindling Progression

Kindling progressed normally in all rats. The number of stimulations required to elicit the first class 5 convulsion was 11.8 ± 1.2 for the BLA-kindled rats and 48.1 ± 3.3 for the dHip-kindled rats. The number of stimulations required to elicit three consecutive class 5 convulsions was 17.3 ± 2.4 for the BLA-kindled rats and 53.4 ± 3.2 for the dHip-kindled rats. Finally, the total number of class 5 convulsions was 76.2 ± 4.0 for the BLA-kindled rats and 43.9 ± 3.2 for the dHip-kindled rats. In contrast to the generalized convulsions elicited by kindling of limbic brain sites, kindling of the CN produced convulsions characterized by brief whole-body tonus and rapid falling to one side with some periods of forelimb clonus (Kalynchuk et al., 1998). In line with previous reports, we did not observe spontaneous convulsions at any point during our study (Pinel & Rovner, 1978).

Figure 4-1A depicts convulsion progression, the time point of the BrdU injections, and the severity of convulsions during the survival and maturation period for BrdU birth-dated cells. The CN-kindled rats were not included in Fig. 4-1A, as these rats did not reliably have limbic seizures. However, electrical stimulation of the CN did elicit some limbic-like convulsions that were less frequent and severe than the convulsions typically seen in limbic-kindled rats $F(2,17)$
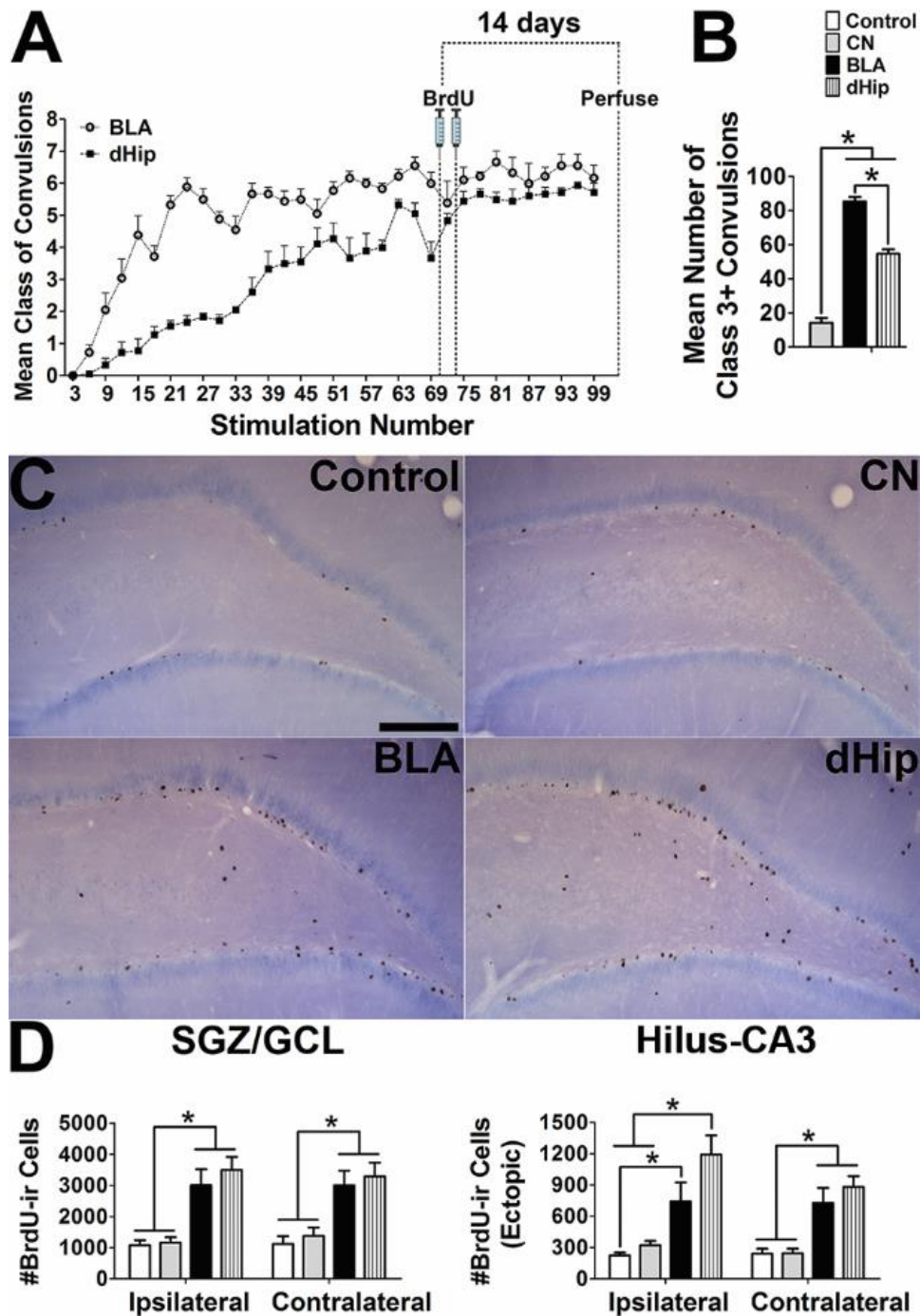
Figure 4-1. Effect of kindling on the mean class of convulsive seizures and the number of adult-generated BrdU-ir cells. **(A)** The mean daily convulsion class displayed by limbic-kindled rats (BLA and dHip) during kindling. Bromodeoxyuridine was injected between kindling stimulations 70-72. Kindling continued for an additional 2 weeks prior to sacrificing the rats. **(B)** Mean number of class 3 or higher convulsions. **(C)** Representative photomicrographs of BrdU-ir in each group, scale bar = 200μm. **(D)** Quantification of the total number of BrdU-ir cells in the SGZ/GCL and hilus-CA3 border. CN, caudate nucleus; BLA, basolateral amygdala; dHip, dorsal hippocampus; SGZ/GCL, subgranular zone, granule cell layer. *$P < 0.05$.

= 171.872, $P < 0.001$. Figure 4-1B shows the mean number of class 3 or above convulsions for the rats in each kindled group. Post hoc analyses indicated that the limbic-kindled rats had significantly more class 3 or greater seizures than the CN-kindled rats ($P < 0.001$) and that the BLA-kindled rats had significantly more class 3 or greater seizures than the dHip-kindled rats ($P < 0.001$) (Fig. 4-1B). This latter finding is typical given that amygdala kindling generally proceeds more rapidly than hippocampal kindling.

4.3.2 Effect of kindling on adult hippocampal neurogenesis

Next, we wanted to determine whether the convulsion profiles associated with limbic and nonlimbic kindling differentially affected adult hippocampal neurogenesis. Figure 4-1C shows a representative example of BrdU-ir in each group. As is typical, there were relatively few BrdU-ir cells in the controls rats, and comparatively more BrdU-ir cells in the BLA-kindled and dHip-kindled rats. Our stereological analyses revealed statistically significant group differences in the ipsilateral $F(3,23) = 12.456$, $P < 0.001$ and contralateral $F(3,23) = 8.860$, $P = 0.001$ SGZ/GCL. This is shown in Fig. 4-1D. Post hoc analyses revealed that bilaterally, the BLA and dHip-kindled rats had significantly more BrdU-ir cells located in the proliferative SGZ/GCL compared to control and CN-kindled rats (P values < 0.27). Furthermore, we found significant group differences in the number of hilar-CA3 ectopic granule cells in both the ipsilateral $F(3,23) = 11.451$, $P < 0.001$ and contralateral $F(3,23) = 12.025$, $P < 0.001$ hemisphere. Post hoc analyses revealed that dHip-kindled rats had significantly more hilar-CA3 ectopic BrdU-ir cells than CN-kindled and control rats, bilaterally (P values < 0.001). Moreover, BLA-kindled rats had significantly more hilar-CA3 ectopic BrdU-ir cells than control rats bilaterally (P values < 0.049) and more hilar-CA3 ectopic BrdU-ir cells than CN-kindled rats within the contralateral hemisphere ($P = 0.009$). The CN-kindled rats did not differ from the control rats in any quantified region (P values > 0.953).

An important distinction to make is that by kindling different brain sites, we manipulated both the severity of the convulsions and the time course over which they developed. In particular, kindling of the BLA occurred quickly and resulted in a large number of severe seizures. Kindling of the dHip occurred slowly, but also ultimately resulted in severe seizures. In contrast, kindling of the CN occurred quickly, but elicited relatively mild seizures. To determine the relationship between neurogenesis, seizure severity, and previous seizure history, we

calculated several correlations. To facilitate the interpretation of these analyses, we averaged the cell counts from the ipsilateral and contralateral hemispheres. First, we determined the relationship between the severity of seizures on the day of BrdU injections and the number of BrdU-ir cells. We found significant positive correlations between seizure severity on the day of BrdU injections and the number of BrdU-ir cells in both the SGZ/GCL ($R = 0.556$, $P < 0.017$) and hilus-CA3 border ($R = 0.561$, $P < 0.016$). Next, we asked whether previous seizure history (i.e., severity of seizures prior to BrdU administration) also had a significant impact on the number of BrdU-ir cells. We found no significant correlation between the number of SGZ/GCL BrdU-ir cells and the number of stage 5 seizures ($R = 0.373$, $P = 0.127$). Furthermore, there were no significant correlations between the number of hilar-CA3 BrdU-ir cells and the number of stage 5 seizures ($R = 0.219$, $P = 0.383$).

Figure 4-2 shows the pattern of DCX-ir in each group, along with our stereological counts of the number of DCX-ir neurons. It is clear from Figure 4-2A that the BLA-kindled and dHip-kindled rats had more DCX-ir neurons with more extensive dendritic processes than did the control rats. Our statistical analyses confirmed these observations. One-way ANOVAs revealed significant group differences within the ipsilateral $F(3,23) = 17.363$, $P < 0.001$ and contralateral $F(3,23) = 14.215$, $P < 0.001$ SGZ/GCL. Post hoc analyses revealed that the BLA-kindled rats had significantly more DCX-ir cells bilaterally than the control or CN-kindled rats (P values $<$ 0.001). Further, the dHip-kindled rats had significantly more DCX-ir cells than the control and CN-kindled rats in the ipsilateral hemisphere (P values < 0.037) and more DCX-ir cells than the CN-kindled rats in the contralateral hemisphere ($P = 0.004$). The CN-kindled rats did not differ from the control rats (P values > 0.378).

4.3.3 Effect of kindling on dendritic complexity of immature neurons.

Close examination of Fig. 4-2A suggested that in addition to increasing the number of DCX-ir cells, BLA and dHip kindling also enhanced dendritic complexity within these DCX-ir neurons. To quantify this qualitative observation, we assigned a subset of DCX-ir neurons into categories based on the extent of their dendritic processes (Lussier et al., 2013; Plumpe et al., 2006) (see Fig. 4-3C). We found significant group differences for categories 1 $F(3,23) = 17.009$, $P < 0.001$, 2 $F(3,23) = 20.149$, $P < 0.001$, 3 $F(3,23) = 4.721$, $P = 0.012$, 4 $F(3,23)= 3.783$, $P =$
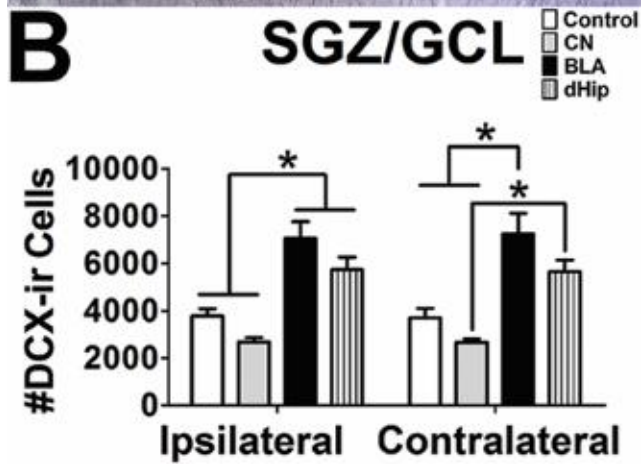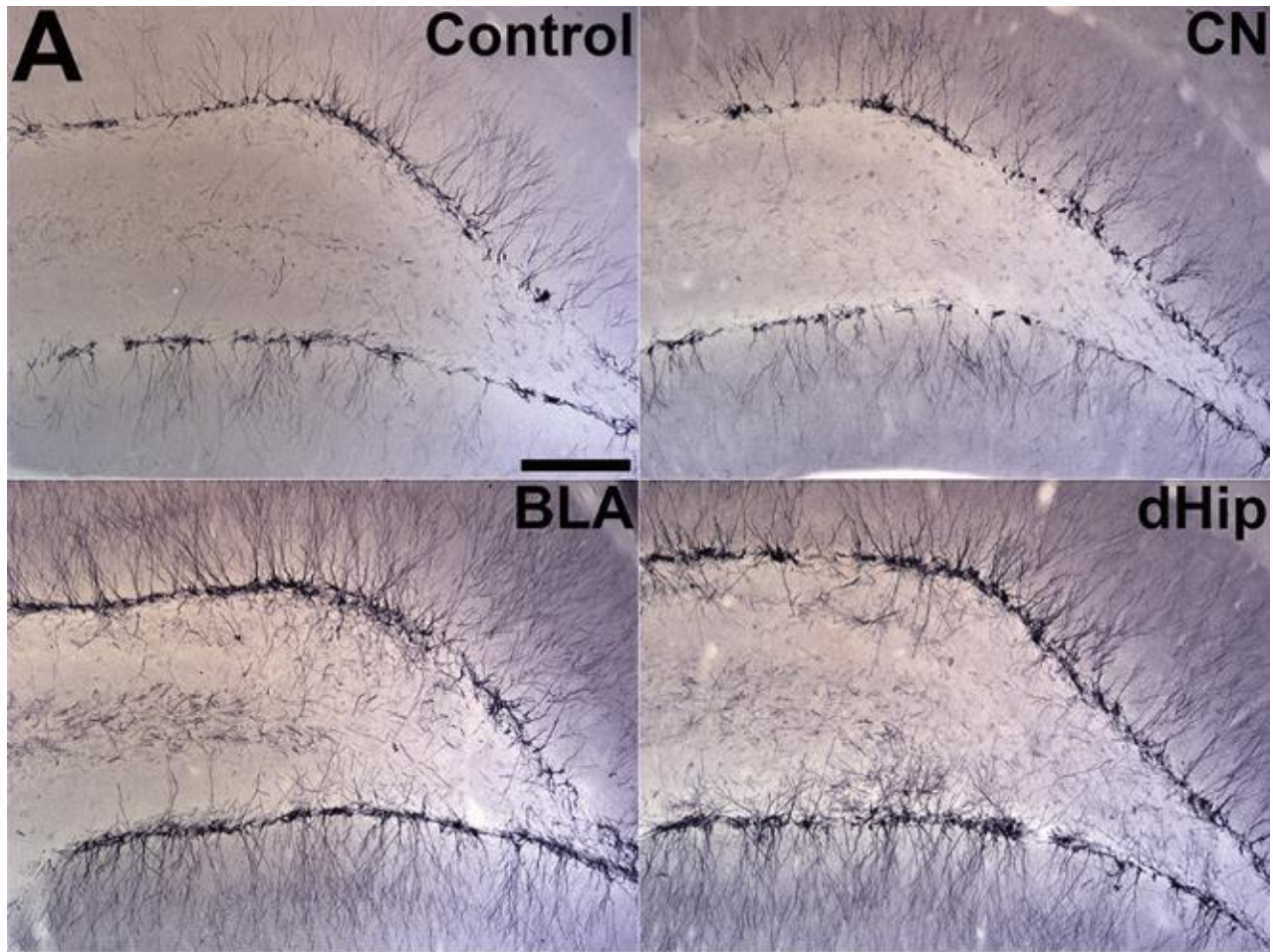
Figure 4-2. Effect of kindling on the number of DCX-ir neurons. **(A)** Representative photomicrographs of DCX-ir in each group, scale bar = 200μm. **(B)** Quantification of the total number of DCX-ir cells in the SGZ/GCL. CN, caudate nucleus; BLA, basolateral amygdala; dHip, dorsal hippocampus; SGZ/GCL, subgranular zone, granule cell layer. *$P < 0.05$.
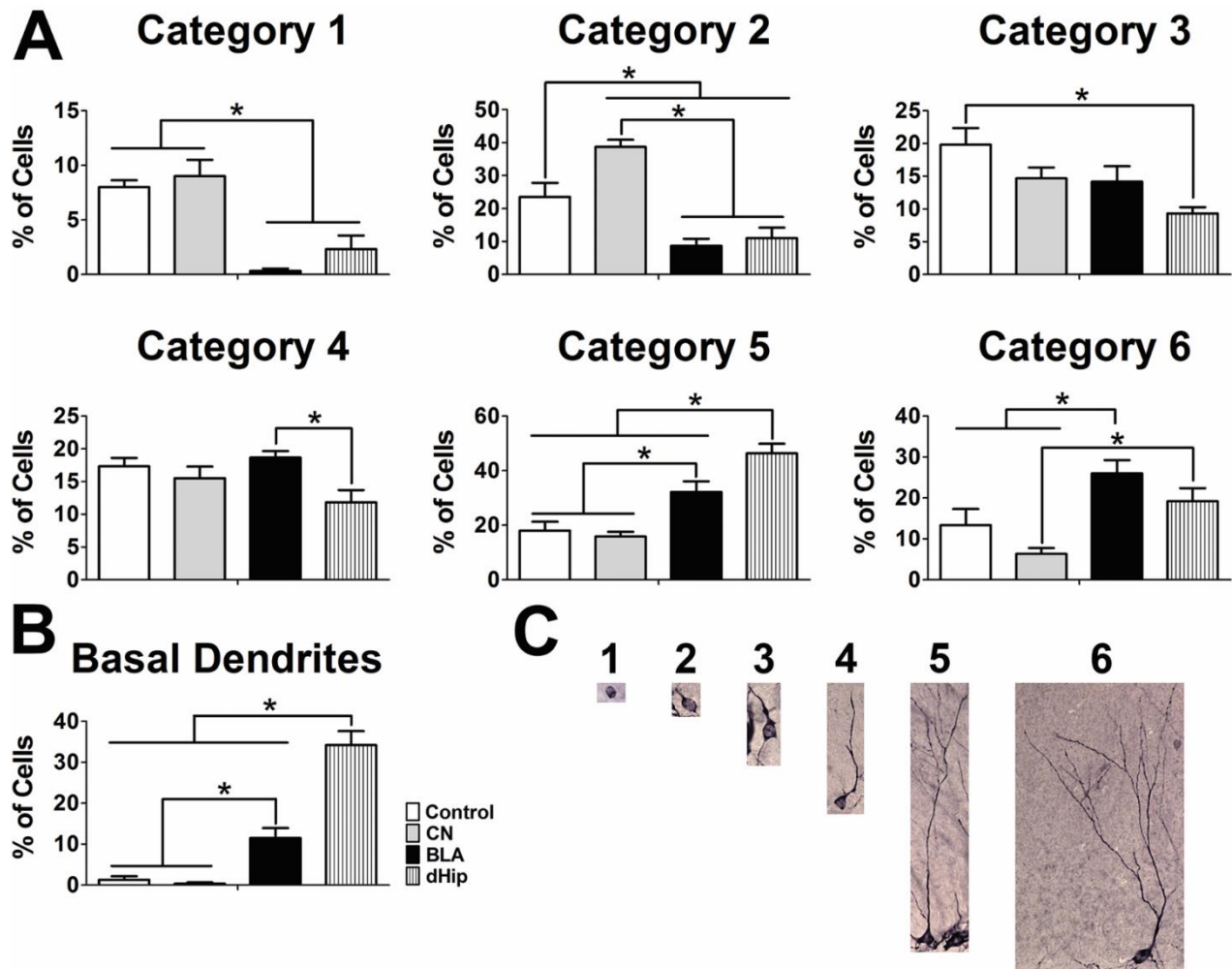
Figure 4-3. Effect of kindling on DCX-ir complexity and the presence of hilar basal dendrites. **(A)** Quantified categorization of dendritic complexity in each group. **(B)** Percentage of sampled cells containing hilar basal dendrites. **(C)** Representative photomicrographs depicting the 6 categories of dendritic complexity. CN, caudate nucleus; BLA, basolateral amygdala; dHip, dorsal hippocampus. *$P < 0.05$.

0.027, 5 $F(3,23) = 19.232$, $P <0.001$, and 6 $F(3,23) = 7.309$, $P = 0.002$ (Fig. 4-3A). For DCX-ir cells in the early stages of maturation, post hoc analyses revealed that the BLA- and dHip-kindled rats had significantly fewer category 1 and 2 cells than the control and CN-kindled rats (P values < 0.042). Interestingly, the CN-kindled rats had significantly more category 2 cells than the control rats ($P = 0.011$). For DCX-ir cells in the intermediate stages of maturation, we found that the dHip-kindled rats had significantly fewer category 3 cells than the control rats ($P = 0.006$) and significantly fewer category 4 cells than the BLA-kindled rats ($P = 0.023$). For DCX-ir cells in the latest stages of maturation, we found that the BLA- and dHip-kindled rats had significantly more category 5 cells than the control and CN-kindled rats (P values < 0.026). The dHip-kindled rats also had significantly more category 5 cells than the BLA-kindled rats ($P = 0.026$). Further, the BLA-kindled rats had significantly more category 6 cells than the control and CN-kindled rats (P values < 0.041) and the dHip-kindled rats had significantly more category 6 cells than the CN-kindled rats ($P = 0.038$). Overall, the pattern of results suggests that DCX-ir cells from rats that underwent BLA and dHip kindling were more complex than DCX-ir cells from the CN-kindled or control rats.

We also noticed a number of DCX-ir neurons with prominent hilar basal dendrites. We found significant group differences in the number of DCX-ir neurons with this characteristic $F(3,23) = 53.627$, $P < 0.001$ (Fig. 4-3B). Post hoc analyses of this effect revealed that the BLA- and dHip-kindled rats had significantly more DCX-ir cells containing hilar basal dendrites than the control and CN-kindled rats (P values < 0.016). Interestingly, the dHip-kindled rats had significantly more DCX-ir cells with hilar basal dendrites than did the BLA-kindled rats ($P < 0.001$).

4.3.4 Effect of kindling on hippocampal BDNF protein

Once we established that limbic, but not nonlimbic, kindling significantly increased adult hippocampus neurogenesis and facilitated the dendritic outgrowth of adult generated neurons, we were interested in determining whether enhancement of hippocampal BDNF protein is a possible mechanism underlying this phenomena. Figure 4-4 shows group differences in hippocampal BDNF protein levels. We found a significant group differences in the amount of BDNF protein as measured by sandwich ELISA $F(3,15) = 42.207$, $P < 0.001$. Post hoc analyses revealed that

Hippocampus
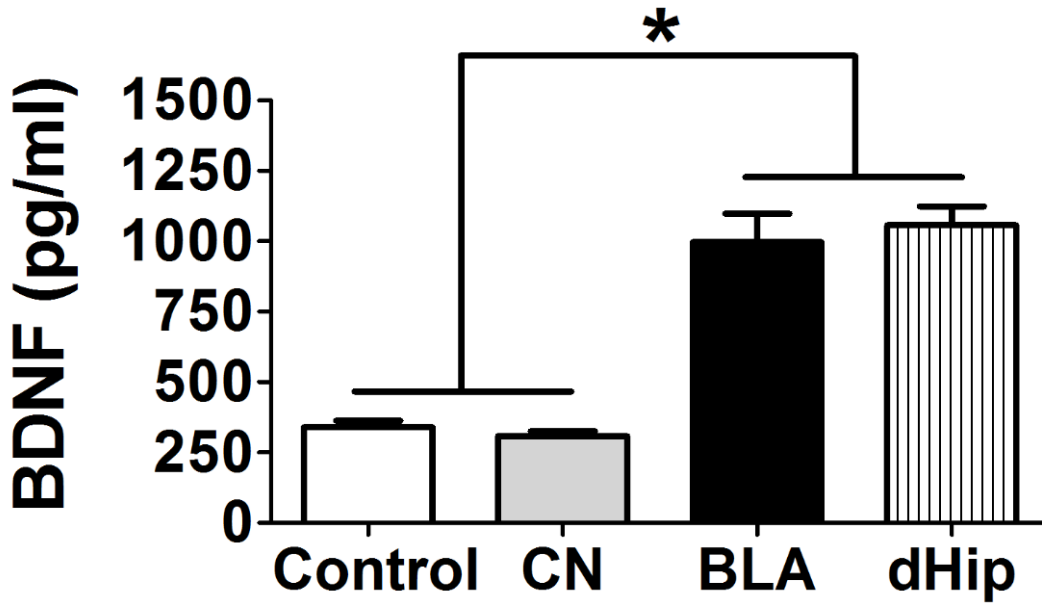
Figure 4-4. Effect of kindling on hippocampal BDNF protein levels. Limbic-kindled rats had significantly higher BDNF protein levels than the CN-kindled or control rats throughout the entire ipsilateral hippocampus. CN, caudate nucleus; BLA, basolateral amygdala; dHip, dorsal hippocampus. *$P < 0.05$.

the BLA- and dHip-kindled rats had significantly more BDNF protein in the hippocampus than did the control or CN-kindled rats (P values < 0.001). Importantly, the CN-kindled rats did not differ from the control rats on this measure ($P = 0.985$), a finding that paralleled normal rates of neurogenesis and dendritic outgrowth in these subjects.

4.3.5 Effect of kindling on hippocampal-dependent trace fear conditioning

To assess whether aberrant neurogenesis following limbic, but not nonlimbic, kindling may contribute to cognitive dysfunction, we conducted hippocampal-dependent trace fear conditioning within 24 h of the final kindling stimulation. Figure 4-5 shows the percent of time spent freezing by the rats in each group during training and testing in the trace fear conditioning task. For the sake of simplicity, we averaged freezing across all tones and post-tone periods. Kindling had no effect on freezing during training as all groups showed similar levels of freezing during the tones $F(3,47) = 0.185$, $P = 0.906$ and the post-tone intervals $F(3,47) = 0.642$, $P = 0.592$ (Fig. 4-5A). However, we found significant group differences during the contextual memory retrieval test $F(3,47) = 13.303$, $P < 0.001$ (Fig. 4-5B). Subsequent post hoc analyses revealed that the BLA- and dHip-kindled rats froze significantly less than the control or CN-kindled rats (P values < 0.009) during this test.

4.4 Discussion

The results of this experiment reveal site-specific effects of kindled seizures on adult hippocampal neurogenesis, hippocampal BDNF protein levels, and hippocampal-dependent learning and memory. We noted several distinct findings. First, our cell counts revealed that the BLA- and dHip-kindled rats had significantly more BrdU-ir cells than did the control or CN-kindled rats. Interestingly, the BLA- and dHip-kindled rats also had more BrdU-ir ectopic granule neurons located in the hilus-CA3 border. Interestingly, the number of BrdU-ir cells in the SGZ/GCL and hilus-CA3 was positively correlated with seizure severity on the day of BrdU injections, but not previous seizure history. Second, we found that the BLA- and dHip-kindled rats had significantly more DCX-ir neurons located in the proliferative SGZ/GCL compared to the control and CN-kindled rats. We also noticed qualitative differences in the appearance of DCX-ir neurons in limbic-kindled rats in that they had enhanced apical dendrite complexity and
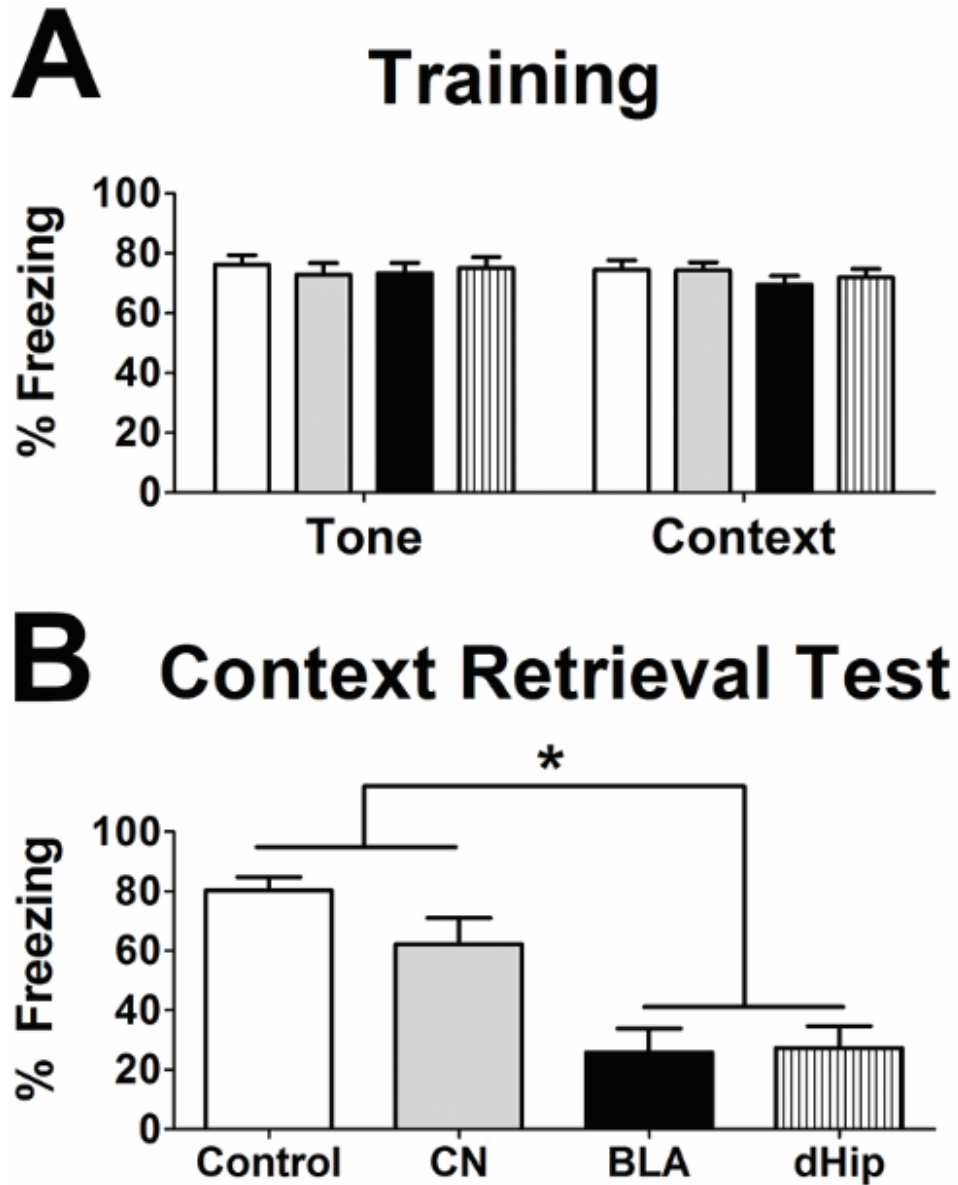
Figure 4-5. Effect of kindling on hippocampal-dependent fear learning and memory. **(A)** Kindling did not affect the acquisition of the fear association. **(B)** Limbic kindling severely impaired the retrieval of contextual fear memories. CN, caudate nucleus; BLA, basolateral amygdala; dHip, dorsal hippocampus.*$P < 0.05$.

they contained hilar basal dendrites, whereas the DCX-ir neurons from control or CN-kindled rats did not. Third, we found that the BLA- and dHip-kindled rats had significantly higher hippocampal BDNF protein levels. And finally, we found that the BLA- and dHip-kindled rats were impaired on a hippocampal-dependent trace fear conditioning task. Importantly, the CN-kindled rats were not significantly different from controls on any of these measures. Overall, these results suggest that limbic kindling promotes aberrant neurogenesis, possibly via enhanced BDNF levels, and that this aberrant neurogenesis may lead to cognitive impairments.

The results of our study suggest a complex relationship between site-specific seizure activity and adult hippocampal neurogenesis. On the suface, the lack of changes in the number of BrdU-ir cells or DCX-ir in the CN-kindled rats seems to suggest that seizure activity outside the limbic region is not sufficient to stimulate adult hippocampal neurogenesis. However, one important variable to consider is that the BLA- and dHip-kindled rats differed from the CN-kindled rats in terms of seizure severity as well as the location of seizure activity in the brain. This raises the question of whether seizure severity might govern the effects of seizures on hippocampal neurogenesis. We calculated several correlations to begin to address this question. Our analyses revealed that seizure severity across groups on the day of BrdU injections was positively correlated with the number of BrdU-ir cells. This suggests that in general, more severe convulsions elicit greater increases in the proliferation of adult hippocampal progenitor cells. Interestingly, our correlational analyses also suggest that previous seizure history was less of a factor in determining the amount of cell proliferation. Specifically, the BLA-kindled rats had significantly more stage 5 convulsions prior to the BrdU injections than did the dHip-kindled rats (see section 3.1), but both groups had roughly the same number of BrdU-ir adult generated neurons at the end of kindling. This likely points to metabolic limits in the degree to which neurogenesis can be increased. That is, the occurrence of severe convulsions on the day of BrdU injections may already maximize the rate of cell proliferation. This interpretation is intriguing because if it is correct, it suggests that enhanced hippocampal cell proliferation is stimulated by acute seizure activity itself and not by any pathology associated with the epileptic state.

Our findings address a central issue in many previous investigations of seizures and hippocampal neurogenesis. Specifically, significant inflammation and overt brain damage associated with status epilepticus in some animal models of epilepsy (Turski et al., 1983) can mask the influence seizures might have on progenitor activation, proliferation, and subsequent

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survival of adult generated neurons. These confounds can make it difficult to determine whether seizures increase neurogenesis because of activation of neural progenitors or whether this is a response to significant cell death. We recently reported that Fluoro-Jade B staining, a marker of neuronal degeneration and apoptosis, is essentially absent in BLA-, dHip-, and CN-kindled rats after 99 electrical stimulations, but it is pronounced in rats treated with lithium pilocarpine (Botterill et al., 2015). Kindling thus provides the opportunity to investigate whether seizures originating within specific brain sites influence hippocampal neurogenesis without the complication of overt brain damage.

The results of this experiment suggest that CN kindling likely activates brain circuits different from the ones activated by BLA or dHip kindling. The caudate nucleus is part of the caudate-putamen, often referred to as the dorsal striatum. The primary outputs of the dorsal striatum are the globus pallidus (entopeduncular nucleus) and substantia nigra pars reticulate (SNr) (Gerfen, 1985). The SNr projects to the anterior thalamus, which innervates the prefrontal cortex. In contrast, the basolateral and central nuclei of the amygdala receive sensory information from the thalamus and cortex and affective/visceral information from the substantia nigra, ventral tegmental area, and nucleus accumbens (Ghiglieri, Sgobio, Costa, Picconi, & Calabresi, 2011). In turn, the amygdala projects to the subiculum, entorhinal cortex, and ventral hippocampus (Pitkanen et al., 2000). Within the hippocampal formation, the entorhinal cortex sends information to the dentate gyrus via the perforant path, which then projects to the CA3 and CA1 areas to the subiculum, and then back to the entorhinal cortex (Ghiglieri et al., 2011). These differences in anatomical projections may explain why CN-kindled rats had convulsions primarily characterized as cortical or neocortical, whereas BLA- and dHip-kindled rats had traditional limbic seizures. Indeed, it is likely that kindling of the CN was ineffective at propagating epileptiform activity in the hippocampus. Instead, epileptiform activity after stimulation of the CN was likely contained within cortical and neocortical circuits. Although it is an oversimplification to suggest that electrical stimulation activates only the immediate downstream targets associated with each of these discrete brain sites, it is not unreasonable to assume that stimulation of a discrete brain site will affect downstream brain areas within its immediate vicinity. With this caveat, it is also probable that afterdischarge activity from kindling stimulations likely propagates into other brain sites not mentioned above. Unfortunately, we were not able to record EEG activity during kindling in this experiment, but this is a logical next

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step to confirm the pattern of afterdischarge spread after kindling of limbic and nonlimbic brain sites.

It is worth noting that the BLA- and dHip-kindled rats groups had a large number of hilar-CA3 ectopic granule neurons, with the dHip-kindled rats having relatively more ectopic neurons than the BLA-kindled rats (albeit a nonsignificant increase). Previous reports have indicated that limbic seizures downregulate the expression of migratory proteins, such as the extracellular matrix protein reelin and the susceptibility gene disrupted in schizophrenia 1 (DISC1), in the SGZ or GCL (Fournier et al., 2010; Frotscher et al., 2003; Gong et al., 2007). This likely contributes to aberrant migration of adult generated neurons after limbic kindling. It would be interesting to investigate whether infusions of recombinant reelin into the hippocampus during the course of kindling would reduce the number of ectopic hilar-CA3 neurons.

Our analyses of DCX-ir complexity revealed that limbic kindling significantly enhanced dendritic outgrowth. We found that limbic-kindled rats had few proliferative category 1 or 2 cells but many postmitotic category 5 or 6 DCX-ir cells. Interestingly, limbic-kindled rats also had significantly more DCX-ir cells containing basal dendrites. These findings are consistent with previous reports that seizures facilitate dendritic outgrowth and the presence of hilar basal dendrites (Fournier et al., 2010; Jessberger et al., 2007b; Overstreet-Wadiche et al., 2006; Shapiro & Ribak, 2006). What mechanisms may account for this hypertrophic dendritic growth? Increases in BDNF protein following seizures are well characterized and known to stimulate hippocampal neurogenesis and the growth of basal dendrites (Danzer et al., 2002; Scharfman et al., 2005). Furthermore, it was demonstrated that locally synthesized hippocampal BDNF from the dendrites of mature granule cells enhances differentiation and maturation of neuronal progenitor cells by enhancing GABA release (Waterhouse et al., 2012). Our findings are consistent with these observations, as the BLA- and dHip-kindled rats had significantly greater hippocampal BDNF protein levels, which paralleled increased proliferation and dendritic outgrowth of immature neurons. In contrast, the CN-kindled rats showed no change in BDNF levels above the control rats, and they also showed no enhancement of neurogenesis or dendritic outgrowth. We have recently reported that limbic, but not nonlimbic, kindling significantly enhances hippocampal neuropeptide Y (NPY) immunoreactivity (Botterill et al., 2015). This finding is particularly relevant, as BDNF is known to induce a long-lasting overexpression of NPY (Reibel et al., 2000), and NPY is known to act as an endogenous anticonvulsant mechanism

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(Vezzani & Sperk, 2004) that protects against cell loss from excitotoxicity (Xapelli, Agasse, Ferreira, Silva, & Malva, 2006; Xapelli et al., 2008), and facilitates adult hippocampal neurogenesis (Howell et al., 2007; Scharfman & Gray, 2006). The interplay between BDNF and NPY in limbic-kindled rats may provide a mechanism through which adult hippocampal neurogenesis is exaggerated following limbic seizures, but not nonlimbic seizures.

The presence of hilar-CA3 ectopic granule cells and hilar basal dendrites represents aberrant features of neurogenesis that may interfere with normal hippocampal function. Our observations that limbic-kindled rats had significantly more BrdU-ir hilar-CA3 ectopic granule cells and DCX-ir neurons containing hilar basal dendrites paralleled significant memory impairments on the trace fear conditioning memory test. It was recently shown that the presence of a small population of hilar-CA3 ectopic granule cells can disrupt pattern separation and completion -- processes that are critical for hippocampal-dependent spatial learning and memory (Myers, Bermudez-Hernandez, & Scharfman, 2013). Our behavioral results in combination with our postmortem analyses support this notion. We have previously reported that mature 4-week-old adult generated hippocampal neurons born under conditions of BLA kindling show reduced colocalization with the behaviorally relevant immediate early gene c-Fos during contextual fear memory retrieval (~1% active), whereas approximately 10% of the same birth-dated population is active in controls (Fournier et al., 2013). These findings suggest that despite significant enhancement of neurogenesis following limbic seizures, the aberrant characteristics these adult generated neurons develop may impair their functional recruitment into memory networks. Normalizing or stabilizing adult hippocampal neurogenesis and limiting its aberrant features may therefore represent a therapeutic approach to ameliorate these deficits. In line with this idea, it was previously shown that blocking seizure-induced neurogenesis with the histone deacetylase (HDAC) inhibitor valproic acid (VPA) significantly attenuated cognitive deficits on a hippocampal-dependent object recognition task (Jessberger et al., 2007a). Future studies should use deletion methods to determine whether ablation of adult hippocampal neurogenesis following seizures can ameliorate cognitive deficits.

4.5 Conclusions

Our results indicate that limbic seizures dramatically increase adult hippocampal neurogenesis and BDNF protein levels, whereas nonlimbic seizures do not. Accelerated

hippocampal neurogenesis and dendritic complexity in limbic-kindled rats paralleled elevated levels of hippocampal BDNF protein and coincided with significant impairments on a hippocampal-dependent memory task. Collectively, these results suggest that limbic kindling facilitates adult hippocampal neurogenesis and promotes aberrant characteristics within newborn neurons. The aberrant features of neurogenesis that are unique to limbic-kindled rats may in part explain the cognitive and behavioral comorbidities associated with TLE and why it is one of the most difficult forms of focal epilepsy.

Acknowledgements

Conflict of interest

The authors declare no conflicts of interest.

CHAPTER 5

Selective plasticity of hippocampal GABAergic interneuron populations following kindling of different brain regions

J.J. Botterill [a], N. Nogovitsyn [b], H.J. Caruncho [c], L.E. Kalynchuk [b]

[a] Department of Psychology, University of Saskatchewan, Saskatoon, SK, S7N 5A5, Canada

[b] Department of Medicine, University of Saskatchewan, Saskatoon, SK, S7N 5E5, Canada

[c] College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, S7N 5E5, Canada

5.1 Introduction

Gamma-aminobutyric acid (GABA) inhibitory interneurons are critical in regulating neuronal excitability in the brain. A myriad of factors can alter interneuron distribution and function, but one noteworthy consideration is epilepsy (Treiman, 2001). Indeed, the vulnerability of hippocampal interneurons is well documented in human patients with intractable seizures and rodent models of temporal lobe epilepsy (Babb, Pretorius, Kupfer, & Crandall, 1989; de Lanerolle et al., 1989; Houser & Esclapez, 1996; Marx, Haas, & Haussler, 2013; Sloviter, 1987). However, the functional consequences of seizures on GABAergic interneuron distribution and their complex rewiring remain topics of vigorous scientific debate. Although numerous studies indicate that seizures cause a reduction of hippocampal GABA-mediated inhibition (Cossart et al., 2001; Hellier et al., 1999; Houser & Esclapez, 1996; Marx et al., 2013; Sloviter, 1987), several reports suggest that seizures paradoxically enhance hippocampal GABA-mediated inhibition (Cossart et al., 2001; Nusser, Hajos, Somogyi, & Mody, 1998; Otis, De, & Mody, 1994; Tuff, Racine, & Mishra, 1983; Tuff, Racine, & Adamec, 1983; Wu & Leung, 2001). One likely explanation for these contradictory results may be the use of different animal models that produce varying degrees of pathology. Moreover, the inconsistent findings further demonstrate the diversity of GABAergic mechanisms that are involved in the development and maintenance of seizure disorders (Houser, 1991). In this regard, our understanding of the vulnerability of specific interneuron types and the complex rewiring they undergo remains poorly understood across different models of epilepsy. This is an important issue to study further, as the selective loss and/or rewiring of different functional interneuron populations has broad implications for the role of GABA in the pathogenesis of epilepsy (Cossart, Bernard, & Ben-Ari, 2005).

Although the majority of interneurons express glutamic acid decarboxylase (GAD), the major enzyme involved in the synthesis of the inhibitory neurotransmitter GABA (Erlander et al., 1991), it is widely recognized that interneurons represent a diverse population of cells that vary in respect to their axonal projections, termination zones, and neuropeptide or calcium-binding protein content. To simplify their diversity, interneurons are often divided into three broad functional groups: dendritic, perisomatic, and interneuron-selective (Bausch, 2005). Dendritic-targeting interneurons primarily innervate the distal dendrites of principal cells to control the generation of calcium spikes and synaptic plasticity (Miles et al., 1996). Interneurons that express the neuropeptide somatostatin (SOM) are classified as dendritic-targeting

interneurons. Perisomatic-targeting interneurons innervate the soma and proximal dendrites (basket cells) or axon initial segments (axo-axonic cells) of principal cells to regulate their output through feedback inhibition. The calcium-binding protein parvalbumin (PV) is the most common type of perisomatic-targeting interneuron. Interneuron-selective interneurons contribute to the regulation of dendritic inhibition through their preferential innervation of other interneurons (Gulyas et al., 1996; Toth & Magloczky, 2014). Cells that express the calcium-binding protein calretinin (CR) are generally classified as interneuron-selective interneurons. We chose to study SOM, PV, and CR interneurons in the present study, as these three different functional classes of interneurons are differentially susceptible to seizures and display varying degrees of seizure-induced plasticity (Long et al., 2011; Marx et al., 2013; Toth & Magloczky, 2014; Zhang et al., 2009).

In this experiment, we utilized the site-specific nature of electrical kindling to determine whether convulsions that originate in different brain regions have differential effects on hippocampal interneuron distribution and morphology. In particular, we kindled limbic (basolateral amygdala; BLA, dorsal hippocampus; dHip) and non-limbic (caudate nucleus; CN) brain regions followed by postmortem immunohistochemistry to evaluate markers of dendritic (SOM), perisomatic (PV), and interneuron-selective (CR) inhibition. The use of these different markers allowed us to ascertain whether certain functional classes of GABA-mediated inhibition are susceptible to kindled seizures. Furthermore, to evaluate if kindling causes sprouting of specific hippocampal interneuron subpopulations as reported in other rodent models of epilepsy (Bausch, 2005; Long et al., 2011; Zhang et al., 2009), we analyzed surface area measurements of SOM, PV, and CR interneurons. Finally, we evaluated GAD67 expression to determine whether kindling had an overall effect on hippocampal interneuron distribution and number. We have published a series of papers describing how kindling of limbic, but not non-limbic, brain regions produce significant alterations in cognitive function and hippocampal plasticity (Botterill, Brymer, Caruncho, & Kalynchuk, 2015; Botterill et al., 2015; Fournier et al., 2013; Kalynchuk et al., 1998). However, we are unaware of any studies that have compared the effects of kindling different brain regions on different functional classes of hippocampal interneurons. This issue is important to further investigate because subfield-specific changes in the number or morphology of interneuron subpopulations can dramatically alter hippocampal circuits, which have broad functional implications for the treatment of epilepsy and its associated cognitive impairments.

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5.2 Materials and Methods

5.2.1 Animals

We used male Long-Evans rats from Charles River (Quebec, Canada) that weighed approximately 200-250g at the time of arrival (approximately 1.5 months old). Rats were individually housed in rectangular polypropylene cages with standard laboratory bedding and provided access to food and water ad libitum. The colony room was maintained at an ambient temperature of 20±1°C with a 12 h light-dark cycle (lights on at 7 a.m.). All procedures were conducted during the light period of the light-dark cycle. All experimental manipulations were in accordance with the guidelines of the Canadian Council on Animal Care and a protocol approved by the University of Saskatchewan Committee on Animal Care and Supply. We made all possible efforts to minimize the number of animals used in the study. A total of two rats were removed from the study due to incorrect electrode placement or head cap loss during kindling.

5.2.2 Surgery

Surgery was conducted as previously described (Botterill et al., 2015). Briefly, each rat was deeply anesthetized with a mixture of isoflurane and oxygen (5% initial, 2-2.5% maintenance) and secured into a stereotaxic apparatus using ear bars. Each rat received a preoperative analgesic (Anafen, Ketoprofen, 10mg/kg s.c.) to reduce pain and inflammation from subsequent procedures. At a flat skull position, a small incision was made down the scalp and surrounding connective tissue was excised. A single bipolar stainless steel electrode (MS-303-2-B-SPC, Plastics One) was stereotaxically implanted into one of three discrete brain regions, relative to bregma: CN (0.2 mm anterior, 3.2 mm lateral, 5.7 mm ventral), BLA (2.8 mm posterior, 5.0 mm lateral, 8.5 mm ventral) or dHip (3.5 mm posterior, 2.6 mm lateral, 3.1 mm ventral) (Paxinos & Watson, 1998). Each electrode assembly was secured to the skull with four stainless steel jeweler screws (0-80 X 3/32, Plastics One) and dental acrylic. To minimize the risk of post-surgical infection, each rat received daily topical administration of antibacterial-antifungal ointment (Chlorhexidine acetate, B.P. 1% w/w) around the incision site for a minimum of one week.

5.2.3 Kindling

All rats received a postsurgical recovery period of 1 week and were then randomly assigned to CN (n = 11), BLA (n = 10), or dHip (n = 10) kindling groups. Control rats (n = 11) included a set of CN, BLA, and dHip electrode-implanted rats. Kindling was conducted as previously described (Botterill et al., 2014). Briefly, electrical stimulations were delivered in a procedures room separate from the room in which the rats were housed. For each kindling session, a wire lead connected to an isolated pulse stimulator (Model 2100, A-M Systems, Sequim, WA, USA) was attached to the electrode assembly of each rat to deliver a 1 s, 60 Hz train of square-wave pulses with a biphasic amplitude of 800 µA (peak-to-peak) and duration of 1 ms. A total of 99 electrical stimulations were delivered on a 3-times-per-day, 5-days-per-week schedule (i.e., 15 stimulations per week), with a minimum of 3 h between consecutive stimulations. Control rats were connected to the electrode lead on the same schedule, but they never received any electrical stimulation. The convulsion elicited by each stimulation was scored using a revised 8 class extension (Pinel & Rovner, 1978) of Racine's original 5 class convulsion scale (Racine, 1972). The convulsion classes were operationally defined as follows: class 0: immobility; class 1: orofacial automatisms; class 2: orofacial automatisms with head nodding; class 3: unilateral forelimb clonus; class 4: rearing with bilateral forelimb clonus; class 5: rearing with bilateral forelimb clonus and loss of equilibrium; class 6: multiple class 5 convulsions and falling episodes; class 7: previous classes with running fit; and class 8: previous classes with intermittent periods of muscle tonus. Under these classification criteria, rats are considered fully "kindled" after 3 consecutive Class 5 convulsions (Pinel & Rovner, 1978).

5.2.4 Perfusions

A subset of rats were sacrificed for postmortem immunohistochemical analyses within 24 h of the final kindling stimulation: control (n = 7), CN (n = 7), BLA (n = 6) and dHip (n = 6). Each rat was deeply anesthetized with sodium pentobarbital (240mg/kg; i.p.) and then transcardially perfused using room-temperature physiological saline, followed by ice-cold 4% (w/v) formaldehyde fixative (pH = 7.4). The brains were then extracted from the cranial vault and immersed in the same formaldehyde fixative for 48 h at 4 °C prior to coronal sectioning at 50 µm on a vibrating microtome (VT1200S, Leica Biosystems, Nussloch, Germany). Sections were collected and stored at -20 °C in a cryoprotectant solution consisting of 30% (w/v) sucrose, 1%(w/v) polyvinylpyrrolidone, and 30% (v/v) ethylene glycol in 0.1 M PBS (pH = 7.4).

5.2.5 Antibody Characterization

The primary antibodies used in this study are listed in Table 5-1.

5.2.5.1 Somatostatin

Polyclonal anti-somatostatin (Bachem/Peninsula Laboratories; catalog No. T-4103, RRID: AB_518614) is raised in rabbit against a synthetic peptide that contains the first 14 amino acids of SOM as the immunogen. Radioimmunoassays indicate this antibody has 100% cross reactivity with SOM-14, SOM-28, and SOM-25 and 0% cross reactivity with substance-P, neuropeptide Y, vasoactive intestinal peptide, insulin, or glucagon (manufacturer's specifications). The specific of this antibody has been further demonstrated in SOM-deficient mice (Saito et al., 2005). We observed a distribution of antigen immunoreactivity that is consistent with previous reports (Gill, Ramsay, & Tasker, 2010; Peng et al., 2013; Saito et al., 2005).

5.2.5.2 Parvalbumin

Mouse monoclonal anti-parvalbumin (Sigma Aldrich; catalog No. P3088; RRID: AB_477329) is derived from the PARV-19 hybridoma produced by the fusion of myeloma cells and splenocytes from an immunized mouse. The immunogen is purified frog muscle PV. This antibody recognizes PV in a Ca^{2+}-ion dependent manner and is reactive with human, bovine, goat, pig, rabbit, dog, cat, rat, frog and fish samples (manufacturer's specifications). This PV antibody does not react with other members of the EF-hand family of calcium binding proteins (manufacturer's specifications). Immunoprecipitation experiments revealed this antibody produces a single 12 kDa band in rat brain extracts and an absence of immunoreactivity following preabsorption of the antiserum with the antigen (Celio & Heizmann, 1981). We observed a distribution of antigen immunoreactivity that is consistent with previous reports (Sun et al., 2007).

5.2.5.3 Calretinin

Polyclonal anti-calretinin (Millipore; catalog No. AB5054; RRID: AB_2068506) is raised in rabbit against a full length sequence of recombinant rat calretinin. This antibody recognizes calcium-bound and calcium-unbound conformations of calretinin in human, rat, and mouse tissues as indicated by western blots (manufacturer's specifications). Specificity of this antibody was shown through detection of a single band of $\approx$ 30 kDa on western blots using hippocampal

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Table 5-1. Primary Antibodies Description and Parameters

Antigen	Abbreviation	Host Species	Clonality	Dilution	Immunogen	Catalog/clone#, RRID	Source
Somatostatin	SOM	Rabbit	Polyclonal	1:1,000	Synthetic peptide against the first 14 amino acids of somatostatin	Cat #T-4103 RRID: AB_518614	Bachem/Peninsula Laboratories, San Carlos, CA, USA
Parvalbumin	PV	Mouse	Monoclonal	1:1,000	Frog muscle parvalbumin	Cat #P3088 clone PARV-19 RRID: AB_477329	Sigma Aldrich, St. Louis, MO, USA
Calretinin	CR	Rabbit	Polyclonal	1:1,000	Full length sequence recombinant rat calretinin	Cat #AB5054 RRID: AB_2068506	Millipore, Bedford, MA, USA
GAD67	GAD67	Mouse	Monoclonal	1:1,000	Recombinant fusion protein containing the N-terminal regions of human GAD67 (amino acids 4-101).	Cat #MAB5406 clone 1G10.2 RRID: AB_2278725	Millipore, Bedford, MA, USA

mouse extracts (Su, Gorse, Ramirez, & Fox, 2010). We observed a distribution of antigen immunoreactivity that is consistent with previous reports (Cho et al., 2011; Su et al., 2010).

5.2.5.4 GAD67

Mouse monoclonal anti-GAD67 (Millipore; catalog No. MAB5406; clone 1G10.2; RRID: AB_2278725) is derived from a recombinant fusion protein containing the N-terminal regions of human GAD67 (amino acids 4-101). This antibody has no cross-reactivity with the 65-kDa isoform of GAD in rat brain lysates (manufacturer's specifications). Western blot analysis of rat hippocampal protein extract was previously reported to produce a single band at 67 kDa (Stranahan, Haberman, & Gallagher, 2011). The distribution of hippocampal antigen immunoreactivity in our tissue was consistent with previous reports (Spiegel, Koh, Vogt, Rapp, & Gallagher, 2013).

5.2.6 Immunohistochemistry

We used a standard immunohistochemistry technique with widely used and commercially available antibodies for all experiments. Immunostaining was conducted on free-floating sections in 6-well tissue culture plates under gentle agitation during every rinse. To ensure consistent immunohistochemical processing, all sections were processed in unison with treatment groups counterbalanced across all tissue plates. To confirm the specificity of each of our antibodies, we omitted the primary antibody from an additional well of free-floating samples. In absence of the primary antibody, we did not detect any immunoreactive cells.

We conducted immunohistochemistry as previously described (Botterill et al., 2015). Briefly, endogenous peroxidase activity was blocked with 0.3% (v/v) H_2O_2 in 0.1 M TBS for 30 min. Following 0.1 M TBS rinses, the sections were then blocked for 1 h in 0.1 M TBS containing 5% normal animal serum, 1% bovine serum albumin (BSA), and 0.3% (v/v) Triton X-100 (PV, SOM, CR) or 0.2% (v/v) Tween-20 (GAD67). The sections were then incubated in mouse monoclonal (1:1,000 GAD67, #MAB5406, Millipore, Bedford, MA, USA; 1:1,000 PV, #P3088, Sigma-Aldrich, St Louis, MO, USA) or rabbit polyclonal (1:1,000 SOM, #T-4103, Bachem/Peninsula Laboratories, San Carlos, CA, USA; CR 1:1,000, #AB5054, Millipore) primary antibodies diluted in blocking solution for 24 h (PV) or 48 h (GAD67, SOM, CR) at 4 °C. Following a series of 0.1 M TBS rinses, the sections were incubated for 2 h in biotinylated horse anti-mouse (1:500; PV, GAD67) or biotinylated goat anti-rabbit (1:500; SOM, CR)

secondary antibodies (Vector Laboratories, Burlingame, CA. USA) in 0.1 M TBS containing 0.3% (v/v) Triton X-100 (PV, SOM, CR) or 0.2% (v/v) Tween-20 (GAD67). The sections were then incubated for 1 h in avidin-biotin peroxidase complex (1:500; Vectastain ABC Elite, Vector Laboratories). Immunoreactivity of PV, SOM, and CR was visualized using the glucose-oxidase DAB (0.05%) method (Botterill et al., 2015; Shu, Ju, & Fan, 1988). Immunoreactivity of GAD67 was visualized with the nickel-enhanced DAB method consisting of 0.033% (w/v) DAB, 2.5% (w/v) $NiSO_4$, and 0.00786% H_2O_2 in 0.175 M sodium acetate (pH = 6.8). Sections were then mounted onto glass slides using 0.2 M PB (pH = 7.4) and left to air dry overnight. On the following morning, the sections were dehydrated using graded alcohols, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA).

5.2.7 Quantification of immunohistochemistry

Researchers blind to the treatment conditions conducted all quantitative analyses. Immunostained sections were examined using a Nikon Eclipse E800 microscope equipped with a motorized stage and digital camera (MicroFire, Optronics, Goleta, CA, USA) that was connected to a computerized stereology system (StereoInvestigator V 9.0, Microbrightfield Inc, Williston, VT, USA). For each analysis, we conducted profile counts bilaterally across 5 sections per brain with 300 μm between sections. We counted immunoreactive cells in the major hippocampal subfields, including the granule cell layer, subgranular zone, hilus, the CA1 and CA3 stratum pyramidale, oriens, and radiatum, and the CA3 stratum lucidum. For every section, we traced contours within the boundaries of each hippocampal subfield at 4X magnification using the brain atlas as a guide (Paxinos & Watson, 1998). Cells were then counted at 40X magnification using a meander scan profile counting method (Botterill et al., 2015; Knapska & Maren, 2009). The meander scan profile counting method is a semi-automated scanning method that allows the user to view and count all cells within the traced contour. To determine the density of immunoreactive cells in a given region of interest, we divided the total number of immunoreactive cells in that region by the total area measurement of the traced contour (in μm^2). Our cell counts are expressed as the average number of cells per mm^2.

In addition to quantitative cell counting, we conducted surface area measurements of immunoreactive cells throughout the dentate gyrus. We used the same slides that were used for cell counting and captured grayscale images at 20X magnification. The surface area of

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immunoreactive cells were traced using ImageJ (V 1.47, National Institutes of Health, Bethesda, MD, USA; RRID: nif-000-30467) across a minimum of 3 sections per subject. We only analyzed cells with clearly defined somas and arborizations that did not overlap with neighboring cells to prevent sampling errors. Due to immunohistochemical differences in the distribution of immunoreactive cells that met this criteria, we analyzed an average of 200 SOM-, 85 CR-, and 75 PV-immunoreactive cells per subject. Values are expressed as the percentage of control.

5.2.8 Protein Extraction & Western Blot analysis

A subset of rats (n = 16; 4 per group) were sacrificed for western blot analysis. Within 24 h of the final kindling stimulation, each rat was deeply anesthetized with an overdose of sodium pentobarbital (240 mg/kg; i.p.) and decapitated with a standard rodent guillotine. Each brain was removed from the cranial vault and the entire hippocampus was rapidly dissected, snap frozen in liquid nitrogen, and stored at -80 °C until use. Hippocampal tissue was mechanically homogenized in a 0.3 M sucrose Tris-EDTA solution containing a protease inhibitor cocktail (cOmplete Mini Protease Inhibitor Cocaktail, Roche Diagnostics, Laval Quebec, Canada). Homogenized tissue samples were then centrifuged at 1000 rcf for 5 min at 4 °C, the supernatant was collected, and protein concentrations were determined using a commercially available BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

A total of 60μg of protein from the left hippocampus (i.e., electrode-implanted hemisphere) of each sample was diluted in Laemmli Sample Buffer (Bio Rad, Mississauga, Ontario, Canada) and 5% 2-mercaptoethanol at a 1:1 ratio. Each sample was denatured for 10 min at 95 °C and resolved on 10% sodium dodecyl sulfate polyacrylamide running gels. The protein was then transferred onto nitrocellulose blotting membranes and blocked for 1 h in tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% non-fat milk to prevent non-specific antibody binding. The membranes were then probed with monoclonal mouse anti-GAD67 primary antibody (1:,1000; Millipore) overnight at 4 °C under gentle agitation. The membranes were then incubated in goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2,000; Santa Cruz Biotechnology; Santa Cruz, CA, USA) diluted in TBST and 5% non-fat milk. The membranes were re-probed for 1 h with mouse anti-β-actin primary antibody (1:10,000; Millipore) and goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10,000; Santa Cruz Biotechnology) to visualize protein loading.

Immunoreactivity of the primary antibodies was visualized with Amersham enhanced chemiluminescence (ECL) plus reagents (GE Healthcare; Baie d'Urfe, Quebec, Canada) on autoradiography X-ray film (Amersham Hyperfilm ECL; GE Healthcare). The films were digitized using a high-powered scanner and optical densities were determined using ImageJ software (National Institutes of Health). Single autoradiographic signal bands of 67 kDa (GAD67) and 42 kDa (β-actin) were identified and quantified. The signal value for each band was normalized against β-actin to verify equivalent loading of protein across samples. Each sample was analyzed in duplicate and the results represent the average score from both experiments. The graph values are expressed as the percentage of control.

5.2.9 Image Processing

All digital photomicrographs were taken on a Nikon Eclipse E800 microscope. For publication purposes, the brightness and contrast of each representative photomicrograph was adjusted equally across treatment conditions using Adobe Photoshop CS6 (Adobe Systems Inc, San Jose, CA, USA; RRID: SCR_014199). To clearly demonstrate the surface area of immunoreactive interneurons in Figures 5-2, 5-4, and 5-6, we generated image stacks (i.e., photomicrographs taken at every 2 μm throughout the focal plane) using the image stacking software CombineZP (Hadley, 2010). No other manipulations were made to the figures.

5.2.10 Statistics

All statistical analyses were conducted with IBM's Statistical Package for Social Sciences (V. 20). Group differences were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc tests when appropriate. The criterion for statistical significance was set at $p < 0.05$. All graphs depict mean ± standard error of the mean.

5.3 Results

5.3.1 Kindling Progression

Kindling progressed normally in all rats. The number of stimulations required to elicit the first class 5 convulsion was 13.9 ± 1.4 for the BLA-kindled rats and 43.4 ± 3.5 for the dHip-kindled rats. The number of electrical stimulations required to achieve the fully kindled state was 17.1 ± 1.5 for the BLA-kindled rats and 49.0 ± 3.1 for the dHip-kindled rats. Finally, the average total number of class 5 or higher convulsions was 76.4 ± 4.0 for the BLA-kindled rats and $46.1 \pm$

3.5 for the dHip-kindled rats. In contrast to the generalized convulsions elicited by kindling of limbic brain regions, kindling of the CN produced convulsions characterized by brief whole-body tonus and rapid falling to one side with some periods of forelimb clonus (Botterill et al., 2015). In line with previous reports, we did not observe spontaneous seizures at any point during our study (Kalynchuk et al., 1997; Kalynchuk & Meaney, 2003; Kalynchuk et al., 2006; Pinel & Rovner, 1978).

5.3.2 Somatostatin

Figure 5-1 shows the effect of different site kindling on the number of SOM-ir interneurons across several regions of the hippocampus. We found significant group differences in the number of SOM-ir cells in the ipsilateral [$F_{(3,25)} = 11.976$, $p < 0.001$] and contralateral [$F_{(3,25)} = 9.646$, $p < 0.001$] CA1 stratum pyramidale. Post hoc analyses revealed that the BLA- and dHip-kindled rats had significantly more SOM-ir cells in this region compared to the control and CN-kindled rats (all p values < 0.011). Furthermore, we found a significant group difference in the ipsilateral CA1 stratum oriens [$F_{(3,25)} = 3.811$, $p = 0.024$], with subsequent post hoc tests revealing that the CN-kindled rats had significantly fewer SOM-ir cells than the BLA-kindled rats ($p = 0.017$). Finally, we found a significant group difference in the contralateral CA3 stratum oriens [$F_{(3,25)} = 6.274$, $p = 0.003$] and post hoc tests revealed that the control rats had significantly more SOM-ir cells than the CN- and dHip-kindled rats (all p values < 0.045). No other group differences were detected (all p values > 0.075).

During our cell counting analyses, we noticed substantial qualitative group differences in the morphology of SOM-ir cells located in the dentate gyrus. This is shown in Fig. 5-2. We therefore analyzed cell surface area measurements to determine whether this qualitative observation was supported by quantitative differences among the groups. We found significant group differences in the surface area of SOM-ir cells in both the ipsilateral [$F_{(3,25)} = 26.863$, $p < 0.001$] and contralateral [$F_{(3,25)} = 28.442$, $p < 0.001$] hemispheres. Post hoc analyses revealed that SOM-ir cells from the BLA- and dHip-kindled rats had a significantly larger surface area than did SOM-ir cells from the control and CN-kindled rats (all p values < 0.001). The surface area of SOM-ir cells was not significantly different between the CN-kindled rats and controls (all p values > 0.865).
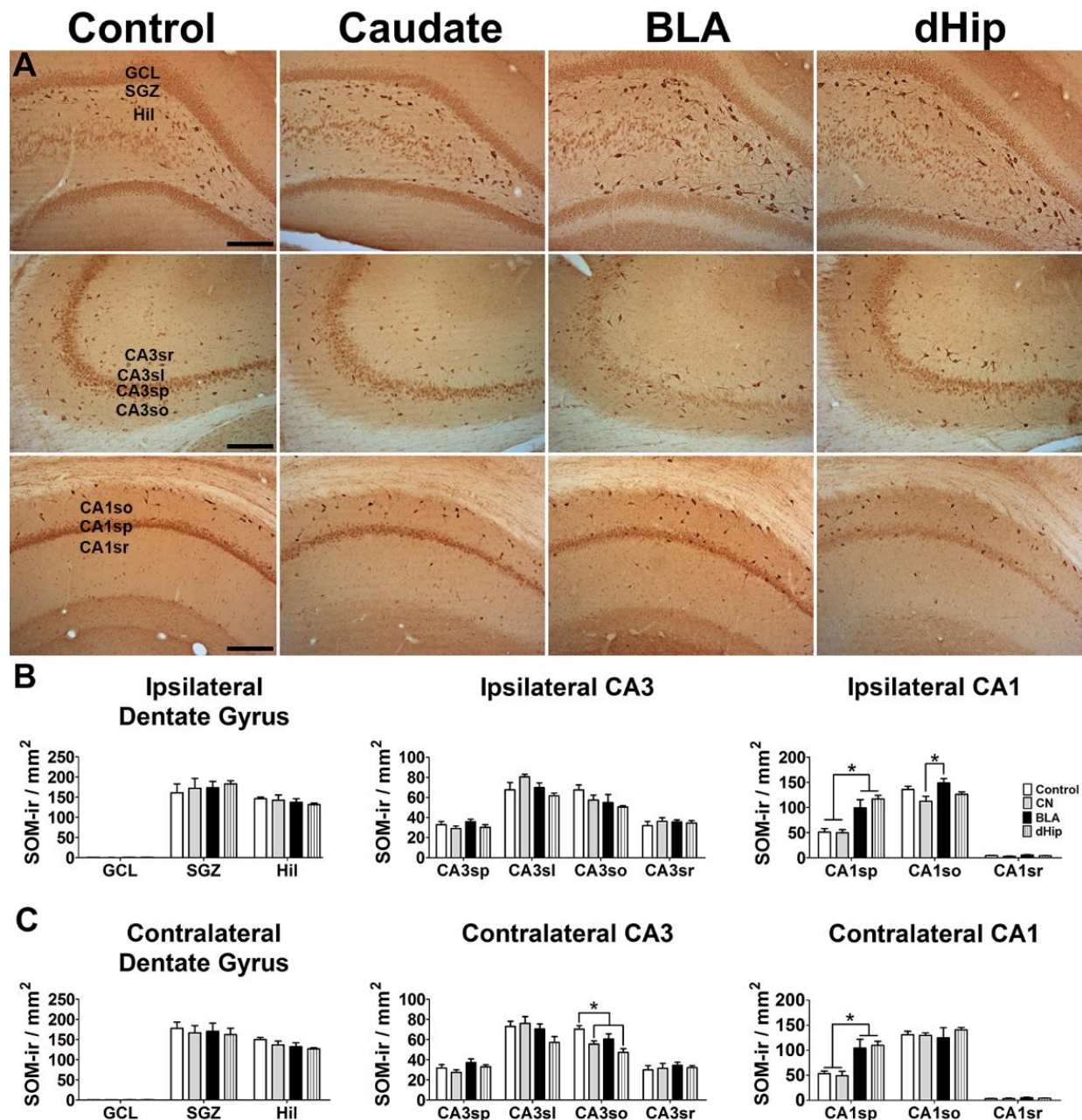
Figure 5-1. Effect of kindling on the number of somatostatin-immunoreactive interneurons in several subregions of the hippocampus. **(A)** Representative photomicrographs of the dentate gyrus, CA3, and CA1 regions from each group (Scale bar = 200μm). **(B-C)** Quantitative cell counts. Note the significant increase in somatostatin-immunoreactive interneurons bilaterally in the CA1 stratum pyramidale of the BLA- and dHip-kindled rats compared to the caudate-kindled or control rats. We also found a significant increase in somatostatin-immunoreactive cells in the BLA-kindled rats compared to the caudate-kindled rats and a significant derease in the number of somatostatin-positive cells in the caudate- and dHip-kindled rats compared to the control rats. GCL, granule cell layer; SGZ, subgranular zone; Hil, hilus; SP, stratum pyramidale; SL, stratum lucidum; SO, stratum oriens; SR, stratum radiatum. *$p < 0.05$

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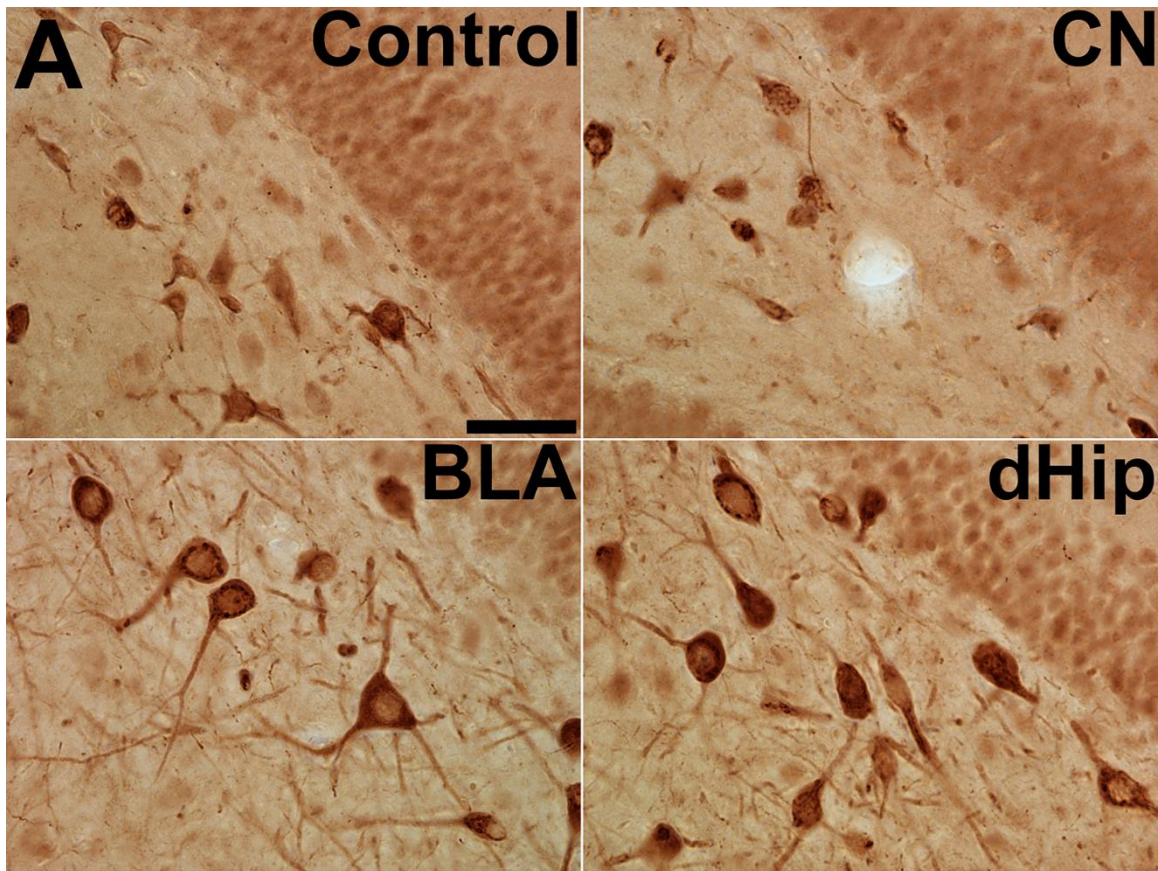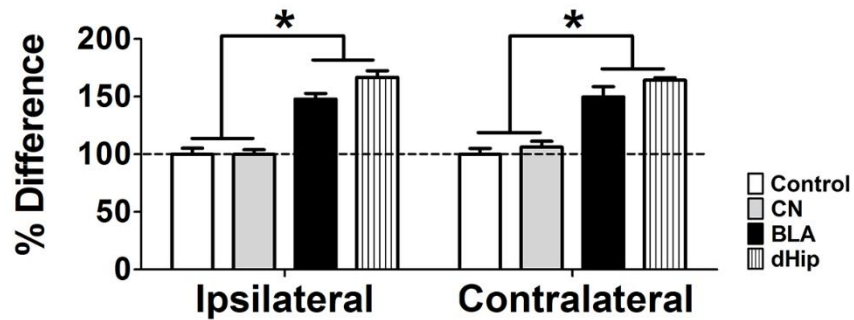
Figure 5-2. Effect of kindling on the surface area measurements of somatostatin-immunoreactive interneurons. **(A)** Representative photomicrographs demonstrate that the soma surface area of somatostatin-immunoreactive interneurons is significantly greater in the BLA- and dHip-kindled rats compared to the control and caudate-kindled rats (Scale bar = 50 μm). **(B)** Quantitative analysis of the surface area of somatostatin-immunoreactive cells normalized to the percentage of control values. * $p < 0.05$

5.3.3 Parvalbumin

Figure 5-3 shows the effect of different site kindling on the number of PV-ir interneurons across the major subregions of the hippocampus. We found a significant group difference in the number of PV-ir interneurons in the ipsilateral CA1 stratum oriens $[F(3,25) = 6.322, p = 0.003]$, with subsequent post hoc tests revealing that the dHip-kindled rats had significantly fewer PV-ir cells than did the control or CN-kindled rats in this region (all p values < 0.045). We also found a main effect in the ipsilateral CA1 stratum pyramidale $[F(3,25) = 3.116, p = 0.047]$; however, post hoc analyses revealed no significant group differences (all p values > 0.067). No other group differences were found (all p values > 0.081).

As with our analysis of SOM-ir, we also investigated whether kindling affected the surface area of PV-ir cells in the dentate gyrus. These data are shown in Fig. 5-4. We found no significant group differences in the ipsilateral $[F(3,25) = 0.835, p = 0.485]$ or contralateral $[F(3,25) = 1.229, p = 0.323]$ hemispheres for this measure.

5.3.4 Calretinin

Figure 5-5 shows the effect of different site kindling on the number of CR-ir interneurons. We found a significant group difference in the ipsilateral granule cell layer $[F(3,25) = 5.587, p < 0.005]$. Post hoc tests revealed that the CN-kindled rats had significantly more CR-ir cells than the BLA- and dHip-kindled rats (all p values < 0.030). We also found a significant group difference in the ipsilateral subgranular zone $[F(3,25) = 4.803, p < 0.010]$, with subsequent post hoc tests revealing that the CN-kindled rats had significantly more CR-ir cells than the dHip-kindled rats ($p = 0.008$). A significant main effect was also found in the ipsilateral hilus $[F(3,25) = 7.898, p < 0.001]$ and in this case post hoc tests revealed that the control and CN-kindled rats had significantly more CR-ir cells than the dHip-kindled rats (all p values < 0.008). No other group differences were found in the ipsilateral hemisphere (all p values > 0.050). In the contralateral hemisphere, we found a significant main effect in the granule cell layer $[F(3,25) = 4.693, p = 0.011]$. Post hoc tests revealed that the CN-kindled rats had significantly more CR-ir cells than dHip-kindled rats ($p = 0.045$). We also found a significant group difference in the contralateral hilus $[F(3,25) = 4.405, p = 0.014]$ and subsequent post hoc tests revealed that the CN-kindled rats had significantly more CR-ir cells than the BLA- and dHip-kindled rats (all p values < 0.048). Finally, we found a significant group difference in the contralateral CA1 stratum
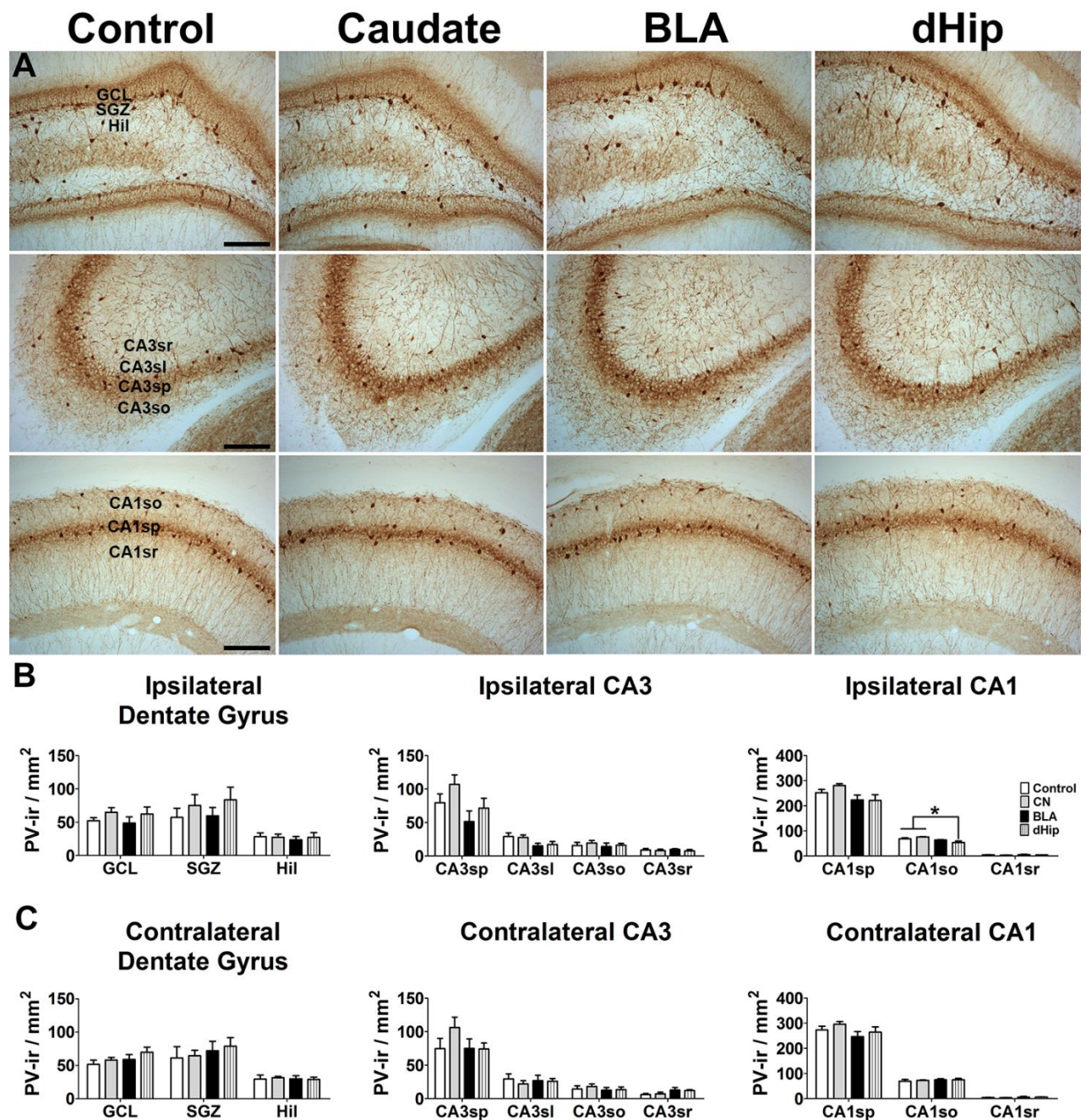
Figure 5-3. Effect of kindling on the number of parvalbumin-immunoreactive interneurons in several hippocampal subregions. **(A)** Representative photomicrographs of the dentate gyrus, CA3, and CA1 regions from each group (Scale bar = 200μm). **(B-C)** Quantitative cell counts. The dHip-kindled rats had significantly fewer parvalbumin immunoreactive interneurons in the CA1 stratum oriens compared to the control and caudate-kindled rats. GCL, granule cell layer; SGZ, subgranular zone; Hil, hilus; SP, stratum pyramidale; SL, stratum lucidum; SO, stratum oriens; SR, stratum radiatum. *$p < 0.05$
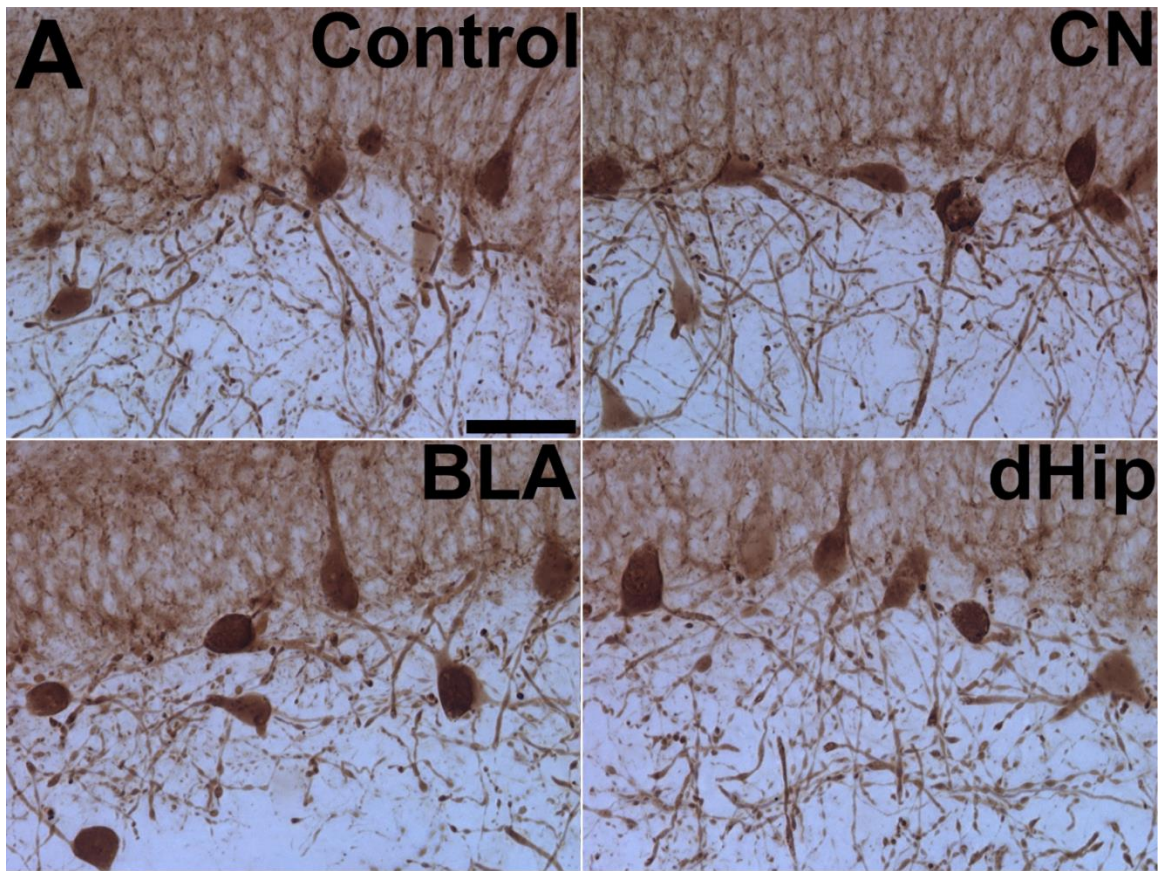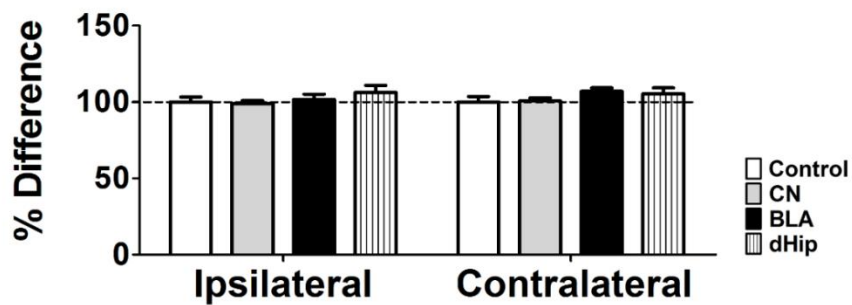
Figure 5-4. Effect of kindling on the surface area measurements of parvalbumin-immunoreactive interneurons. **(A)** Representative photomicrographs indicate that the soma surface area of parvalbumin-immunoreactive interneurons is similar across groups (Scale bar = 50 μm). **(B)** Quantitative analysis of parvalbumin-immunoreactive surface area normalized to the percentage of control values.
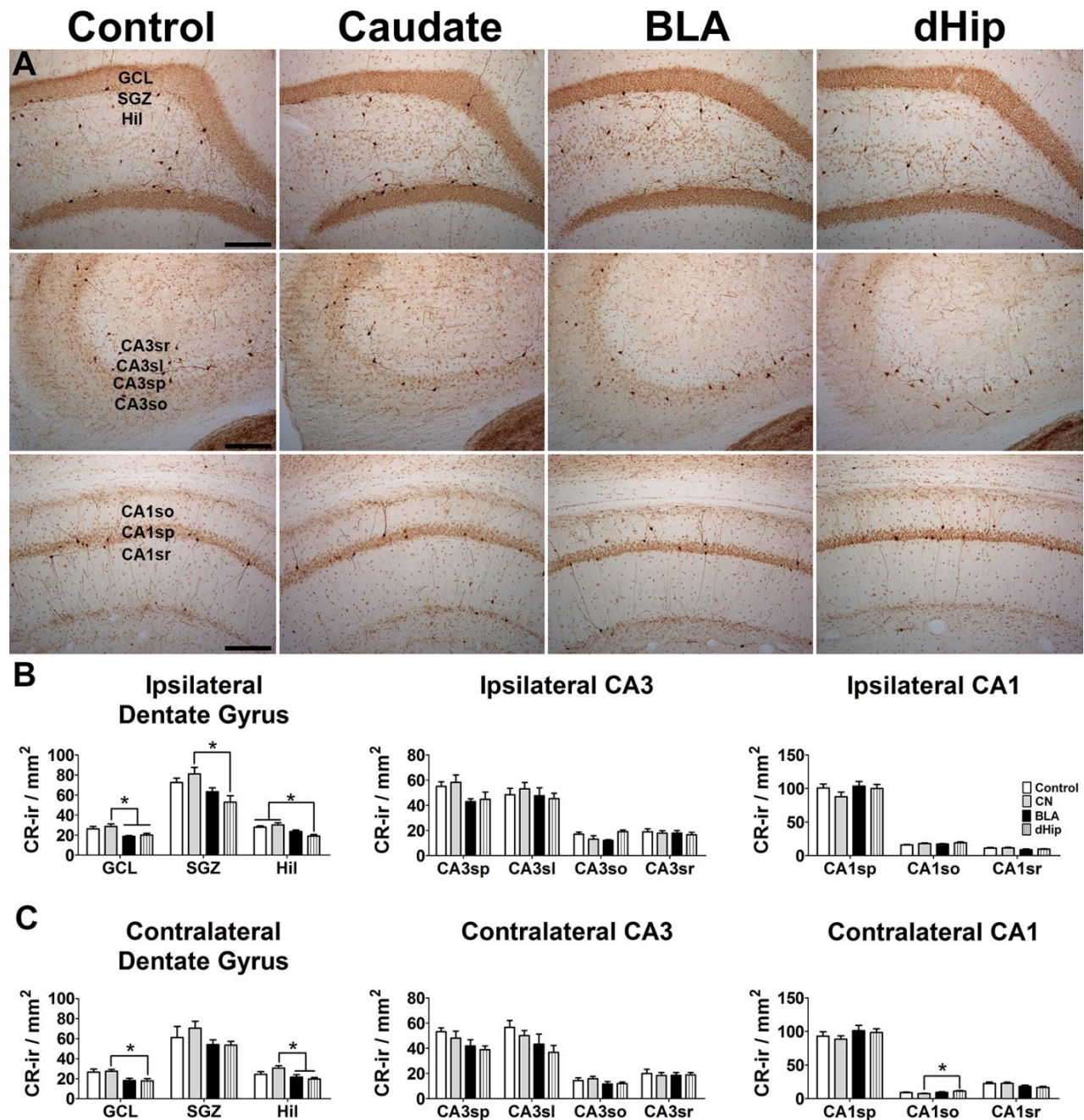
Figure 5-5. Effect of kindling on the number of calretinin-immunoreactive interneurons in several hippocampal subregions. **(A)** Representative photomicrographs of the dentate gyrus, CA3, and CA1 regions from each group (Scale bar = 200μm). **(B-C)** Quantitative cell counts revealed that the dHip-kindled rats had significantly fewer calretinin-immunoreactive interneurons in the ipsilateral hilus compared to the caudate-kindled or control rats. We also found several between-treatment differences in the bilateral dentate gyrus and contralateral CA1. GCL, granule cell layer; SGZ, subgranular zone; Hil, hilus; SP, stratum pyramidale; SL, stratum lucidum; SO, stratum oriens; SR, stratum radiatum. *$p < 0.05$

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oriens [$F_{(3,25)} = 3.740$, p = 0.026] and post hoc tests indicated that the dHip-kindled rats had significantly more CR-ir cells than the CN-kindled rats in this area (p > 0.015). We found no other significant differences in the remaining hippocampal subfields (all p values > 0.065).

Figure 5-6 shows the effect of different site kindling on CR-ir surface area. We found no significant group differences in CR-ir surface area in either the ipsilateral [$F_{(3,25)} = 0.629$, p = 0.604] or contralateral [$F_{(3,25)} = 0.416$, p = 0.743] hemispheres.

5.3.5 GAD67

In addition to examining the number and morphology of interneuron subpopulations, we also wondered whether kindling had an overall effect on the majority of hippocampal GABAergic interneurons, as indicated by GAD67-ir. Figure 5-7 shows the effect of kindling different brain regions on GAD67-ir in quantified regions of the hippocampus. We found a significant group difference in the ipsilateral granule cell layer [$F_{(3,25)} = 5.629$, p = 0.005] and subsequent post hoc tests revealed that the CN-kindled rats had significantly more GAD67-ir cells than did the dHip-kindled rats (p = 0.003). We also found several significant group differences in the contralateral hemisphere. In particular, we found significant main effects in the contralateral granule cell layer [$F_{(3,25)} = 10.715$, p < 0.001], hilus [$F_{(3,25)} = 11.624$, p < 0.001] and stratum radiatum [$F_{(3,25)} = 8.854$, p < 0.001]. Post hoc tests revealed that the CN-kindled rats had significantly more GAD67-ir cells than the control, BLA- and dHip-kindled rats in each of these regions (all p values > 0.013). We also found a significant main effect in the CA3 stratum lucidum [$F_{(3,25)} = 3.573$, p < 0.030] and subsequent post hoc tests revealed that the CN-kindled rats had significantly more GAD67-ir cells than the dHip-kindled rats (p = 0.040). We also found a significant group difference in the contralateral CA1 stratum pyramidale [$F_{(3,25)} = 4.387$, p = 0.015] and post hoc tests revealed that the CN-kindled rats had significantly more GAD67-ir cells than the dHip-kindled rats. Finally, we found a significant group difference in the contralateral CA1 stratum oriens [$F_{(3,25)} = 3.581$, p = 0.030]. Post hoc tests revealed that the BLA-kindled rats had significantly more GAD67-ir cells than the control rats (p = 0.023). No other group differences were observed (all p values > 0.073).

In addition to counting the total number of GAD67-ir cells, we also used western blots to determine general levels of GAD67 protein in the entire hippocampus (Fig. 5-8). There were no
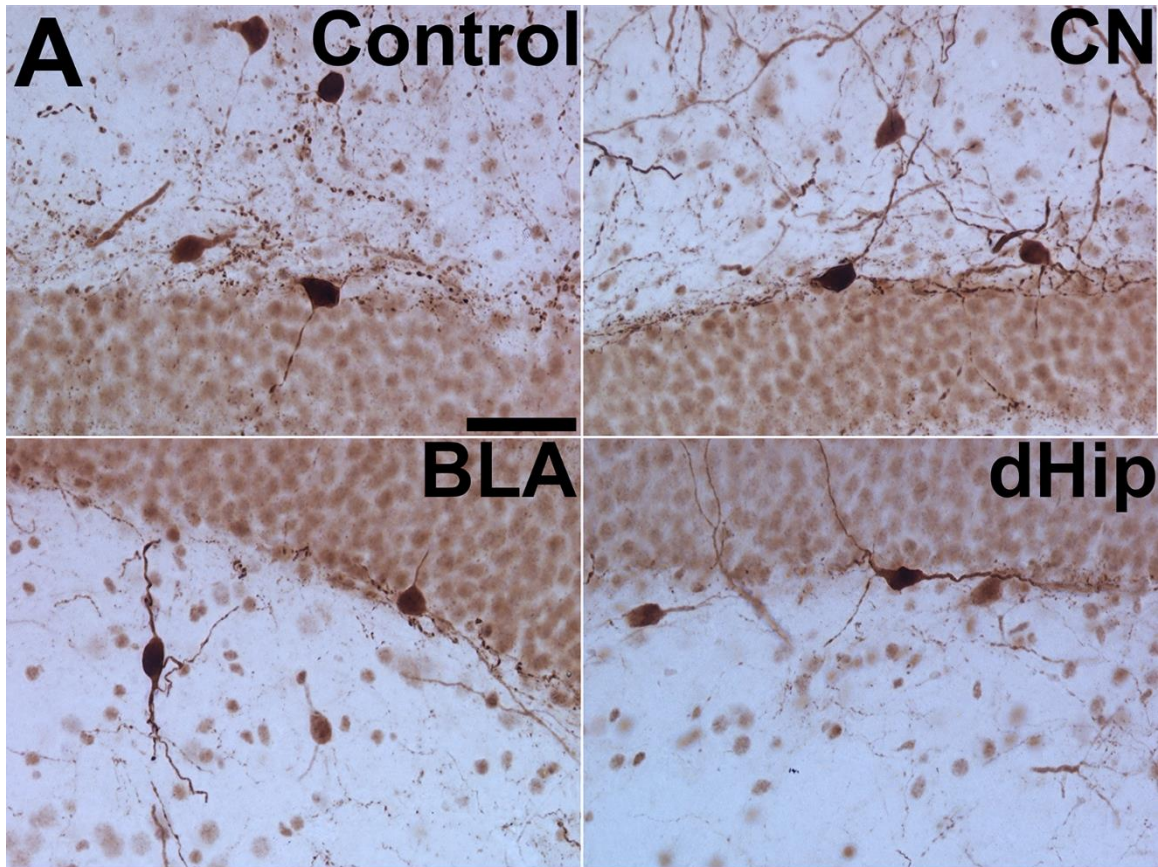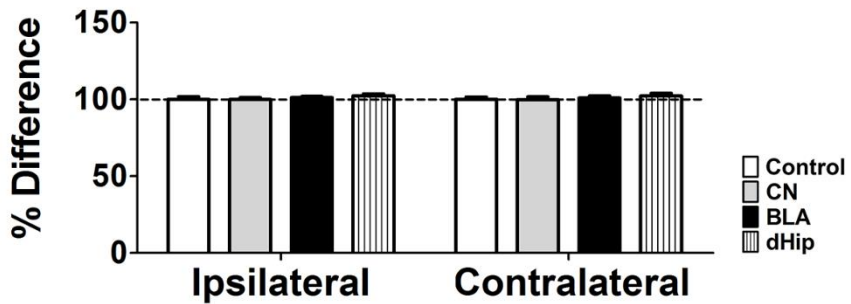
Figure 5-6. Effect of kindling on the surface area measurements of calretinin-immunoreactive interneurons. **(A)** Representative photomicrographs indicate that the soma surface area of calretinin-immunoreactive interneurons is comparable across groups (Scale bar = 50 μm). **(B)** Quantitative analysis of surface area normalized to percentage of control values.
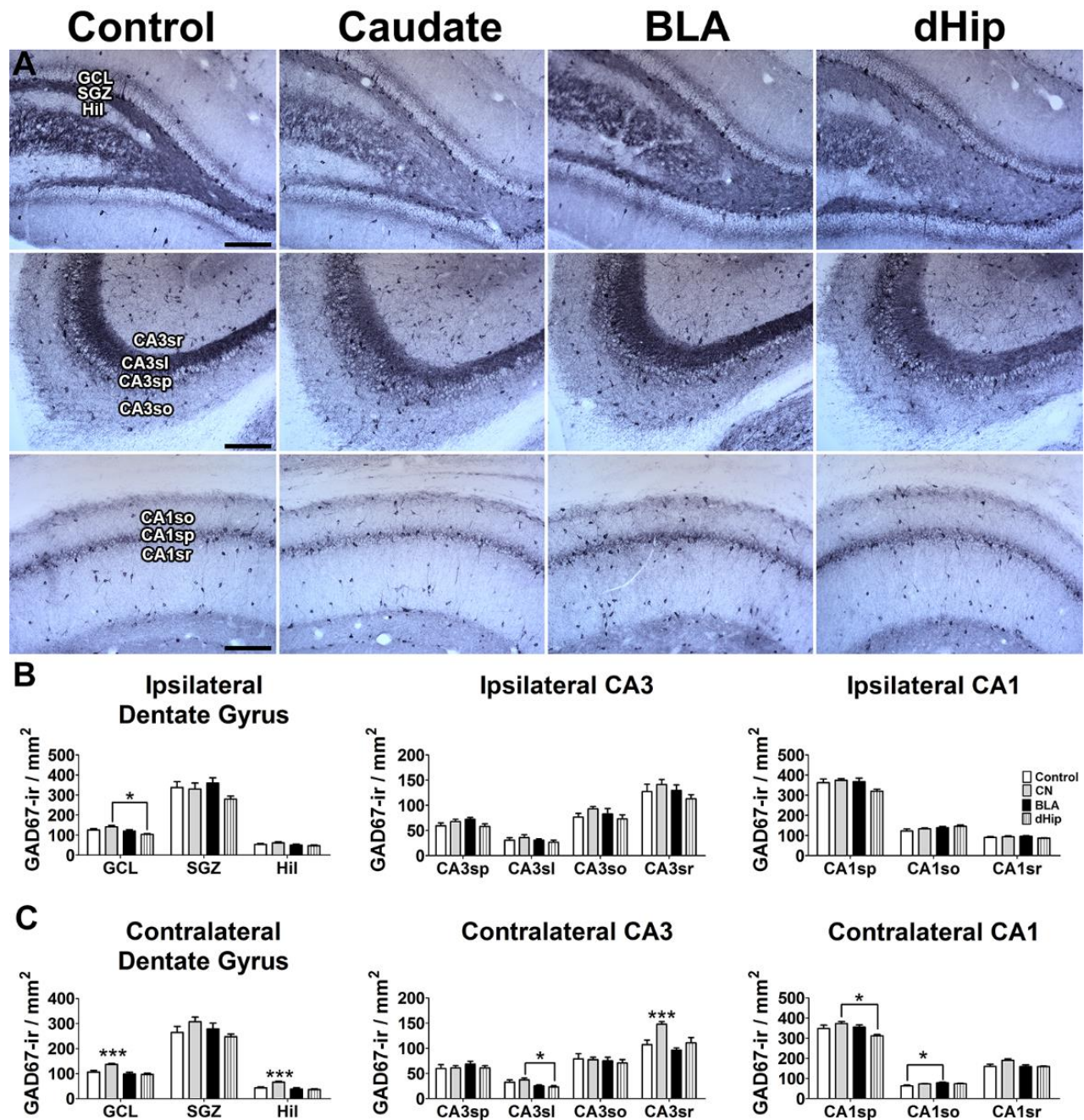
Figure 5-7. Effect of kindling on the number of GAD67-immunoreactive interneurons in several subregions of the hippocampus. **(A)** Representative photomicrographs of the dentate gyrus, CA3, and CA1 regions from each group (Scale bar = 200μm). **(B-C)** Quantitative cell counts revealed a significant increase in GAD67-immunoreactivity in the caudate-kindled rats in selected regions within the dentate gyrus, CA3, and CA1 subfields. We also noted that the BLA-kindled rats had more GAD67-immunoreactive cells than the control rats. GCL, granule cell layer; SGZ, subgranular zone; Hil, hilus; SP, stratum pyramidale; SL, stratum lucidum; SO, stratum oriens; SR, stratum radiatum. *$p < 0.05$

A GAD67 Protein
(Hippocampus)
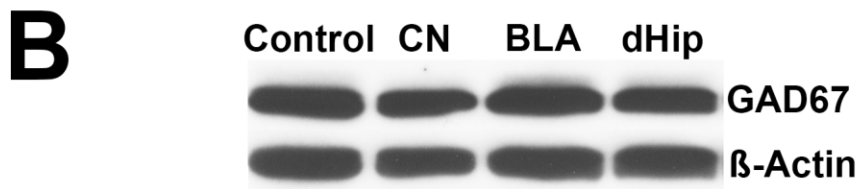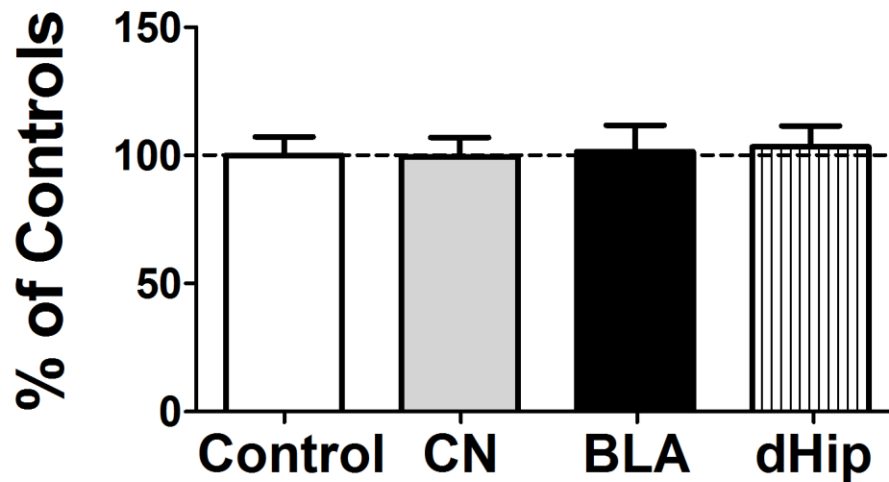
Figure 5-8. Hippocampal protein expression of GAD67. **(A)** Mean optical density scores of hippocampal GAD67 protein expression shown as a percentage change of control values. **(B)** Representative scanned western blots for GAD67 and β-Actin. There were no significant group differences among the groups. CN, caudate nucleus; BLA, basolateral amygdala; dHip, dorsal hippocampus.

significant group differences in hippocampal GAD67 protein expression [F(3,15) = 0.043, p = 0.987].

5.4 Discussion

This experiment evaluated dendritic, perisomatic, and interneuron-selective hippocampal interneuron subpopulations following kindling of different brain regions. Our results clearly demonstrate that kindling has selective effects on the number of GABAergic interneurons detected throughout the different subfields of the hippocampus. These results can be summarized as follows. First, we found an increase in the number of dendritic-targeting SOM interneurons in the CA1 pyramidal cell layer of limbic-kindled rats compared to controls. However, we also found a decrease in the number of SOM interneurons in the CA3 stratum oriens of the CN- and dHip-kindled rats compared to the controls. Second, we found a decrease in the number of perisomatic-targeting PV interneurons in the CA1 stratum oriens of dHip-kindled rats. Third, we found that the dHip-kindled rats had fewer CR-ir interneurons in the ipsilateral hilus compared to the control and CN-kindled rats; the CN-kindled rats had significantly more CR-ir interneurons than the limbic-kindled rats in several dentate gyrus subfields, including the granule cell layer, subgranular zone, and hilus; and the dHip-kindled rats had more CR-ir cells than the CN-kindled rats in the CA1 stratum oriens. Finally, we found that the CN-kindled rats had more GAD67-ir interneurons than the control, BLA-, and dHip-kindled rats in the contralateral granule cell layer, hilus, and CA3 stratum radiatum, and the CN-kindled rats also had more GAD67-ir interneurons than the dHip-kindled rats in the ipsilateral granule cell layer and the contralateral CA3 stratum lucidum and CA1 stratum pyramidale. The BLA-kindled rats had more GAD67-ir interneurons than did the control rats in the contralateral CA1 stratum oriens. Interestingly, there were no group differences in hippocampal GAD67 protein expression as measured by western blots.

In addition to quantitative cell counting, we also analyzed surface area measurements to determine whether kindling altered the morphology of dendritic, perisomatic, or interneuron-selective targeting interneurons. Interestingly, we found that dendritic targeting SOM-ir interneurons had a significantly greater surface area in the limbic-kindled rats, indicative of soma enlargement and axonal sprouting (Peng et al., 2013; Zhang et al., 2009). However, we did not detect any differences in the surface area of perisomatic-targeting PV or interneuron-selective CR interneurons, which suggests that these interneuron populations are resilient to kindling-

induced morphological changes. Collectively our results indicate that hippocampal GABAergic interneuron subpopulations undergo selective plasticity in number and morphology following kindling of different brain regions.

A reduction in the number of hippocampal SOM interneurons is well documented in resected tissue samples from human epilepsy patients (de Lanerolle et al., 1989; Sundstrom et al., 2001) and status epilepticus models (Houser & Esclapez, 1996; Magloczky & Freund, 1993; Schwarzer et al., 1995; Sloviter, 1991; Sperk et al., 1992; Sun et al., 2007). SOM interneurons in the hilus appear particularly sensitive to excitotoxic insults, as more than 50% of these cells are reported to die following pilocarpine-induced seizures (Buckmaster & Wen, 2011; Zhang et al., 2009). However, SOM interneurons appear resilient to neuronal degeneration in response to kindling and instead undergo kindling-induced upregulation (Schwarzer et al., 1996). The results of our experiment support these observations. In particular, kindling of limbic sites has previously been reported to cause a transient increase in preprosomatostatin mRNA and increased SOM immunoreactivity in cell bodies within the hilus (Shinoda, Schwartz, & Nadi, 1989; Tonder, Kragh, Finsen, Bolwig, & Zimmer, 1994; Vezzani, Schwarzer, Lothman, Williamson, & Sperk, 1996; Wanscher, Kragh, Barry, Bolwig, & Zimmer, 1990). Moreover, seizures induced by kainic acid transiently increase the expression of preprosomatostatin mRNA in hippocampal pyramidal cells which can be subsequently detected as SOM-ir cells (Hashimoto & Obata, 1991). Although the function of seizure-induced upregulation of SOM remains to be determined, one possibility is that this phenomenon represents an endogenous antiepileptic mechanism to inhibit hippocampal excitability and protect against seizure-induced cell death (Hashimoto & Obata, 1991; Tallent & Qiu, 2008). This hypothesis is consistent with reports that demonstrate seizures enhance activity-dependent release of SOM (Vezzani & Hoyer, 1999) and induce Fos expression in the majority of hilar and CA1 SOM interneurons (Pretel, Applegate, & Piekut, 1995).

The reduction of SOM interneurons in status epilepticus models is hypothesized to reduce dendritic inhibition and contribute to the pathogenesis of epilepsy (Cossart et al., 2001). However, it is important to note that not all SOM interneurons die following status epilepticus. The surviving SOM interneurons enlarge, sprout axons, and form new synapses (Buckmaster & Wen, 2011; Mathern, Babb, Pretorius, & Leite, 1995; Zhang et al., 2009). The sprouting of

surviving SOM interneurons could compensate for interneuron loss, in that sprouted SOM interneurons defined by immunoreactive cells with a larger somatic area and dendritic length display a moderate decrease in resting membrane potential and increased whole cell capacitance, increased frequency of slowly rising spontaneously excitatory postsynaptic currents (sEPSCs), increased evoked EPSC amplitude, and increased spontaneous action potential generation (Halabisky, Parada, Buckmaster, & Prince, 2010). The sprouting of SOM interneurons raises several functional considerations. Is the sprouting of SOM interneurons merely a response to stabilize hippocampal inhibition following hilar cell loss? The electrophysiological data mentioned above supports this hypothesis. However, the results of our experiment indicate that morphological changes in SOM-ir cells occur independently of hilar cell loss. Although it is possible that the sprouting of SOM interneurons in absence of neuronal loss may retain inhibitory properties, the development of excessively connected SOM interneuron populations could also confer proconvulsant properties (Buckmaster & Wen, 2011). For example, sprouted SOM interneurons could promote the hypersynchronization of excitatory granule neurons (Babb et al., 1989), generate depolarizing GABA responses in principal cells (Fujiwara-Tsukamoto et al., 2010; Staley, Soldo, & Proctor, 1995), or act as network hubs that promote seizure activity (Morgan & Soltesz, 2008). More research is needed to determine the effects of sprouted SOM interneurons on hippocampal function.

The results of our perisomatic-targeting interneuron analyses revealed a selective reduction of PV-ir interneurons in the CA3 stratum oriens of the dHip-kindled rats compared to the control and CN-kindled rats. This finding indicates a subfield-specific reduction of perisomatic inhibition in the dHip-kindled rats. However, we did not observe any other significant differences in the number of PV-ir interneurons or morphology following kindled seizures. PV interneurons represent pyramidal basket cells or axo-axonic cells in the dentate gyrus and the strata oriens or pyramidale in the CA3-CA1 subfields of the hippocampus (Freund & Buzsaki, 1996; Kosaka et al., 1987). PV interneurons are characterized by a fast-spiking phenotype that provides a major source of perisomatic inhibition onto hippocampal principal cells (Freund & Buzsaki, 1996). Moreover, the fast synaptic inhibition of PV interneurons contributes to hippocampal network oscillations critical for information processing (Bartos et al., 2007; Klausberger et al., 2005). A reduction in the number of hippocampal PV pyramidal basket cells therefore reduces inhibition of principal cells (Kobayashi & Buckmaster, 2003; Sloviter,

1987) and facilitates hypersynchronous activity that increases the susceptibility of behavioral and electrographic seizures (Schwaller et al., 2004). This evidence is further supported by the finding that fewer PV-expressing interneurons are detected in resected hippocampal samples in human patients with intractable TLE (Andrioli et al., 2007). However, there is also evidence that suggests pyramidal basket cells may have decreased neurochemical expression of PV but do not degenerate following repeated seizures (Sloviter et al., 2003). This observation is consistent with human studies where even though a significant reduction of PV is reported, there is no reduction in perisomatic inhibition (Wittner et al., 2001). Taken together, our immunohistochemical data suggests that perisomatic-mediated inhibition mediated by PV interneurons remains largely intact in the kindled brain; however, electrophysiological evidence is required to further validate this interpretation.

The results of our CR-ir analyses revealed a selective reduction in the number of detectable CR-ir cells in the dentate gyrus of the dHip-kindled rats. In particular, the dHip-kindled rats had fewer CR-ir cells in the ipsilateral hilus compared to the control and CN-kindled rats. Moreover, the CN-kindled rats had more CR-ir cells than the limbic-kindled rats in the various subfields of the dentate gyrus. Our results are interesting because CR interneurons innervate other interneurons to regulate dendritic inhibition (Gulyas et al., 1996). A reduction in the number of CR-ir cells could disrupt regulatory control over interneuron-selective dendritic inhibition and therefore stimulate the synchronous discharge of excitatory principal cells to facilitate epileptiform activity (Toth et al., 2010; Toth & Magloczky, 2014). Therefore, our results indicate that the dHip-kindled rats may have compromised interneuron-selective dendritic inhibition in a region-specific manner.

However, it is important to note that CR-ir is detected in morphologically distinct spiny and spine-free cell types in the hippocampus. Approximately 89% of spiny CR-ir cells are GABA-negative and located exclusively in the dentate gyrus or CA3 stratum lucidum where they receive the majority of their inputs from the granule cell mossy fibers (Gulyas et al., 1992). In contrast, spine-free CR-ir cells are distributed throughout all subfields of the hippocampus and approximately 92% of these cells are GABA-positive (Miettinen et al., 1992). This indicates that a portion of CR-ir cells in the dentate gyrus are not interneurons and that our results may not necessarily represent a reduction in the number of GABA-positive CR interneurons. One

explanation for this finding is that CR is transiently expressed by immature adult generated neurons in the dentate gyrus during their transition to a mature phenotype (Brandt et al., 2003). However, seizures rapidly accelerate the maturation of adult generated neurons (Overstreet-Wadiche et al., 2006). One intriguing possibility from our findings is that the reduction of CR-ir in the dentate gyrus of the limbic-kindled rats may be partially attributed to a reduction in the number of detectable immature adult generated neurons that transiently express CR. Because the morphologically distinct spiny and spine-free CR cells are not mutually exclusive for their presence or absence of GABA, we did not differentiate between them during our cell counts. Therefore, although our results indicate that there is a reduction of CR-ir in the dentate gyrus of dHip-kindled rats, it remains unresolved whether this represents a reduction in the number of GABA-negative or GABA-positive CR-ir cells or both. Despite this shortcoming, it is important to note that in all other hippocampal subfields where the overwhelming majority of CR-ir cells are GABA-positive, we did not find any differences in the number of CR-ir interneurons compared to controls. Future studies should be done using double labelling analyses to determine if the reduction of CR-ir in the dentate gyrus of limbic kindled rats is specific to GABA-positive or GABA-negative cells.

Finally, we analyzed the number of GAD67-ir interneurons as a general indicator of interneuron distribution following kindling. Interestingly, we found that the CN-kindled rats had significantly more GAD67-ir interneurons within the dentate gyrus and CA3 stratum radiatum of compared to all other groups. This finding suggests that CN-kindled seizures may result in an increase in the number of GAD67 interneurons, or possibly reflect a transient induction of GAD67 protein. In addition, we also found that the BLA-kindled rats had a greater number of GAD67-ir interneurons in the CA1 stratum oriens compared to controls. We also observed significant differences between kindling treatments in the number of GAD67-ir interneurons in the CA3 stratum lucidum and the CA1 stratum pyramidale and oriens. We did not conduct surface area measurements of GAD67 interneurons because they reflect heterogeneous populations of dendritic, perisomatic, and interneuron-selective interneuron populations. Instead, a subset of rats were sacrificed for western blot analyses to determine hippocampal protein expression of GAD67. We found no group differences in the amount of hippocampal GAD67 protein across groups. Taken together, our results demonstrate a subfield-selective induction in

the number of GAD67-ir cells in the CN-kindled rats, but no significant group differences in the overall hippocampal expression of this protein.

GAD converts glutamate to GABA and is expressed in two isoforms, GAD65 and GAD67. GAD67 is constitutively active and produces approximately 90% of the GABA used by the central nervous system (Kanaani, Kolibachuk, Martinez, & Baekkeskov, 2010). Importantly, GAD67 is conducive for cell counting, as it is expressed in the cell bodies and processes of interneurons. GAD67-ir can therefore be used as a general indication of interneuron survival or loss following experimentally-induced seizures. In contrast, GAD65 is transiently activated in axon terminals for rapid conversion of glutamate to vesicular GABA for immediate release during periods of intense network activity (Patel, de Graaf, Martin, Battaglioli, & Behar, 2006). GAD65 is therefore not representative of interneuron number and may only reflect transient fluctuations of GABA. GAD67-ir is transiently induced in hippocampal granule cells and mossy fibers following seizures induced by kainic acid or kindling (Gutierrez et al., 2003; Gutierrez & Heinemann, 2006; Ramirez & Gutierrez, 2001; Schwarzer & Sperk, 1995). More recently, GAD67 expression was discovered in hippocampal mossy fibers of human patients with temporal lobe epilepsy (Sperk et al., 2012). The transient upregulation of GAD67 is thought to act as an endogenous neuroprotective mechanism that could convert excessive and potentially excitotoxcic levels of intracellular glutamate into GABA (Sperk et al., 2012). The subfield-specific enhancement of GAD67-ir in the CN-kindled rats support this hypothesis. However, we were surprised that we did not detect a similar upregulation of GAD67 in our limbic-kindled rats.

Collectively, the results of the present study indicate that dendritic, perisomatic, and interneuron-selective inhibition undergoes selective plasticity following kindling. In particular, dendritic inhibition appears to be markedly enhanced in limbic-kindled rats as indicated by the increased number of SOM-ir interneurons and morphological sprouting of those interneurons. In contrast, perisomatic inhibition appears to be largely resilient to the effects of kindling. Interneuron-selective dendritic inhibition appears to be selectively reduced in dHip-kindled rats, although more research is required to determine whether the reduction represents GABA-positive or GABA-negative CR-ir cells. And finally, we found a significant subfield-selective induction of GAD67-ir in CN-kindled rats, which may indicate a greater synthesis of GABA following seizure activity.

Acknowledgements

Conflict of interest

The authors declare no conflicts of interest.

CHAPTER 6

Intrahippocampal infusions of the extracellular matrix glycoprotein reelin ameliorates fear memory impairment associated with kindling of the basolateral amygdala

J.J. Botterill [a], D.M. Ferguson [a], H.J. Caruncho [b], L.E. Kalynchuk [c]

[a] Department of Psychology, University of Saskatchewan, Saskatoon, SK, S7N 5A5, Canada

[b] College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, S7N 5E5, Canada

[c] Department of Medicine, University of Saskatchewan, Saskatoon, SK, S7N 5E5, Canada

6.1 Introduction

Temporal lobe epilepsy (TLE) is associated with broad neuropathology and cognitive dysfunction. Although many factors contribute to the neuropathology of TLE, one interesting candidate gene is reelin. Reelin is expressed in Cajal-Retzius cells during development and contributes to the proper lamination of the hippocampus and cortex (Forster et al., 2006; Frotscher et al., 2009; Ogawa et al., 1995). In adulthood, reelin is secreted by GABAergic interneurons in the neocortex and hippocampus to regulate structural and functional plasticity (Alcantara et al., 1998; Pesold et al., 1998). In particular, reelin mediates dendritic spine development and maturation (Chameau et al., 2009; Niu et al., 2008), synaptic plasticity (Beffert et al., 2005; Beffert et al., 2006; Pesold et al., 1998; Weeber et al., 2002), and postnatal neurogenesis (Pujadas et al., 2010; Won et al., 2006). TLE is associated with numerous structural changes that are associated with a reduction in reelin expression in the hippocampus (Frotscher et al., 2003; Gong et al., 2007; Haas et al., 2002). For example, decreased reelin expression is associated with an increase in the number of adult generated neurons that migrate abnormally into the hilus (Fournier et al., 2010; Parent et al., 2006). Moreover, there is a strong relationship between the extent of reelin downregulation and hippocampal granule cell dispersion in human patients with TLE (Frotscher et al., 2003; Haas et al., 2002). Remarkably, intrahippocampal infusions of exogenous reelin prevents dentate granule cell dispersion in experimental models of epilepsy (Muller et al., 2009). This finding suggests that exogenous reelin may confer therapeutic benefits for individuals with epilepsy.

The therapeutic potential of reelin supplementation through genetic overexpression or infusions has been demonstrated in several studies. In otherwise naïve experimental animals, a single bilateral injection of recombinant reelin into the lateral ventricles was reported to increase dendritic spine density, hippocampal long-term potentiation, and behavioral performance on contextual fear conditioning and the Morris water maze (Rogers et al., 2011). A study from the same laboratory recently reported that intraventricular injections of reelin rescued synaptic plasticity and contextual fear conditioning deficits in a mouse model of Angelman syndrome (Hethorn et al., 2015). Moreover, reelin overexpressing mice were protected from the development of behavioral phenotypes associated with schizophrenia and bipolar disorder induced by NMDA antagonists (Teixeira et al., 2011). Finally, reelin overexpression is also reported to delay the formation of amyloid beta fibrils and rescue deficits of recognition memory

in a mouse model of Alzheimer's disease (Pujadas et al., 2014). Collectively, these studies demonstrate the numerous cognitive and therapeutic benefits of reelin in the naïve and compromised brain.

To determine whether recombinant reelin can ameliorate cognitive deficits associated with repeated convulsions, we used the kindling model of epilepsy. Kindling refers to the gradual development and intensification of elicited motor seizures resulting from daily electrical stimulation of a discrete brain site (Goddard et al., 1969). We have previously reported that kindling of the basolateral amygdala (BLA) reduces the number of reelin immunoreactive cells in the dentate gyrus (Fournier et al., 2010) and impairs hippocampal-dependent fear memory retrieval (Botterill et al., 2014; Fournier et al., 2010). However, we are unaware of any studies that have evaluated the cognitive effects of intrahippocampal reelin infusions following repeated convulsions. To study this issue, we conducted 99 stimulations of the BLA as previously described (Botterill et al., 2014). Upon the completion of kindling, we conducted a total of two post-kindling ipsilateral (i.e., stimulation-side) intrahippocampal infusions of recombinant reelin followed by hippocampal-dependent auditory trace fear conditioning.

6.2 Materials and Methods

6.2.1 Subjects

A total of 18 Long-Evans rats weighing 200-250g at time of arrival were purchased from Charles River (Quebec, Canada). Each rat was housed individually in a rectangular polypropylene home cage containing standard laboratory bedding and given free access to food and water. The colony room was maintained at a temperature of 20 ± 1 °C on a 12:12 h light-dark cycle (lights on at 7 am). All experimental procedures were in accordance with the guidelines of the Canadian Council of Animal Care and an animal care protocol approved by the University of Saskatchewan Committee on Animal Care and Supply.

6.2.2 Surgery

Surgery was conducted as previously reported. Rats were deeply anesthetized with isoflurane (5% initial, 2-2.5% maintenance) and secured into a stereotaxic apparatus via ear bars. At a flat skull position, a single bipolar stimulating electrode (MS-303-2-B-SPC, Plastics One, Roanoke, VA, USA) was chronically implanted into the left BLA using the following

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coordinates from a stereotaxic brain atlas: -2.8 mm anteroposterior, +5.0 mm mediolateral, -8.5 mm dorsoventral, relative to bregma. A subset of electrode-implanted rats (N=6) also received a single cannula implanted into the left dorsal hippocampus (-3.5 mm anteroposterior, +2.1 mm mediolateral, -3.0 mm dorsoventral) for post-kindling infusions of recombinant reelin (BLA-reelin). The electrode and electrode-cannula assemblies were secured to the skull with 4 jeweler screws and dental acrylic.

6.2.3 Kindling

All rats received a postsurgical recovery period of at least 1 week prior to kindling. Rats then underwent 99 kindling stimulations over the course of 6.5 weeks. Kindling was conducted as previously described. Briefly, the electrode assembly of each rat was connected to a wire lead connected to an isolated pulse stimulator (model 210, A-M Systems, Sequim, WA, USA) to deliver a 1s, 60-Hz train of square-wave pulses, with each pulse having a duration of 1 ms and biphasic amplitude of 800 μA (peak-to-peak). A total of 99 electrical stimulations were delivered on a 3 times per day, 5 days per week schedule (i.e., 15 stimulations per week), with a minimum of 3 hours between consecutive kindling stimulations. Control rats (N=6) were connected to the simulation lead but did not receive any electrical current at any point in the experiment. The convulsion elicited by each stimulation was scored as previously described. The convulsions classes were operationally defined as class 0: immobility, class 1: orofacial automatisms, class 2: previous class with head nodding, class 3: unilateral forelimb clonus, class 4: rearing with bilateral forelimb clonus, class 5: rearing with bilateral forelimb clonus and loss of equilibrium, class 6: multiple class 5 convulsions and falling episodes, class 7: previous classes with running fit, and class 8: previous classes with intermittent muscle tonus. Under these classification criteria, rats are considered to be fully "kindled" following 3 consecutive stage 5 convulsions.

6.2.4 Reelin infusions

A total of two intrahippocampal infusions of recombinant reelin were administered over a period of 24 hours following the 99th kindling stimulation. We ensured that reelin infusions occured at least 3 hours after the last kindling session. Lyophilized recombinant reelin (3820-MR-025/CF; R&D Systems; Minneapolis, MN, USA) was reconstituted immediately prior to infusions to a working concentration of 1 μg of protein per 1 μl in 0.1 M PBS (pH = 7.4). A total of 1 μl of reelin was infused at a rate of 0.5 μl per minute over 2 minutes using a Hamilton

syringe connected to a Harvard Apparatus infusion pump. The infusion needle remained in the cannula for an additional minute to allow for diffusion of the solution into tissue.

6.2.5 Auditory trace fear conditioning

Within 24 hours of the final kindling stimulation, all rats underwent an auditory trace fear conditioning paradigm as previously described (Botterill et al., 2015). The behavioral analyses included one habituation day, a 7 CS-US trace training session, 4 tones in a novel context, and a training context memory retrieval test. We used four identical operant chambers (ENV-008, Med Associates Inc, St. Albans, VT, USA) encased in sound-attenuated cubicles for all fear conditioning procedures. Each operant chamber was equipped with infrared video cameras that recorded all behaviors for offline analyses. Auditory stimuli were presented through a loudspeaker mounted in the chamber wall (ENV-224BM; Med Associates). Footshocks were delivered to aluminum grid floor controlled by a shock generator running Video Freeze software (Med Associates Inc). The primary behavioral measure was conditioned freezing, operationally defined as the absence of motor movements except those necessary for respiration. Freezing behavior was scored every 2 s during tone presentations and every 4 s otherwise. The freezing observations were transformed into a percentage of time spent freezing, by summing the total number of freezing observations and dividing by the total number of observations for each measure and multiplying the resultant number by 100. The chambers were cleaned thoroughly with 0.6 % (v/v) acetic acid between subjects unless mentioned otherwise.

During the habituation period (day 1), we assessed baseline ambulatory activity (midline crossing), operationally defined as all four limbs crossing the center of the chamber (Quinn et al., 2005). For the training day (day 2), each rat was placed in the same operant chamber as day 1. The training began with a 180 s acclimiation period followed by 7 CS-US pairings. Each pairing consisted of an auditory tone (16 s, 85 db, 2 kHz) followed by a 30 s trace interval that co-terminated with a 2 s footshock (0.9 mA). The inter-trial interval was set at 180 s between tone onsets. For the novel context tone test (day 3), each rat was placed in a novel operant chamber. The operant chamber was altered by replacing the aluminum floor with a plastic white floor and installing an oval insert on the back side of the operant chamber to alter the physical shape inside the chamber. In addition, the chamber was cleaned with 30% EtOH and scented with vanilla extract. Testing was conducted in the dark and monitored using infrared cameras. Following a

180 s baseline period, each rat received a total of four 16 s tones identical to the training day. For the contextual memory test (day 4), each rat was returned to the operant chamber they were originally trained in and measured conditioned freezing over 8 min. In addition to quantifying contextual conditioned freezing, we also evaluated fecal boli counts as an additional measure of fear expression.

6.2.6 Postmortem Analyses

Each rat was sacrificed within 60 minutes of completing the contextual fear memory task (e.g., day 4) using room temperature physiological saline followed by ice cold (4% w/v) formaldehyde fixative. Each rat was then decapitated and the brain was excised from the cranial vault and stored in the same formaldehyde fixative for up to 48 h and transfered into a sodium azide solution for long-term storage. Each brain was sectioned at 30 μm in the coronal plane using a vibrating microtome (Leica VT 1200S, Leica Biosystems, Nussloch, Germany). Sections were stored at -20 °C in a cryoprotectant solution comprised of 30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, and 30% (v/v) ethylene glycol in 0.1 M PBS (pH = 7.4).

To evaluate behaviorally relevant immediate early gene activity, we conducted Fos immunohistochemistry as previously described (Botterill et al., 2015). Immunostaining was conducted on free-floating sections in 6-well tissue plates. All sections were processed in unison to keep incubation periods and visualization steps constant for all animals. To confirm specificity of the antibody, we included one additional well that did not receive the primary antibody. In absence of the primary antibody, we did not detect any Fos immunoreactivity. All rinses and incubations were done using in 0.1 M Tris buffered saline (TBS, pH 7.4). Sections were initially rinsed in 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity and then blocked in 5% normal goat serum with 0.3 % Triton X-100 in 0.1 M TBS for 1 hour. Sections were incubated in the primary antibody (1:1000; Fos, #ABE457, Millipore, Boston, MA, USA) in blocking solution for 72 hours at 4°C. Sections were then incubated in biotinylated goat anti-rabbit IgG secondary antibody (1:500; Vector Laboratories, Burlingame, CA, USA) and treated with avidin-biotin-peroxidase complex (1:500, Vector Laboratories). Immunoreactivity of Fos was visualized with nickel-intensified DAB comprised of 0.02 % (w/v) 3,3'-diaminobenzidine, 2.5 % (w/v) nickel ammonium sulfate and 0.00786 % (v/v) H_2O_2 diluted in 0.175 M sodium acetate. The sections were then mounted onto glass slides and left to dry overnight. The mounted sections

were then dehydrated in a graded alcohol series, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA).

6.2.7 Statistics

Group differences were analyzed using IBM's statistical package for the social sciences. We conducted one-way ANOVAs followed by Fisher LSD *post hoc* tests when appropriate. Significance was set at $P < 0.05$. Graphs depict mean ± SEM.

6.3 Results

6.3.1 Kindling Progression

Kindling progressed normally in all rats. The number of stimulations required to elicit the first stage 5 convulsion was 8.83 ± 2.35 in the BLA-kindled and 10.66 ± 1.9 in the BLA-reelin-kindled rats. The number of stimulations required to elicit three consecutive stage 5 convulsions was 12.33 ± 2.31 in the BLA-kindled and 13.00 ± 1.91 in the BLA-reelin-kindled rats. Finally, the total number of stage 5 or greater convulsions was 82.83 ± 8.02 for the BLA-kindled and 83.83 ± 2.36 for the BLA-reelin-kindled rats.

6.3.2 Trace Fear Conditioning

For the habituation day, we found no significant group differences in the number of midline crosses $F(2,17) = 0.028$, $P = 0.972$ (Fig. 6-1). On the training day, we found no significant differences in freezing during the pre-training baseline period $F(2,17) = 0.117$, $P = 0.891$. Furthermore, all groups successfully learned the fear association, indicated by increased levels of conditioned freezing with successive CS-US pairings. Indeed, we found no significant group differences in conditioned freezing to the training tones $F(2,17) = 0.105$, $P = 0.901$, the trace interval $F(2,17) = 0.610$, $P = 0.556$, or the post-shock interval $F(2,17) = 0.240$, $P = 0.790$. In contrast, we found significant group differences during subsequent memory tests. On the novel context tone test, we observed low levels of baseline freezing that were not significant different across groups $F(2,17) = 0.361$, $P = 0.703$, indicating there was minimal generalization to the novel context. However, we found that there were significant group differences of conditioned freezing during the CS $F(2,17) = 8.181$, $P = 0.004$. Post hoc analyses revealed that

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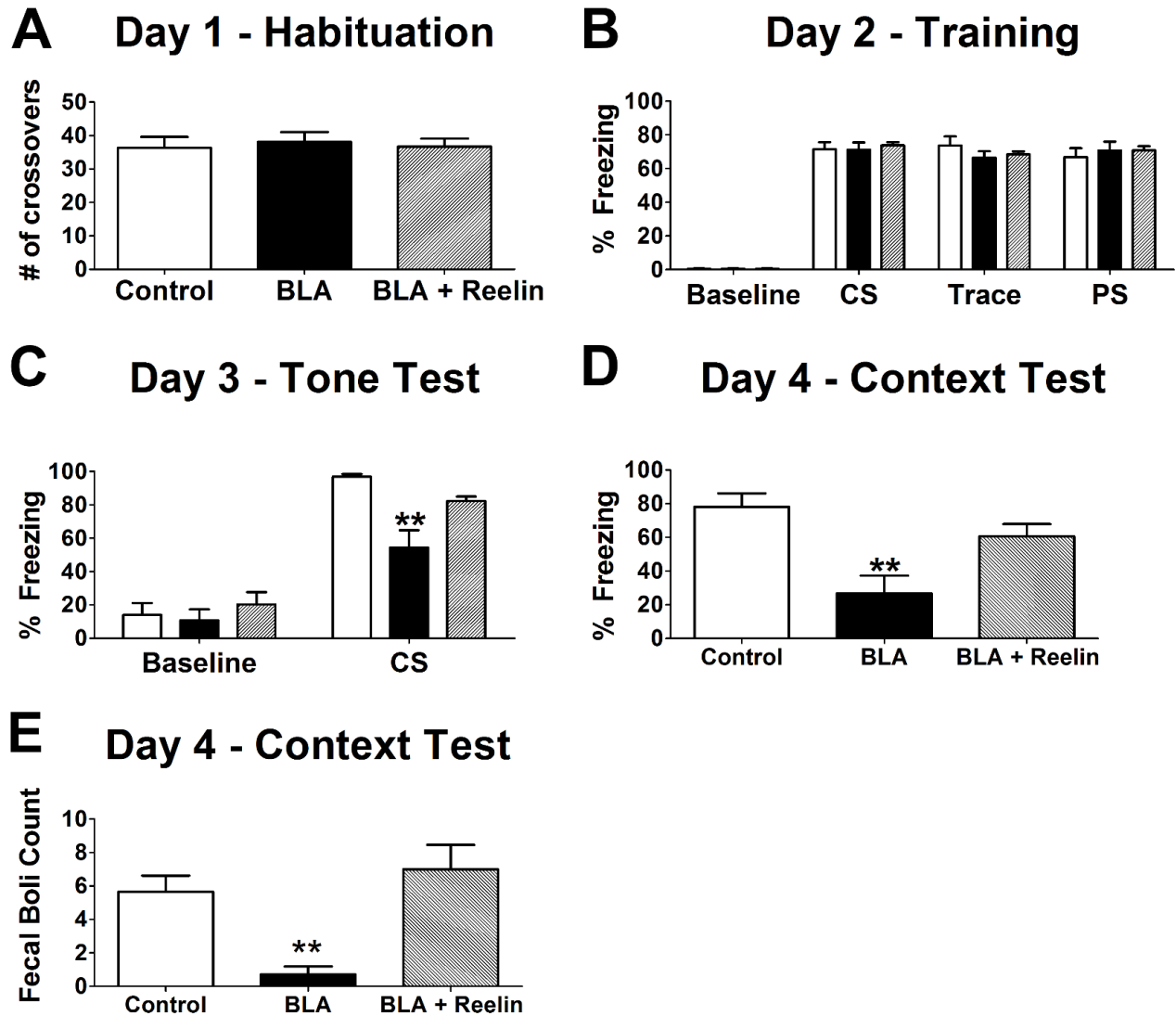
Figure 6-1. Effect of amygdala kindling and intrahippocampal reelin infusions on trace fear conditioning. **(A)** We found no effect of kindling or reelin infusions on baseline locomotor activity. **(B)** During the training period, we found no group differences in baseline or conditioned freezing during tone presentations (CS), the trace period, or post-shock intervals (PS), indicating acquisition was normal for all groups. **(C)** We found no differences in baseline freezing during the acclimation period in a novel context. However, the BLA-kindled rats displayed significantly less freezing than the control and BLA-reelin-kindled rats during subsequent tone presentations. **(D)** The BLA-kindled rats froze significantly less than the control and BLA-reelin-kindled rats on the context memory retrieval test. **(E)** Upon completing the context memory retrieval test, the BLA-kindled rats were found to have lower numbers of fecal boli in the operant chamber than the control or BLA-reelin-kindled rats. BLA, basolateral amygdala; CS, conditioned stimulus; PS, post-shock. $*p < 0.05$

the BLA-kindled rats displayed significantly less conditioned freezing than the control and BLA-reelin-kindled rats (all P values < 0.025). Interestingly, the control rats were not significantly different than the BLA-reelin kindled rats on this measure ($P = 0.150$).

On the context memory test, we found significant group differences in conditioned freezing $F(2,17) = 7.001$, $P = 0.007$. Post hoc analyses revealed that the BLA-kindled rats displayed significantly less conditioned freezing than the control and BLA-reelin-kindled rats (all P values < 0.032). Importantly, the BLA-reelin-kindled rats were not significantly different from the control rats ($P = 0.206$). As a secondary measure of fear memory, we also evaluated fecal boli counts during contextual memory retrieval. We found significant group differences in the number of fecal boli following fear memory retrieval $F(2,17) = 9.459$, $P = 0.002$. Post hoc analyses revealed that the BLA-kindled rats had significantly fewer fecal boli numbers in the operant chamber than did the control and BLA-reelin-kindled rats (all P values < 0.005). The control rats did not differ from the BLA-reelin-kindled rats on this measure ($P = 0.386$).

6.4 Discussion

The results of this experiment provide novel evidence that intrahippocampal infusions of recombinant reelin can ameliorate cognitive and behavioral impairments associated with kindling of the BLA. The behavioral results demonstrate that kindling had no effect on baseline locomotor activity during the habituation day, which is consistent with previous reports (Botterill et al., 2014; Botterill et al., 2015). Importantly, the combined treatment of kindling and intrahippocampal infusions of reelin also had no effect on baseline locomotor activity. This finding indicates that subsequent measures of conditioned freezing are not due to kindling- or reelin-induced hyperlocomotion. The results of the training session also revealed that kindling and intrahippocampal infusions of reelin had no effect on the acquisition of hippocampal-dependent learning. Indeed, all treatment groups showed comparable conditioned freezing during tones, trace, and post-shock intervals. However, we noticed significant group differences on the novel context (day 3) and context memory retrieval (day 4) tests. On the novel context test, there were no group difference in baseline freezing, which suggests that there was minimal generalization to the novel context. Upon presentation of the training tones in the novel context, the BLA-kindled rats displayed significantly less conditioned freezing compared to the control and BLA-reelin-kindled rats. We found a similar result in the context memory retrieval test,

wherein the BLA-kindled rats displayed significantly less conditioning freezing to the original training environment than the control and BLA-reelin-kindled rats. In addition to analyzing conditioned freezing, we also counted the number of fecal boli in the chamber upon the completion of the contextual memory retrieval test. We found that the BLA-kindled rats had significantly fewer fecal boli in the operant chamber compared to the control and BLA-reelin-kindled rats. Previous studies have indicated that intrahippocampal infusions of recombinant reelin improve contextual-dependent freezing at 24 and 72 hours post-infusion, but not at 1 hour post-infusion (Rogers et al., 2011). This finding suggests that the restoration of conditioned freezing and fecal boli number we observed in the BLA-reelin-kindled rats likely reflects improved cognitive performance rather than non-specific effects of reelin on conditioned freezing.

Our preliminary results (Fig. 6-2) reveal a pattern of hippocampal Fos expression that is consistent with previous reports from our laboratory (Botterill et al., 2014; Botterill et al., 2015). Specifically, the control rats that have a high percentages of conditioned freezing behavior show robust Fos immunoreactivity in the dentate gyrus, whereas the BLA-kindled rats with impaired freezing behavior have substantially less Fos immunoreactivity. Interestingly, the BLA-reelin-kindled rats appear to have a pattern of Fos immunoreactivity that closely resembles the control rats. These preliminary observations indicate that intrahippocampal infusions of reelin may restore deficits of neural activity during periods of fear memory retrieval.

We have previously reported that BLA-kindled rats that undergo hippocampal-dependent trace fear conditioning display impaired contextual fear retrieval with a parallel reduction of Fos immunoreactivity in hippocampal and adjacent limbic structures (Botterill et al., 2014). Moreover, post-mortem analyses have shown that BLA-kindled rats with hippocampal-dependent memory deficits show a parallel enhancement of NPY and NPY2R immunoreactivity in the hippocampus (Botterill et al., 2015). Interactions between NPY and NPY2R potently suppresses presynaptic glutamate release through inhibition of N-type, P/Q-type, and voltage-dependent calcium channels (Qian et al., 1997). This led us to propose that plasticity of NPY and NPY2R following limbic seizures may act as an endogenous anticonvulsant mechanism to increase hippocampal inhibition, but that these changes could inadvertently limit glutamate-dependent functions such as cognition (Botterill et al., 2015). Interestingly, reelin is a potent
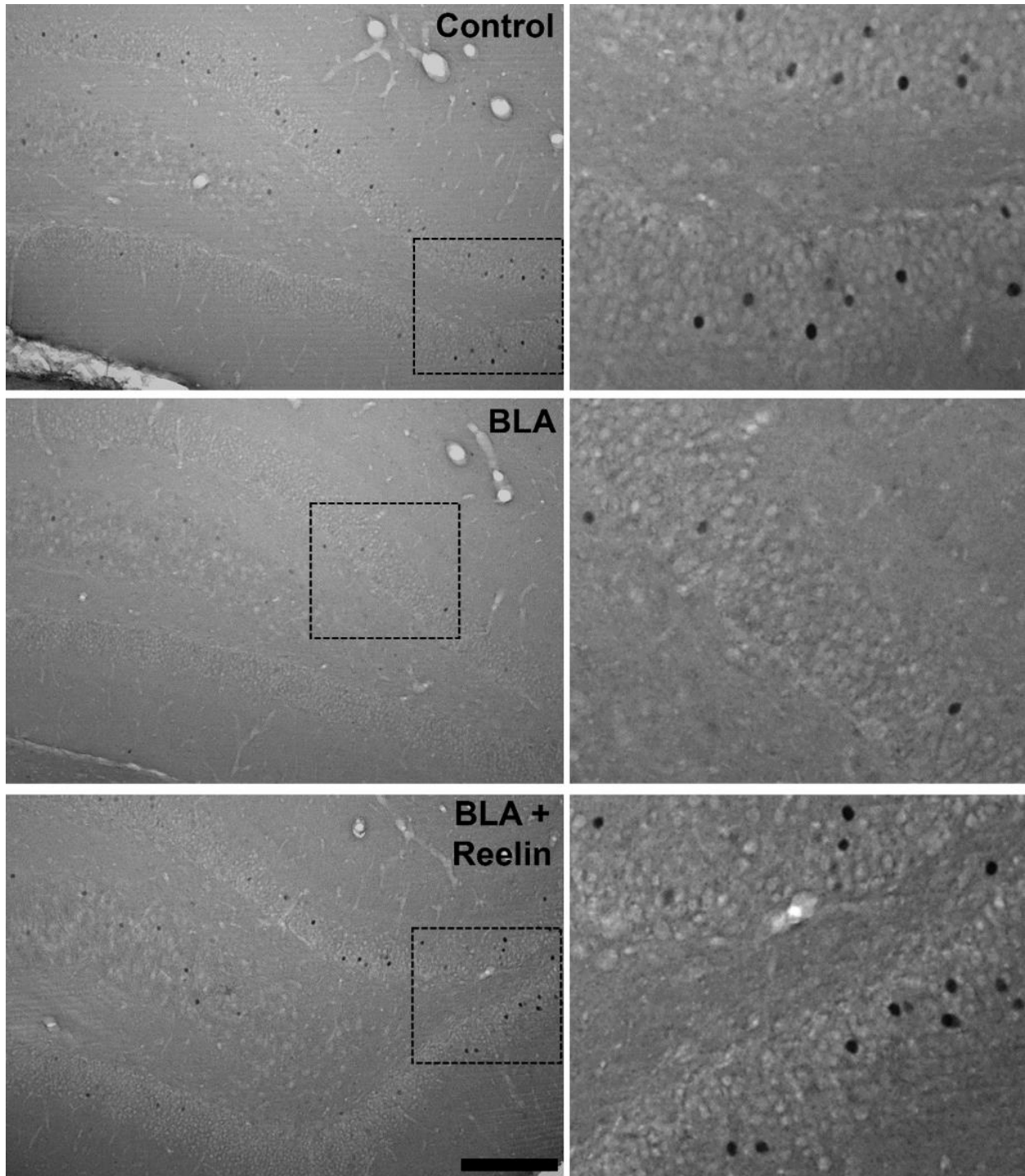
Figure 6-2. Pattern of Fos immunoreactivity following contextual fear memory retrieval. Preliminary photomicrographs indicate that the control and BLA-kindled rats have patterns of Fos immunoreactivity following memory retrieval similar to those shown in *Chapters 2 & 3*. Interestingly, the pattern of Fos expression in the BLA-reelin-kindled rats appears comparable to the control rats. BLA, basolateral amygdala. (Scale bar = 200 μm).

modulator of glutamatergic function (Qiu & Weeber, 2007). In particular, reelin modulates calcium influx through interactions with the NR2B subunit of the NMDA receptor, which in turn increases phosphorylation and nuclear translocation of the transcription factor cAMP-response element binding protein (CREB) (Chen et al., 2005). The ability of reelin to regulate glutamate function through NR2B receptors is a particularly enticing mechanism to study, because seizures downregulate the hippocampal expression of NR2B (Auzmendi, Gonzalez, & Girardi, 2009; Pratt et al., 1993) and reelin (Fournier et al., 2010), whereas recombinant reelin increases NR2B phosphorylation and function (Qiu, Zhao, Korwek, & Weeber, 2006). The preliminary results of this study suggests that intrahippocampal infusions of recombinant reelin may restore disruptions of glutamatergic function associated with kindling of the BLA. However, a series of immunohistochemical and electrophysiological experiments is required to study this hypothesis further.

Acknowledgements

Conflict of interest

The authors declare no conflicts of interest.

CHAPTER 7

General Discussion

7.1 Overview of the Main Findings

The objective of this dissertation was to develop a greater understanding of the relationship between cognitive impairment and aberrant neural plasticity resulting from repeated convulsions. In order to study this relationship, I ran a series of experiments that investigated the effect of repeated kindling on operant fear behavior and behaviorally relevant neural activity markers, hippocampal neurogenesis, inhibitory interneurons, and intrahippocampal infusions of reelin.

In *Chapter 2*, I evaluated the effect of short- and long-term amygdaloid kindling on amygdala- and hippocampal-dependent fear conditioning. I found that amygdaloid kindling disrupted conditioned fear behavior in a stimulation-dependent and task-specific manner. Specifically, the acquisition of hippocampal-dependent trace fear conditioning was comparable for all groups; however, the long-term BLA-kindled rats displayed marked reductions of conditioned freezing on subsequent tone and context memory tests. In contrast, the short- and long-term BLA-kindled rats that underwent amygdala-dependent delay fear conditioning displayed mild acquisition deficits and pronounced conditioned freezing deficits on subsequent tone and context tests. Post-mortem analyses revealed that amygdaloid kindling reduced Fos immunoreactivity throughout limbic fear circuits in a manner that paralleled conditioned freezing deficits on the contextual memory test. Specifically, I found a reduction of conditioned freezing and limbic Fos immunoreactivity in the long-term BLA-kindled rats that were subjected to trace fear conditioning. In contrast, in rats that were subjected to delay fear conditioning, I found a reduction in conditioned freezing and limbic Fos immunoreactivity in both the short- and long-term BLA-kindled rats. Moreover, correlational analyses revealed a positive relationship between conditioned freezing and Fos immunoreactivity in hippocampal and parahippocampal circuits in rats subjected to hippocampal-dependent trace fear conditioning. In contrast, correlational analyses revealed a positive relationship between conditioned freezing and Fos immunoreactivity in amygdaloid, hippocampal, and parahippocampal circuits in rats subjected to amygdala-dependent delay fear conditioning.

Chapter 2 provided a strong basis for my subsequent studies. The results of this study revealed that long-term amygdaloid kindling produced greater impairments in fear learning and memory than short-term kindling and that amygdala-dependent tasks are more likely to be disrupted by short-term amygdaloid kindling than hippocampal-dependent tasks. These findings also indicate that kindling is a useful model to study the relationship between plasticity within fear-related circuits and cognition. Furthermore, *Chapter 2* demonstrated that hippocampal-dependent trace fear conditioning could be utilized in subsequent studies without the concern of acquisition deficits associated with amygdala-dependent fear conditioning. This finding is important because it eliminates the difficulty of interpreting mild acquisition deficits on subsequent memory performance. Therefore, I focused my efforts to study the consequences of long-term kindling on aberrant hippocampal plasticity and hippocampal-dependent memory deficits in subsequent experiments.

In *Chapter 3,* I examined the effect of kindling different brain regions on hippocampal-dependent fear conditioning and markers of hippocampal plasticity and degeneration. To study this question, I administered 99 kindling stimulations of limbic (e.g., BLA, dHip) and non-limbic (e.g., CN) brain regions followed by hippocampal-dependent auditory trace fear conditioning. My results revealed that kindling had no effect on the acquisition of trace fear conditioning. All rats showed normal acquisition of the task, as seen by comparable conditioned freezing to tone presentations, trace intervals, and post-shock periods. However, limbic but not non-limbic kindling impaired the retrieval of fear memories, evidenced by a reduction in conditioned freezing on subsequent tone and context memory tests. Post-mortem evaluations revealed that impaired conditioned freezing in the limbic-kindled rats coincided with a general reduction of the neural activity marker Fos in several hippocampal subfields. Next, I evaluated markers of hippocampal plasticity to support my behavioral observations. Although I found that the limbic-kindled rats had fewer detectable NPY-immunoreactive cells, I subsequently detected that the limbic-kindled rats had greater immunoreactivity of NPY and NPY2R throughout the dentate gyrus and CA3. Analysis of the neurodegenerative marker Fluoro-Jade B further revealed that kindling did not appear to cause neuronal loss or degeneration, which was in stark contrast with a subset of rats that received a standard dose of lithium-pilocarpine. This finding indicated that a reduction of detectable NPY-immunoreactive cells following kindling may not reflect a loss of these cells, but rather an accumulation of NPY in the mossy fiber nerve terminals and neuropil

instead of the cell body. The changes to the NPY system that I observed in *Chapter 3* are reported to increase the effectiveness of inhibitory neurotransmission by presynaptically inhibiting glutamate release (Colmers & El, 2003; Vezzani & Sperk, 2004). Collectively, my findings indicate that limbic kindling promotes plasticity of NPY and the NPY2R, resulting in an augmented inhibitory tone that could negatively impact cognition.

In *Chapter 4*, I investigated the effect of limbic and non-limbic kindling on hippocampal neurogenesis and hippocampal-dependent fear conditioning. Hippocampal neurogenesis was assessed by immunohistochemical labelling for BrdU and DCX. My cell count analyses revealed that limbic-kindled rats had more BrdU-ir cells than control or CN-kindled rats in the proliferative SGZ/GCL and more BrdU-ir ectopic granule neurons in the hilar-CA3 border. Interestingly, correlational analyses revealed that the number of BrdU-ir cells in the SGZ/GCL and hilar-CA3 border positively correlated with convulsion severity on the day of BrdU injections, but not previous seizure history. Moreover, the limbic-kindled rats had more DCX-ir neurons located in the proliferative SGZ/GCL compared to the control and CN-kindled rats. In addition to a greater number of DCX-ir neurons, the limbic-kindled rats also had greater apical dendrite complexity and a greater frequency of hilar basal dendrites compared to the control and CN-kindled rats. This finding suggested that limbic kindling may accelerate neuronal maturation. A series of ELISA experiments further revealed that the limbic-kindled rats had greater hippocampal protein expression of the pro-neurogenic factor BDNF than the control and CN-kindled rats. Finally, kindling had no effect on the acquisition of hippocampal-dependent trace fear conditioning; however, the limbic-kindled rats were found to have impaired conditioned freezing on subsequent contextual memory retrieval test. Taken together, my results suggest that limbic kindling facilitates adult hippocampal neurogenesis, promotes aberrant characteristics within newborn neurons, increases hippocampal BDNF protein, and impairs hippocampal-dependent memory. The aberrant features of neurogenesis that are unique to the limbic-kindled rats likely contributes to the cognitive and behavioral comorbidities observed in these subjects (Cho et al., 2015).

In *Chapter 5*, I investigated the effect of limbic and non-limbic kindling on hippocampal GABAergic interneuron distribution and morphological plasticity. I used immunohistochemistry to evaluate the effects of kindling on dendritic (SOM), perisomatic (PV), and interneuron-

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selective (CR) inhibitory interneurons and the major GABA synthesizing enzyme (GAD67) as a general indicator of the entire interneuron population. I also used western blots to evaluate GAD67 protein expression throughout the entire hippocampus. The results of this experiment indicated that kindling produces selective effects on the number and morphology of different function classes of GABAergic interneurons. In particular, limbic kindling promoted an induction of dendritic-targeting SOM in the CA1 pyramidal cell layer and increased the surface area of SOM-ir interneurons in the dentate gyrus compared to the control and CN-kindled rats. My results further indicated that the dHip-kindled rats had a subfield-specific reduction of perisomatic-targeting (PV) interneurons in the CA3 stratum oriens. However, I detected no other group differences in terms of PV-ir number or surface area. Moreover, my analysis of interneuron-selective (CR) interneurons indicated that the number of CR-ir interneurons was reduced in the dentate gyrus hilus of the dHip-kindled rats, but that there were no differences in the surface area of CR-ir cells between groups. Finally, I found that the CN-kindled rats had a significant increase in the number of GAD67-ir cells in the contralateral dentate gyrus and CA3 stratum radiatum compared to the control and limbic-kindled rats. Despite the subfield-specific induction of GAD67-ir in the CN-kindled rats, I was unable to detect any group differences in total hippocampal protein expression of GAD67 in western blot analyses. Collectively, these findings indicate that dendritic, perisomatic, and interneuron-selective inhibition undergo selective plasticity following kindling. Specifically, dendritic inhibition appeared to be markedly enhanced in the limbic-kindled rats, as indicated by a significant induction of SOM-ir interneurons in the CA1 pyramidal layer and morphological sprouting in the hilus. In contrast, perisomatic inhibition appears largely resilient to the effects of kindling. Moreover, interneuron-selective dendritic inhibition appears to be compromised in the dentate gyrus of dHip-kindled rats. The induction of GAD67-ir in the CN-kindled rats may indicate a subfield-specific enhancement of hippocampal GABA synthesis following kindling of the CN.

Chapter 6 was a preliminary investigation to determine whether intrahippocampal infusions of the extracellular matrix protein reelin could ameliorate fear memory impairments associated with kindling of the BLA. In line with my previous studies (*Chapters 2-4*), I found no group differences on the habituation or acquisition days of auditory trace fear conditioning. However, the BLA-kindled rats had impairments of conditioned freezing on subsequent tone and context tests compared to the control rats. Remarkably, intrahippocampal infusion of

recombinant reelin (BLA-reelin-kindled) restored conditioned freezing deficits on tone and context tests to control levels. Importantly, the BLA-reelin-kindled rats were not significantly different from the control rats on either test. Finally, the BLA-kindled rats had fewer fecal boli in the operant chamber following context testing compared to the control and BLA-reelin-kindled rats, indicating the reduction in conditioned freezing is likely a memory deficit rather than an anxiety phenotype. Collectively these preliminary findings indicate that intrahippocampal infusion of recombinant reelin can ameliorate fear memory impairment in BLA-kindled rats.

The overall findings of this dissertation demonstrate that repeated convulsions elicited within specific brain regions promotes aberrant plasticity and cognitive deficits. Specifically, my experimental data indicates that limbic kindling causes hippocampal-dependent memory impairments that coincide with robust plasticity to the NPY system, aberrant hippocampal neurogenesis, and selective plasticity of hippocampal GABAergic inhibitory interneurons. In contrast, kindling of non-limbic brain regions had relatively minor effects on neural plasticity and cognition. The final experimental chapter of my thesis provides preliminary evidence that the extracellular matrix protein reelin can ameliorate cognitive impairments associated with kindling of the BLA.

7.2 Interictal Behavioral Comorbidities Associated with Kindling

Kindling is widely recognized as a model for studying interictal behavioral comorbidities associated with epilepsy (Kalynchuk, 2000; Teskey, 2009). Historically, the majority of studies have characterized the effects of kindling on unconditioned behaviors. In particular, kindling of the BLA dramatically and reliably increases fear and anxiety behaviors in the open field test and elevated plus maze (Adamec, 1990; Depaulis et al., 1997; Fournier et al., 2009; Hannesson et al., 2008; Kalynchuk, 2000; Wintink et al., 2003). The effect of BLA kindling on spatial memory performance is less consistent, with some reports indicating a range of impairments (Beldhuis et al., 1992; Cammisuli et al., 1997), whereas others do not find any deficits (Hannesson et al., 2008; Nieminen et al., 1992). Moreover, kindling of the hippocampus is commonly used to study mnemonic function, where it is frequently reported to disrupt memory performance in the Morris water maze and radial arm maze (Gilbert et al., 2000; Hannesson et al., 2001; Leung et al., 1990; Lopes da Silva et al., 1986). In contrast, there is considerably less information regarding the behavioral effects of CN kindling. In the few studies that have evaluated CN kindling, there are

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no substantial behavioral disturbances on the elevated plus maze, resistance to capture in an open field, or reactionary responses to pencil taps on the tail (Kalynchuk et al., 1998; Pinel et al., 1977). Collectively, these observations indicate that limbic, but not non-limbic kindling produces significant interictal comorbidities on a range of unconditioned behaviors.

Few studies have investigated the effect of kindling on conditioned behaviors. Kindling has been reported to condition interictal anticipatory behaviors and place aversion associated with the environment where kindling procedures are delivered (Barnes, Pinel, Francis, & Wig, 2001; Wagner & Corcoran, 2008). In contrast, the effect of seizures on conditioned learning and memory has received substantially less attention. However, several independent studies have reported that conditioned fear is impaired following kindling (Henderson, Galic, & Teskey, 2009), kainic acid (Kemppainen et al., 2006), and pilocarpine (Cardoso et al., 2009; Dos Santos, Longo, Blanco, Menezes de Oliveira, & Mello, 2005; McKay & Persinger, 2004; Szyndler et al., 2005). The results of *Chapters 2-4* support these observations, but also add several novel findings. In particular, my results indicate that limbic kindling significantly impairs conditioned fear behaviors, whereas non-limbic kindling does not (*Chapter 3*). These observations are consistent with previous reports that limbic, but not non-limbic kindling disrupts unconditioned behaviors (Kalynchuk et al., 1998). The results of *Chapter 2 and 3* further reveal that impairments of conditioned freezing parallel reductions of neural activity in limbic circuits mediating fear learning and memory. Specifically, my results indicate that amygdala-dependent fear conditioning is rapidly impaired by amygdaloid kindling. In contrast, hippocampal-dependent fear conditioning is only impaired following long-term kindling. These results indicate that amygdalar circuits are quickly compromised following amygdala kindling, whereas hippocampal circuits become disrupted much more slowly. These dissociations clearly demonstrate that operant fear conditioning is a useful model to investigate neural circuits involved in learning and memory. In line with this, I found that deficits in conditioned freezing of limbic, but not non-limbic-kindled rats paralleled plasticity of the hippocampal NPY system and hippocampal neurogenesis (*Chapters 3 and 4, respectively*). Collectively, my results suggest that operant fear conditioning is an ideal assay to evaluate the relationship between interictal comorbidities and the aberrant plasticity of neural circuits associated with kindling.

7.3 The Role of NPY in Seizures and Cognition

Hippocampal NPY expression is dramatically increased following experimentally induced seizures (Rizzi et al., 1993; Sperk et al., 1992; Tonder et al., 1994; Vezzani et al., 1994; Vezzani et al., 1996) and in human patients with TLE (Furtinger et al., 2001). In particular, NPY expression is greatly increased in the hippocampal mossy fibers and termination zones in the CA3 stratum lucidum. Under basal conditions, NPY is stored in large dense core vesicles and released during periods of intense neuronal activity (Hokfelt, 1991; Ramamoorthy, Wang, & Whim, 2011). However, NPY release is greatly increased under basal and depolarizing conditions in rats that experience recurrent seizures (Rizzi et al., 1993; Vezzani et al., 1994). Seizures also induce *de novo* expression of NPY2R in mossy fibers and dentate granule cells (Furtinger et al., 2001; Gobbi et al., 1998; Roder et al., 1996; Schwarzer et al., 1998) and increase NPY2R receptor binding affinity (Gobbi et al., 1998; Schwarzer et al., 1998). This seizure-induced upregulation of the hippocampal NPY system is important because interactions between NPY and NPY2R presynaptically inhibit glutamate release by reducing Ca^{2+} influx through inhibition of several calcium channels (Qian et al., 1997; Schwarzer et al., 1998). The presynaptic blockade of glutamate mediated by interactions between NPY and NPY2R potently inhibits epileptiform activity and protects against seizure-induced excitotoxicity (Colmers & El, 2003; Noe et al., 2010; Silva et al., 2003; Sorensen et al., 2008). These findings have led researchers to posit that seizure-induced upregulation of NPY represents an endogenous anticonvulsant mechanism. The results of *Chapter 3* are consistent with these observations, in that I also observed significant induction of NPY and NPY2R in the dentate gyrus and CA3. However, my findings provide novel evidence that is necessary to elicit seizures within limbic structures to induce plasticity of NPY and NPY2R. This finding is interesting because it suggests that severe seizures that propagate throughout limbic circuitry are necessary for the compensatory changes of the NPY system to occur. My findings also revealed a reduction in the number of detectable NPY-ir interneurons in CN-kindled rats. One possibility of this observation is that I studied a period where CN seizures begin to induce hippocampal NPY plasticity, albeit to a lesser degree than limbic kindling. It would be interesting to do time-course studies that evaluate NPY immunoreactivity in response to varying numbers of kindling stimulations.

Although NPY is most widely studied for its behavioral effects on appetite (Batterham & Bloom, 2003; Kalra, Dube, Sahu, Phelps, & Kalra, 1991), the critical role of NPY in hippocampal learning and memory is becoming increasingly recognized (Redrobe et al., 1999).

Indeed, several studies have demonstrated that gain or loss of function manipulations of NPY and its receptors have broad effects on hippocampal-dependent learning and memory. For example, NPY overexpressing transgenic rats display substantial acquisition and spatial memory deficits in the Morris water maze (Thorsell et al., 2000). Similarly, NPY2R knockout mice (NPY Y2R -/-) show normal learning in the acquisition phase of the Morris water maze, but display spatial memory deficits on subsequent probe trials (Redrobe, Dumont, Herzog, & Quirion, 2004). Moreover, systemic injections of the NPY2R agonist PYY3-36 disrupts spatial memory performance in the Morris water maze (Stadlbauer et al., 2013). Most recently, it was reported that methamphetamine rapidly increases hippocampal mRNA expression of NPY, NPY2R, and NPY5R and induces a parallel enhancement of NPY2R binding affinity (Goncalves et al., 2012). The plasticity of NPY and its receptors following methamphetamine exposure coincides with deficits on spatial and objection recognition memory. Remarkably, these cognitive impairments were abolished with pre-treatment with the selective NPY2R antagonist BIIE0246 prior to methamphetamine exposure. These results demonstrate a critical role for the NPY2R in the spatial memory deficits associated with methamphetamine intoxication (Goncalves et al., 2012). Collectively, these studies demonstrate that alterations of NPY and its receptors have broad effects on hippocampal-dependent learning and memory. These findings are particularly exciting in relation to *Chapter 3*, where the increased immunoreactivity of NPY and NPY2R in limbic-kindled rats coincided with substantial hippocampal-dependent memory impairments. One possibility of my findings is that the upregulation of NPY and NPY2R in limbic-kindled rats potently restricts hippocampal glutamate function during interictal periods and thereby inadvertently disrupts cognitive function. More research is required to develop a better understanding of the functional consequences of NPY and NPY2R plasticity on memory function in the epileptic brain.

7.4 Aberrant Hippocampal Neurogenesis and Cognition

It is widely recognized that seizures cause a robust induction of aberrant hippocampal neurogenesis (Kokaia, 2011; Parent & Lowenstein, 2002; Parent et al., 2006). In particular, seizure-generated neurons have a tendency to migrate abnormally into the hilus (HEGCs) and develop HBDs that persist into maturity (Ribak et al., 2000; Scharfman, Goodman, & McCloskey, 2007). HEGCs and HBDs are particularly problematic because they form recurrent

excitatory circuits that contribute to hippocampal dysfunction. The results of *Chapter 4* are consistent with these findings; however, they further demonstrate that limbic convulsions are necessary to increase hippocampal neurogenesis and induce aberrant characteristics. Due to the overwhelming preference to study limbic kindling and status epilepticus models of generalized seizures, there has been minimal consideration in the epilepsy field regarding the site-specific effects of seizures on hippocampal neurogenesis prior to my report. The site-specific differences of seizures on hippocampal neurogenesis is important to consider, as it may help explain the extensive behavioral comorbidities that are most prevalent and severe in TLE. Furthermore, my results also demonstrate that limbic but not non-limbic kindling results in an upregulation of hippocampal BDNF protein expression. This observation is interesting because BDNF is reported to enhance neuronal differentiation and maturation of adult generated neurons (Waterhouse et al., 2012), induce the formation of HBDs (Danzer et al., 2002), contribute to the formation of HEGCs (Scharfman et al., 2005), and cause a long-lasting induction of NPY (Reibel et al., 2000); findings which are consistent with the results of *Chapters 3 and 4*. Taken together, my results indicate that limbic but not non-limbic kindling promotes aberrant hippocampal neurogenesis and cognitive dysfunction, possibly through seizure-induced upregulation of BDNF.

The relationship between aberrant seizure-induced neurogenesis and cognition remains a complex issue. A limited number of studies have demonstrated that ablation or normalization of seizure-induced neurogenesis coincides with improved cognitive outcomes (Cho et al., 2015; Jessberger et al., 2007a; Pekcec et al., 2008). However, these findings raise several important considerations. The ablation of seizure-induced neurogenesis also reduces the frequency of spontaneous seizures, presumably because of the proconvulsant properties of these neurons. Therefore, a reduction in seizure frequency likely provides numerous cognitive benefits that may be independent of neurogenesis (Cho et al., 2015). Similarly, VPA and endoN have numerous downstream cellular targets that could potentially enhance cognitive function independent of stabilizing neurogenesis (Jessberger et al., 2007a). Despite these concerns, the ablation or restoration of seizure-induced neurogenesis would also reduce the number of recurrent excitatory circuits formed by seizure-generated neurons (e.g., HBDs, HEGCs). This consideration is important, because computational modeling experiments suggest that the recurrent excitation of dentate granule cells interferes with the sparse coding functions of the dentate gyrus, which

could impair pattern separation and presumably spatial memories (Yim, Hanuschkin, & Wolfart, 2015).

One caveat of the previously described results may be that it is an oversimplification to exclusively classify adult neurogenesis as aberrant. Rather, seizures produce a heterogeneous population of adult generated neurons, with some neurons featuring neuroprotective properties whereas others confer proconvulsant features (Murphy et al., 2011). In line with this, the results of *Chapter 4* reported that only a subset of seizure-generated neurons migrate abnormally into the hilus (i.e., HEGCs), where they are reported to form recurrent excitatory circuits that facilitate epileptiform activity (Scharfman et al., 2000). The remaining population of seizure-generated neurons appear to migrate normally into the GCL. Of the adult generated neurons that migrate appropriately, as many as 40% feature HBDs that form recurrent excitatory circuits (Ribak et al., 2000). The results of *Chapter 4* are consistent with these observations, as I found that approximately 34% of DCX-ir neurons in dHip-kindled rats featured HBDs. However, these observations do not account for all of the seizure-generated neurons that migrate correctly into the GCL. Indeed, some seizure-generated neurons migrate and integrate into the hippocampal circuitry without any overt aberrant morphological features. These morphologically normal cells are reported to have anti-epileptic properties, indicated by a reduction of excitatory inputs and an enhancement of inhibitory synaptic drive (Jakubs et al., 2006). These contradictory findings suggest that adult generated neurons can limit local network activity to provide potent anticonvulsant effects. In line with an inhibitory role, it was recently reported that prior ablation of hippocampal neurogenesis increased the severity and duration of kainic acid-induced status epilepticus (Iyengar et al., 2015). This observation suggests that the ablation of neurogenesis results in greater network excitability that may facilitate seizure activity. One interesting possibility is that the inhibitory properties of adult generated neurons may inhibit adjacent mature granule cell populations. In particular, extracellular field recordings of the dentate gyrus in neurogenesis ablated mice were reported to show a greater frequency of gamma-frequency bursts and synchronization of dentate granule cell firing compared to controls (Lacefield, Itskov, Reardon, Hen, & Gordon, 2012). Moreover, neurogenesis ablated mice were found to have greater induction of the IEG ARC within the granule cell layer following active place avoidance tasks (Burghardt, Park, Hen, & Fenton, 2012). These observations are particularly exciting because my results indicate that the reduction of IEG activity occurs exclusively in limbic-

kindled rats that have increased hippocampal neurogenesis. Whether seizure-generated neurons produce similar net inhibitory effects remains to be determined.

Unfortunately, researchers currently lack molecular or genetic tools to selectively manipulate the aberrant or neuroprotective subcategories of seizure-generated neurons *in vivo*. Indeed, all current manipulations of adult generated neurons (e.g., irradiation, genetic deletion, pharmacological agents) target the entire population of cells regardless of their morphological or electrophysiological features. This issue poses a major barrier for developing novel therapeutic options, where it would be ideal to ablate aberrant proconvulsant seizure-generated neurons while retaining neuroprotective neurons. More research is required to elucidate molecular or genetic factors that can discriminate between these two different populations of seizure-generated neurons.

7.5 Kindling and Cell Loss

The relationship between kindling and cell loss is a contentious issue. Several studies have reported that electrical stimulation of the perforant path or limbic structures results in a pattern of hippocampal cell loss that is representative of end folium sclerosis (Cavazos & Sutula, 1990; Cavazos, Das, & Sutula, 1994; Sloviter, 1987). In these reports, as many as 50% of dentate hilar cells (e.g., mossy cells and GABAergic interneurons) die following 150 electrical stimulations of limbic sites (Cavazos et al., 1994). In contrast, several studies have also reported that there is no detectable cell loss or tissue damage even after periods of extended kindling (i.e., 200 - 300 stimulations) (Brandt et al., 2004; Mathern et al., 1997; Michalakis et al., 1998). As an extreme example, it was reported that 1500 kindling stimulations of the ventral hippocampus over 9 months had no effect on the total number of hilar neurons compared to controls (Bertram, III & Lothman, 1993). However, extended hippocampal kindling in this study resulted in the expansion of the dentate hilus which lead to an overall reduction in hilar neuronal density. Despite this caveat, this same study reported that a single episode of status epilepticus drastically reduced the total number of hilar neurons and hilar neuronal density compared to controls due to cell death (Bertram, III & Lothman, 1993). Taken together, these findings suggest that kindling simply does not cause the same extent of neuronal damage as status epilepticus models (Bertram, 2007; Morimoto, Fahnestock, & Racine, 2004). The inconsistent reports on kindling-induced

neuronal loss between laboratories may reflect differences in stimulation parameters, rat strains, and methods of analysis.

The results of this dissertation are consistent with previous observations that kindling does not cause gross neuronal loss (Brandt et al., 2003; Mathern et al., 1997; Michalakis et al., 1998). The results of *Chapter 3* support this notion, as I found minimal immunoreactivity of the neurodegenerative marker FluoroJade B in the hippocampus of limbic and non-limbic kindled rats following 99 electrical stimulations. In stark contrast, a single episode of pilocarpine-induced status epilepticus produced robust FluoroJade B immunoreactivity throughout the dentate gyrus. My observations in *Chapter 5* are also consistent with previous reports that kindling does not produce overt degeneration of PV or SOM interneurons (Kamphuis, Huisman, Wadman, Heizmann, & Lopes da Silva, 1989; Schwarzer et al., 1996). Moreover, if kindling causes extensive neuronal degeneration of GABAergic interneurons, my results should have detected significant reductions in the number of hippocampal GAD67-ir interneurons throughout the various subfields of the hippocampus. However, I was unable to find a single instance where GAD67-ir was reduced in kindled rats compared to controls. Indeed, my results indicate that caudate-kindling caused an induction in the number of GAD67-ir cells in several hippocampal subfields. Taken together, these findings indicate that the kindling model I used does not cause extensive hippocampal damage or neuronal loss. Instead, my results suggest that kindling induces subfield-specific reductions of perisomatic and interneuron-selective inhibition, and an enhancement of dendritic inhibition. It is becoming increasingly recognized that researchers should consider the profound reorganization of excitatory and inhibitory networks associated with seizures rather than focusing exclusively on the absolute number of cells that may be lost (Cossart et al., 2005). In line with this, the robust morphological plasticity of SOM interneurons following limbic kindling (*Chapter 5*) likely has broad functional consequences for dendritic-mediated inhibition in the kindled brain.

My results further indicate that interictal behavioral comorbidities in limbic-kindled rats (*Chapters 2-4*) occur independently of gross neuronal loss (*Chapters 3 & 5*) These observations suggest that the cognitive impairments associated with limbic kindling may reflect alterations in synaptic function and/or plasticity rather than overt neural damage. The rapid restoration of cognitive function following acute intrahippocampal infusions of reelin supports this notion

(*Chapter 6*). In particular, it is unlikely that acute intrahippocampal reelin infusions provides ample time for the restoration of aberrant structural features induced by kindling (e.g., mossy fiber sprouting, HEGCs and HBDs). Rather, it is more likely that exogenous reelin induces rapid morphological (e.g., dendritic spines) or functional (e.g., increased neurotransmitter release or receptor binding) changes that may account for its acute effects on cognitive function. Although my preliminary observations cannot explain the mechanisms through which reelin restores cognition, one intriguing possibility is through interactions with the extracellular matrix (ECM).

During developmental periods, the ECM facilitates neural plasticity by increasing cell proliferation, guiding neuronal migration, and promoting synaptogenesis. As the nervous system matures, the ECM restricts neural plasticity to provide nervous system stability (McRae & Porter, 2012; McRae, Baranov, Rogers, & Porter, 2012). The perineuronal net (PNN) is a core component of the ECM that contributes to synaptic stability by enveloping mature CNS neurons. Importantly, reelin is preferentially secreted into the PNN, where it interacts with other ECM molecules to provide synaptic stability (Pesold et al., 1998). These findings are important, because seizures reduce the hippocampal expression of reelin (Fournier et al., 2010) and compromise the integrity of the ECM and the PNN in particular (McRae & Porter, 2012; McRae et al., 2012). The epilepsy-induced dysfunction of the ECM in adulthood may explain why seizure-induced plasticity recapitulates developmental periods (Galanopoulou & Moshe, 2014). One intriguing hypothesis is that the exogenous reelin infusions restores the seizure-induced downregulation of hippocampal reelin in BLA-kindled rats to rescue PNN function and provide synaptic stability. More research is needed evaluate to this hypothesis.

7.6 Limitations

7.6.1 Clinical Relevance of Kindling

Kindling provides researchers a level of experimental control that is simply not available in other models of epilepsy. In particular, kindling allows researchers to select the focal site of seizure initiation and control both the number and the intensity of seizures that are experienced by a subject. Kindling therefore provides the ability to study time-sensitive factors in the process of epileptogenesis. The ability to study early versus late factors involved in epileptogenesis provides immense scientific value for studying neural circuits involved in seizure initiation and

propagation. In this regard, kindling is arguably one of the best models to study neural plasticity in a controlled manner (Scharfman, 2002).

Despite the many advantages of kindling, one of the most widely criticized limitations of this model is that it does not readily produce the defining feature of human epilepsy: spontaneous recurrent seizures (Bertram, 2007). Although spontaneous seizures can be elicited with extended kindling stimulations under certain circumstances, this process is arduous and does not occur in every subject. This issue is best exemplified by the work of Juhn Wada, who first reported that daily amygdaloid kindling of Senegalese baboons over a period of 2 – 3 months resulted in the development of spontaneous complex partial and primary generalized seizures (Wada & Osawa, 1976). However, subsequent work in Wada's laboratory revealed that rhesus macaques never fully "kindled" (i.e., experienced class 5 convulsive seizures) or developed spontaneous seizures despite receiving approximately 400 amygdaloid stimulations over a period of 6 months (Wada, Mizoguchi, & Osawa, 1978). These seminal studies by Wada revealed that certain subjects have a greater resistance to the "kindled" state and the subsequent development of spontaneous seizures. Although the literature on spontaneous seizures evoked by kindling in primates is inconsistent, several independent laboratories have reported that rats readily develop spontaneous seizures following 200 - 300 amygdaloid stimulations (Brandt et al., 2004; Michalakis et al., 1998; Pinel & Rovner, 1978).

A second major issue with the use of kindling as a model of epilepsy is whether a kindling-like process actually occurs in human cases of epilepsy (Post, 2002). The best support for a kindling-like process in humans stem from cases where patients with a history of epilepsy develop seizures that become increasingly complex over time, evidenced by increases in seizure severity and duration (Bertram, 2007). However, some cases of epilepsy do not follow a kindling-like progression. For instance, benign Rolandic epilepsy during childhood ends by the onset of puberty and has no adverse long-term consequences into adulthood (Camfield & Camfield, 2014).

One possible reason for these discrepancies is that kindling requires a stimulus to elicit a seizure. With the exception of using electroconvulsive therapy (ECT) for treatment-resistant depression, there are few instances where humans experience inducible recurrent seizures. Retrospective studies from ECT patient populations reliably report that patients almost never

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develop epilepsy or spontaneous seizures despite numerous ECT procedures (Blackwood, Cull, Freeman, Evans, & Mawdsley, 1980; Ray, 2013). As an extreme example to support this notion, a patient that reportedly received 1250 ECT procedures over a 26 year period did not develop spontaneous seizures or display any evidence of gross neurological damage upon post-mortem evaluation (Lippman et al., 1985). However, there are rare cases where patients that underwent ECT procedures were subsequently diagnosed with epilepsy (Rasmussen & Lunde, 2007). In these rare cases, the majority of patients were able to continue ECT treatments without further complications or the development of spontaneous seizures. Collectively, these findings suggest that although a kindling-like phenomena can occur in humans, it is extremely rare. This point raises the issue of whether kindling is truly a model of human epilepsy, or whether it is better described as a model of controlled neural plasticity.

One final limitation of the kindling model relates to the region-specific differences involved in the initiation and propagation of elicited seizures (McIntyre & Gilby, 2008). An important distinction to make is that by kindling different brain regions, I manipulated both the severity of the convulsions and the time course over which they developed (e.g., *see Fig. 4-1*). In particular, kindling of the BLA occurred quickly, which resulted in a large number of severe convulsions throughout the duration of the study. Kindling of the dHip occurred much more slowly (e.g., approximately 3 X more stimulations to yield the first class 5 seizure), yet also ultimately resulted in severe convulsions. In contrast, kindling of the CN elicited convulsions on the first stimulation, but the severity and duration of the convulsions remained comparatively mild throughout the duration of the study. In this regard, the present collection of studies did not control for the total number of severe convulsions experienced by each experimental group. Moreover, because I did not record EEG during kindling stimulations, there is no data on the total afterdischarge duration that proceeded beyond the convulsion. This issue is an important omission from the collection of studies discussed in this thesis because it would further complement our understanding of how seizure severity and/or afterdischarge duration relates to epileptogenesis, cognitive dysfunction, and neural plasticity. Despite these aforementioned issues, the inherent differences in the local circuitry of each targeted brain region would make it nearly impossible to control for the duration and severity of elicited motor seizures between groups. This last point reflects the fact that seizures represent heterogeneous phenomena that are difficult to compare between subjects.

7.6.2 Sex Differences

A limitation of my current findings is that all experiments were done exclusively in male rats. This issue warrants discussion because there are well-known sex differences involved in the neurobiology of epilepsy (Scharfman & MacLusky, 2014). An obvious concern is that female sex hormones can broadly influence hippocampal plasticity and function. For example, hippocampal excitability and BDNF immunoreactivity are influenced by the estrus cycle, and peak during during periods of proestrus and estrus compared to metestrus (Scharfman, Mercurio, Goodman, Wilson, & MacLusky, 2003). Although these findings indicate that estrogens may confer proconvulsant properties at specific time points, estrogens also exert anticonvulsant effects (Velisek & Veliskova, 2008). In particular, beta-estradiol appears to protect the hippocampus against seizure-induced damage by increasing expression of NPY throughout the hilus of the dentate gyrus (Veliskova & Velisek, 2007). Finally, estrogens also increase the proliferation and survival of adult generated neurons in the dentate gyrus (Duarte-Guterman, Yagi, Chow, & Galea, 2015). Whether sex-specific differences in hippocampal excitability, growth factors, and hippocampal neurogenesis would drastically alter the results of my behavioral and post-mortem evaluations remains to be determined.

Although I acknowledge sex-specific differences must be carefully considered in my research, it is important to emphasize that seizures also induce robust changes with minor sex-specific differences. For example, NPY immunoreactivity is upregulated in both sexes following electroconvulsive shocks, albeit to a slightly greater degree in male rats (Jimenez-Vasquez, Overstreet, & Mathe, 2000). Moreover, cortical kindling induces similar plasticity of layer III pyramidal neurons in female and male rats, but these morphological changes occur more rapidly in female rats (Teskey, Hutchinson, & Kolb, 1999). Finally, my laboratory has previously characterized fearful behaviors of male and female kindled rats in the open field test. These studies revealed that although kindling increases fearfulness compared to controls, the sex-specific differences in fearful behavior were relatively subtle (e.g., more wall jumping in females) (Fournier et al., 2009; Wintink et al., 2003). Taken together, these findings indicate that seizures induce many changes that are relatively consistent between sexes.

7.6.3 Fos as a Marker of Neural Activity

In the present collection of studies, I used the IEG Fos as a marker of behaviorally relevant neural activity. IEGs are widely used in neuroscience applications because of their low detection rates under resting conditions and robust induction following patterned neural activity typically associated with memory consolidation or retrieval (Guzowski et al., 2005). However, it is important to note that there are two functional classes of IEGs that produce activity-dependent responses over different time courses. In particular, the class of IEGs known as regulatory transcription factors (RTFs) represent late-response genes that have broad control over global cellular function by controlling transcription of numerous downstream target genes (Kubik, Miyashita, & Guzowski, 2007). Fos is the most widely used RTF and has a maximal induction time of approximately 90 – 120 minutes after behavioral testing. In contrast, the class of IEGs known as effector proteins influence cellular functions, including intracellular signaling and structural modifications (e.g., synapse formation). ARC is the most widely used effector protein and has maximal induction times of 5 minutes (RNA) or 60 minutes (protein) after behavioral testing. One possible limitation of my post-mortem observations is the exclusive use of Fos. Although comparative studies report that behavioral tasks induce similar patterns of RTF and effector protein expression (Guzowski et al., 2005), I did not make these comparisons. However, my preliminary observations suggest that patterns of Fos immunoreactivity following behavioral testing is comparable with ARC immunoreactivity (Fig. 7-1).

7. Future Directions

7.7.1 The Relationship Between Seizure-Induced Plasticity of NPY and Cognition

The results of *Chapter 3* revealed that limbic, but not non-limbic kindling impaired hippocampal-dependent fear conditioning and reduced immunoreactivity of the behaviorally relevant neural activity marker Fos. The reduction of conditioned freezing and Fos immunoreactivity coincided with parallel enhancement of hippocampal immunoreactivity for NPY and NPY2R. Because interactions between NPY and NPY2R presynaptically inhibit glutamate release (Greber, Schwarzer, & Sperk, 1994; Vezzani & Sperk, 2004), I proposed that plasticity of the NPY system following limbic kindling may limit glutamatergic function during interictal periods effects, thereby inadvertently impairing cognitive functions. Due to the correlative nature of my findings, I did not provide evidence whether manipulations of the NPY system can ameliorate deficits on hippocampal-dependent fear conditioning or restore markers of
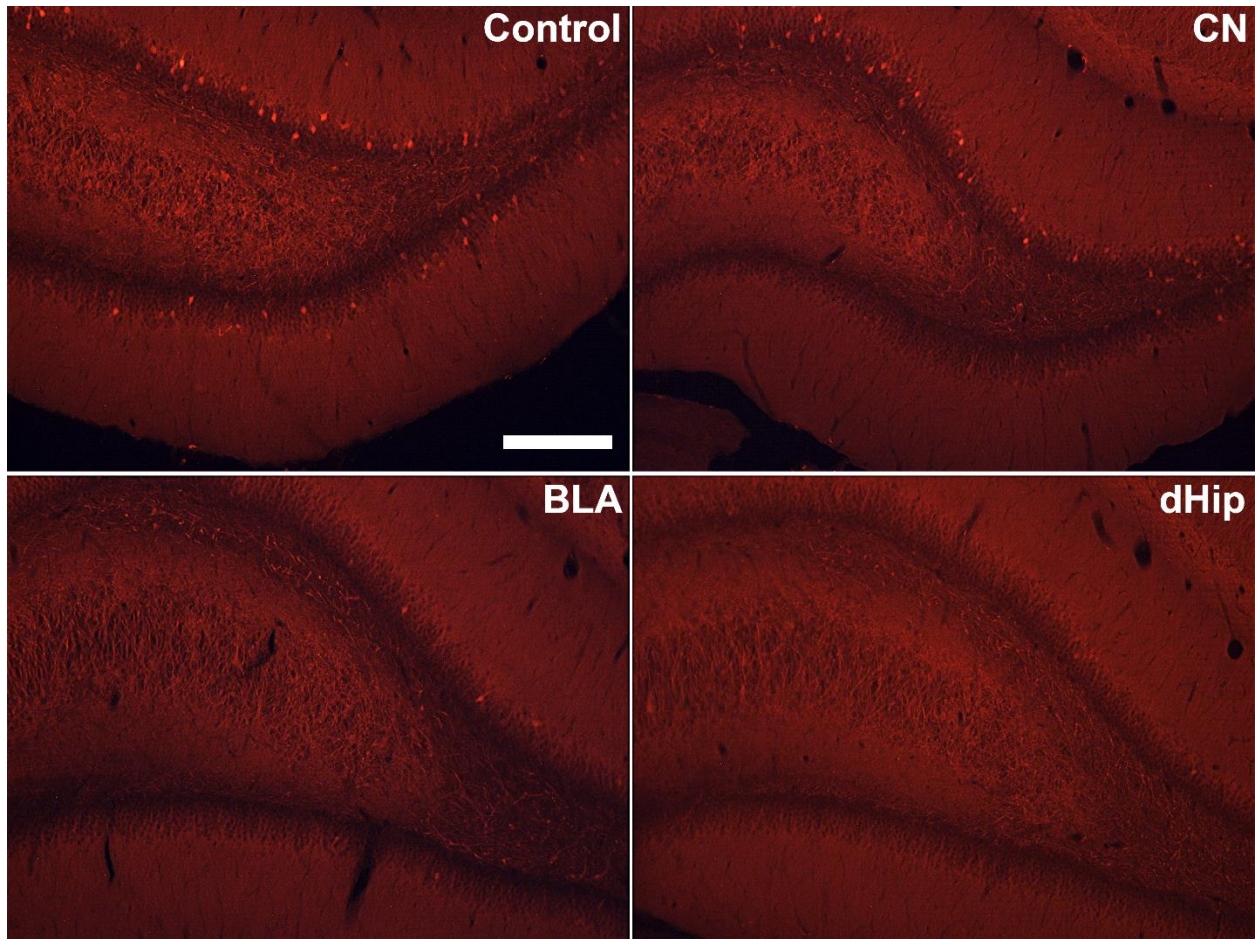
Figure 7-1. Pattern of ARC immunoreactivity following contextual fear memory retrieval. Note the significant induction of ARC immunoreactivity in control and CN-kindled rats approximately 60 minutes following fear memory retrieval. In contrast, BLA- and dHip-kindled rats show marked reductions of ARC immunoreactivity. CN, caudate nucleus; BLA, basolateral amygdala; dHip, dorsal hippocampus. (Botterill , Nogovitsyn, Caruncho, & Kalynchuk, Unpublished observations, 2016). (Scale bar = 200 μm).

behaviorally relevant neural activity to control levels following behavioral testing. To address this issue, future experiments could directly manipulate the NPY system during fear conditioning procedures with commercially available and highly specific brain-penetrant small molecule NPY2R antagonists (Bonaventure et al., 2004; Brothers et al., 2010; Jablonowski et al., 2004). In addition to determining whether antagonism of the NPY2R can ameliorate hippocampal-dependent fear conditioning deficits in limbic-kindled rats, these experiments could also provide valuable information regarding the functional consequences of seizure-induced hippocampal NPY upregulation. For example, NPY overexpressing mice have significant disruptions of hippocampal LTP that can be rapidly reversed through antagonism of the NPY2R (Sorensen et al., 2008). A series of slice electrophysiology experiments could be done to determine whether antagonism of the NPY2R ameliorates kindling-induced disruptions of LTP (Schubert, Siegmund, Pape, & Albrecht, 2005; Sherafat et al., 2013).

7.7.2 The Effects of Kindling on Striatal-Dependent Cognition and Plasticity

The present collection of studies suggests that CN kindling has minimal effects on hippocampal-dependent fear conditioning and hippocampal plasticity. However, my analyses did not determine whether CN kindling impairs striatal-dependent behavioral tasks or induces aberrant striatal plasticity. To resolve this issue, future studies should conduct a series of behavioral tasks that are designed to dissociate hippocampal- and striatal-dependent forms of cognition. For example, the two-platform Morris water maze features two visually distinct balls (e.g., horizontal vs vertical stripes) emanating from the water that are indicative of possible platform locations. The hippocampal-dependent version of this task involves learning to swim to the same platform location on each session regardless of alternating visual ball cues. In contrast, the striatal-dependent version of this task involves alternating the correct platform choice by relying on one of the visual ball cues in particular (i.e., stimulus-response habit learning) (Packard & McGaugh, 1992). Moreover, an alternative striatal-dependent behavioral assay is the win-stay version of the radial arm maze. This version of the radial arm maze cues the location of 4 random food-baited arms with light and requires rats to revisit arms they have already visited within a single trial (McDonald & White, 1993; Packard, Hirsh, & White, 1989).

Kindling of several discrete brain regions (e.g., the amygdala, hippocampus, and corpus callosum) leads to cortical motor map expansion and interictal motor impairments (Henderson et

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al., 2011; Teskey et al., 2008; van, Young, Larson, & Teskey, 2006). Because the caudate-putamen is part of the basal ganglia motor system, it would be interesting to determine whether CN kindling also leads to cortical motor map expansion. In line with this, one could determine if CN kindling impairs fine motor skill learning on the single pellet reaching task (Klein & Dunnett, 2012). To compliment any of the aforementioned behavioral assays, a series of post-mortem analyses could be included to evaluate aberrant striatal plasticity (e.g., plasticity of interneuron subpopulations, patterns of Fos immunoreactivity following behavioral testing, or measures of neurogenesis).

7.7.3 The Relationship Between Aberrant Neurogenesis & Cognitive Dysfunction

Although the aberrant characteristics of seizure-generated neurons are well characterized for their proconvulsant effects (Parent et al., 2006; Parent & Murphy, 2008), the contribution of these neurons in cognitive functions remain poorly understood. My laboratory has previously shown that mature seizure-generated neurons of BLA-kindled rats show minimal colocalization with the neural activity marker Fos following hippocampal-dependent fear memory retrieval (Fournier et al., 2013). This finding suggests that seizure-generated neurons are not properly recruited into memory networks. To evaluate this issue further, it would be interesting to determine whether kindling of other brain regions (e.g., CN and dHip) also impairs the recruitment of seizure-generated neurons into functional memory networks. Moreover, because VPA has been shown to rescue aberrant neurogenesis and cognitive function in kainic acid models of epilepsy (Jessberger et al., 2007a), it would be interesting to determine whether this effect can be recapitulated in limbic-kindled rats. Alternatively, it would be important to do a series of ablation studies to provide causal evidence demonstrating aberrant neurogenesis contributes to cognitive dysfunction. Although this approach is more challenging in rats due to the lack of available transgenic lines, recent advances in clustered regularly-interspaced short palindromic repeats (CRISPR) is conducive to genome editing in rats (Shao et al., 2014) and recent studies have manipulated murine neural stem cells *in vivo* using this approach (Kalebic et al., 2016).

Brain insults such as seizures or stroke are known to reliably increase SVZ neurogenesis (Parent, Vexler, Gong, Derugin, & Ferriero, 2002; Parent, Valentin, & Lowenstein, 2002). Interestingly, some of these SVZ-generated neurons migrate into the striatum and appear to serve

reparative functions by replacing damaged neurons (Parent, 2003). Future studies should determine whether there are differences in limbic and non-limbic kindling on measures of SVZ neurogenesis, with particular emphasis towards neurogenesis in the dorsal striatum.

7.7.4 Reelin to the Rescue! Mechanisms of Action?

The preliminary evidence discussed in *Chapter 6* revealed that acute intrahippocampal infusions of recombinant reelin rescued fear memory impairments associated with BLA kindling. Remarkably, my preliminary Fos observations indicate that the restoration of memory impairments may coincide with the normalization of hippocampal neural activity. These observations pose many questions that remain to be answered. First, a series of western blot analyses should confirm that reelin infusions result in a net increase of reelin protein expression in the hippocampus during behavioral testing. Second, because exogenous reelin alters the hippocampal expression of several glutamatergic receptors (e.g., GluR1, NR1, NR2A, NR2B) (Qiu et al., 2006; Qiu & Weeber, 2007) and my preliminary observations indicate that reelin may restore behaviorally relevant neural activity, a series of western blots should determine the effects of exogenous reelin on the hippocampal expression of glutamatergic receptors in the kindled brain. A series of electrophysiological experiments could further determine whether exogenous reelin is able to reverse kindling-induced deficits of hippocampal LTP (Sherafat et al., 2013). These series of experiments could be particularly fruitful because reelin has been shown to enhance LTP under basal conditions (Weeber et al., 2002) and restore deficits of hippocampal LTP in a mouse model of Angelman syndrome (Hethorn et al., 2015).

7.8 Conclusions

This goal of this dissertation was to develop a better understanding of the relationship between aberrant plasticity and cognitive dysfunction in the kindling model of epilepsy. The collection of experiments revealed that kindling impairs operant fear conditioning in a task- and stimulation-dependent manner. Moreover, I demonstrated that kindling of limbic, but not non-limbic brain regions cause impairments of conditioned fear that parallel changes to the NPY system, hippocampal neurogenesis, and specific hippocampal GABAergic inhibitory interneurons. Finally, my preliminary data suggests that kindling-induced cognitive dysfunction is rescued through intrahippocampal infusions of recombinant reelin. Taken together, my findings reveal multiple mechanisms through which repeated convulsions promote aberrant

plasticity and cognitive dysfunction. These preclinical findings represent novel therapeutic targets that may lead to the development of more effective treatments for epilepsy and its associated cognitive decline.

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