ABERRANT STRUCTURAL AND FUNCTIONAL PLASTICITY IN THE ADULT HIPPOCAMPUS OF AMYGDALA KINDLED RATS

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ABSTRACT

Amygdala kindling is commonly used to study the neural mechanisms of temporal lobe epilepsy and its behavioral consequences. The repetitive seizure activity that occurs during kindling is thought to induce an extensive array of structural and functional modifications within the brain, particularly in the hippocampus and dentate gyrus regions. Some of these changes include the growth or sprouting of new axonal connections as well as the birth and integration of new neurons into hippocampal circuits. Previous work has shown that these changes in structural and functional plasticity are not necessarily beneficial events. For instance, the growth and reorganization of synaptic terminals in the hippocampus and other brain regions might serve as a substrate that enhances hyperexcitability and seizure generation. In addition, although seizures induce the birth of new neurons, many of these newly generated cells migrate and function improperly within the hippocampal networks. Considering the prominent role of the hippocampus in a variety of behaviours, including learning, memory, and mood regulation, it would appear that alterations involving the structural and functional properties of both mature and newly born neurons in this region could impact these hippocampal-dependent functions. However, to date, the role of kindling-induced changes in hippocampal structural plasticity and neurogenesis on behaviour is incomplete, and the molecular mechanisms that govern these pathological events are poorly understood.

The aim of this dissertation is to gain a better understanding of the changes in synaptic plasticity and neurogenesis within the hippocampus that occur after amygdala kindling. In chapter 2, we will examine if kindling alters the expression of synapsin I, a molecular marker of synaptic growth and activity, in both the hippocampus and other brain regions. In addition, we will also set out to determine if changes in synapsin I are related to the development of behavioural impairments associated with kindling. In chapter 3, the effect of kindling on hippocampal neurogenesis will be examined. In addition, we will also evaluate the effect of kindling on the expression of Reelin and Disrupted-in-Schizophrenia 1 (DISC1), two proteins instrumental for mediating proper neuronal migrational and maturation during development. In chapter 4, the effect of altered DISC1 expression in the dentate gyrus after kindling will be examined more

extensively. We will examine whether altered DISC1 expression in the dentate contributes to some of the pathological features associated with seizure-induced hippocampal neurogenesis, such as ectopic cell migration and dentate granule cell layer dispersion. Finally, in chapter 5, the impact of aberrant seizure-induced neurogenesis on behaviour will be examined by determining if seizure-generated neurons functionally integrate and participate in hippocampal circuits related to memory processing. The results of this dissertation enhances our understanding of the functional consequences that altered hippocampal synaptic plasticity and neurogenesis may have on the development of epilepsy and emergence of cognitive impairments associated with chronic seizures.

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DEDICATION

I dedicate this work to my mother (Mary Fournier) and to my Nana (Joan Mary Patrick) who I wish could both be here with me to celebrate the completion of my Doctorate.

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LIST OF ABBREVIATIONS

5-HT	serotonin
AD	after-discharge
BDNF	brain-derived neurotrophic factor
BLA	basolateral amygdaloid complex
BrdU	bromodeoxyuridine
CA	Cornu Ammonis
CNS	central nervous system
CR	Cajal-Retzius cells
DCX	doublecortin
DG	dentate gyrus
DISC1	disrupted-in-schizophrenia 1
DISC2	disrupted-in-schizophrenia 2
EC	entorhinal cortex
EEG	electroencephalographic
EPSC	excitatory post-synaptic current
GABA	γ-aminobutyric acid
GAP43	growth associated protein 43 kDa
GCL	granule cell layer
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
LIS1	lissencephaly 1
LTP	long-term potentiation
MAM	methylazoxymethanol acetate
NeuN	neuron-specific nuclear marker
NDEL1	nuclear distribution gene E homolog-like 1
NO	nitric oxide
NMDA	N-methyl-D-aspartate
Prox1	prospero-homeobox 1 transcription factor
PSA-NCAM	polysialylated form of the neural adhesion molecule
RELN	reelin gene

SGZ	subgranular zone
s.l.	straum lucidum
s.l.m.	stratum lacunosum-moleculare
s.o.	stratum oriens
s.r.	stratum radiatum
SPSS	statistical package for the social sciences
SVZ	subventricular zone
TLE	temporal lobe epilepsy

The truth is rarely pure and never simple
Oscar Wilde, The Importance of Being Earnest, 1895

CHAPTER 1

General Introduction

1. Outline of the Thesis

Many of the diverse functions carried out by the nervous system—from the perception of sensory input and the control of motor output to cognitive functions such as learning and memory—depend on the correct placement and wiring of many millions of neurons that are formed during embryonic and early postnatal periods. It was generally believed that following a lengthy and elaborate developmental process, neurons in the adult mammalian brain become irreplaceable elements in a network with limited capacity for structural change. The consequent longevity of neurons compared to other cell types within the body and the inability of neural circuits to spontaneously recover function after injury to the nervous system appeared to support the idea that neurons had little or no capacity for renewal (Kuhn et al., 2001). However, the brain must also accommodate the growth of the organism and the acquisition of new behavioural or cognitive capacities that are conducive for its survival and adaptability. As a result, a fundamental question emerged in neuroscience: How can an organ incapable of macroscopic structural changes adapt its function to meet the challenging demands imposed on an adult organism?

The problem outlined above has been answered, in part, by the discovery that a network of molecular and structural changes at the level of the neuronal synapse are critically involved with the long lasting representation of experience, a process commonly referred to as "synaptic plasticity". However, the mature nervous system also faces two difficult challenges. First, the adult brain must maintain normal behaviour through the preservation of its underlying circuitry, and second, it must also permit

defined circuits to adapt to unforeseen challenges or pressures that may be encountered on a day-to-day basis.

The capacity for neurocircuits to engage in activity-dependent plastic changes also comes at a great cost. For example, in the mammalian central nervous system (CNS), the affliction of epilepsy, which involves excessive, synchronous discharge of neurons, emerges from a relatively large assembly of highly interconnected cells. If we consider that a key feature of the cellular machinery of CNS neurons is to undergo activity-dependent modifications in response to extrinsic or intrinsic synaptic stimulation (Bliss and Collingridge, 1993; Hebb, 1949)—a condition that also serves to promote normal learning and memory—then it should be no surprise that an extreme manifestation of this process would involve the formation of circuits that promote hyperexcitability and hypersynchronicity. Consequently, it follows that the same mechanism important for promoting adaptive changes in the brain might also provide the basis for abnormal discharges (Schwartzkroin, 1997), especially within brain regions that are well suited for rapid activity-dependent plastic responses. One such brain region is the hippocampus, which contains specialized cell populations that must deal with a continually changing environment. Thus, there is considerable evidence that the hippocampus and surrounding structures are centrally involved in the manifestation of many forms of epilepsy.

For decades, neurobiologists have known that neuronal network organization as well as individual performance can be shaped by a variety of environmental events, experiences, or by internal signals such as hormones or aging (Kolb and Whishaw, 1998; McEwen, 1997). In addition, various forms of brain injury, including stroke and traumatic brain injuries, promote synaptic rewiring in specific brain regions affected by

cell loss (Cohen et al., 2007). However, most traditional neurorehabilitative procedures assumed that the adult CNS was extremely limited in its self-regenerative potential and most treatment strategies focused on providing forms of stimulation that promoted the functional adaptation of the remaining uninjured neurons.

The belief that new functional neurons could not be stimulated to grow limited the use of cellular replacement strategies as an effective means for combating various CNS injuries and neurodegenerative diseases (Bithell and Williams, 2005). However, a turning point came when it was unequivocally demonstrated that the adult mammalian brain was capable of mitosis and that the newly generated cells differentiated into neurons that migrated and integrated into the surrounding circuitry. Interestingly, this new form of structural brain plasticity seems to be restricted to discrete brain regions, namely the subventricular zone and the dentate gyrus of the hippocampal formation. Around the same time that hippocampal neurogenesis was demonstrated in the adult human brain, it was also demonstrated that epileptic seizures could increase the production of new neurons (Kuruba and Shetty, 2007; Parent et al., 1997; Parent, 2007). Because the epileptic brain is often characterized by alterations in normal tissue characteristics that have been in some way modified, damaged, or released from direct inhibitory control, it was speculated that these new neurons might represent an endogenous repair system that served to replace dysfunctional or lost neurons. However, most of the available evidence suggests that many of these newly born neurons do not form normally and often possess aberrant connections with other cells. As a result, recent thinking on this topic has shifted to consider that structural and functional abnormalities

of the new neurons and the neural circuits that they synapse on may actually contribute to the development of epilepsy.

The present dissertation comprises a collection of studies that attempt to further our understanding of the relationship between structural changes in the hippocampus, one of the primary sites of epileptogenesis in the adult brain, and their consequences on brain function, such as learning and memory. To begin addressing this question, I will examine how repeated epileptic seizures bring about changes in synaptic plasticity and the generation of new neurons in the hippocampus. In addition, I will attempt to identify specific molecular factors that influence the maturation and migration of new neurons in the epileptic brain. To address the main questions outlined in this dissertation, the kindling model of epilepsy, in which repeated application of electrical stimulation to specific brains regions provoke the generation of seizure activity, will be used.

The remaining section of this chapter will provide a brief introduction to the field of epilepsy and neurogenesis. First, I will provide an overview of the clinical factors related to temporal lobe epilepsy and how the use of animal models has greatly enhanced our understanding of the pathophysiological mechanisms responsible for producing epilepsy. Second, I will provide a short discussion regarding the neuroanatomy of the limbic system and its relationship to epilepsy. Following this, I will discuss the role and function of hippocampal neurogenesis in the adult brain and the direct contribution of the dentate gyrus to epilepsy. I will then conclude this chapter with a discussion of specific topics that remain unanswered in the context of neurogenesis and epilepsy research.

Chapters 2, 3, 4, and 5 will provide experimental data that address the specific aims outlined in Chapter 1. Chapter 6 will provide a general overview and discussion of

the implications of these results. In addition, specific areas in which further research is necessary will be outlined.

2. Classification and Mechanisms of Epilepsy

Epilepsy remains a major, unsolved health problem affecting 1-2% of the population. Approximately, 50 million people suffer from epilepsy worldwide, making it the third most common neurological disorder. In the United States, the estimated cost associated with epilepsy has been calculated to be 12.5 billion dollars each year with direct costs (e.g., the direct cost of medical resources devoted to diagnose and treatment of this disorder) accounting for 14% and indirect costs (e.g., the indirect cost from foregone earnings and reductions in household activities because of epilepsy-related morbidity and mortality) accounting for 86% (Begley et al., 2000). Epilepsy is also unique among chronic conditions in terms of the relative high percentage of indirect morbidity-related costs: 70% for persons with intractable epilepsy in comparison to an average of 11% for all persons with other chronic diseases (Begley and Beghi, 2002). This is because epilepsy strikes all ages, including the young, who are disabled during the most productive periods of their lives.

One of the most debilitating aspects of epilepsy is the unpredictable nature of seizures. If seizures cannot be controlled, the patient experiences major limitations in family, social, educational, and vocational activities. Although seizures can be controlled in about two-thirds of patients, the other third of patients does not respond to the present repertoire of anticonvulsant medications and continues to experience seizures. Individuals suffering from uncontrolled seizures or pharmacoresistant epilepsy show evidence of progressive neurodegeneration as well as an elevated risk for sudden unexpected death.

For this group – comprising millions of people, many of whom are children – epilepsy can be a formidable barrier towards living a normal life.

Epilepsy is best characterized by recurrent seizures, which can manifest as motor, sensory, cognitive or autonomic disturbances. The word epilepsy is derived from the Greek verb *epilambanein*, meaning “to be seized”, “to be taken hold of”, or “to be attacked” (Temkin, 1971). Epileptic seizures reflect a transient occurrence of signs and/or symptoms due to abnormally excessive or synchronous neuronal activity in the brain (Fisher et al., 2005). By convention, the diagnosis of epilepsy requires that the patient has had at least two unprovoked seizures. The seizures, which can last between a few seconds or a few minutes, can be isolated or occur in series. However, as a rule, a seizure must have a clear start and finish point, although the termination may be less evident than its onset (Rodin et al., 2009).

Because epilepsy is not a specific disease entity, or even a single syndrome, it is important to remember that epilepsy is a general name given to a broad range of symptoms that arise from any number of disordered brain functions. Furthermore, many different kinds of seizures exist, each occurring under various circumstances and differing considerably in their severity, appearance, cause, and management (Devinsky et al., 1996; Noachtar and Peters, 2009). Finally, it is also important to recognize that for patients with epilepsy, seizures are only part of the problem. This condition also comprises many social, behavioural, and cognitive disabilities that together contribute to an overall poor quality of life for the patient (Devinsky, 2004).

2.1. Classification of the Epilepsies

It has long been known that some epileptic disorders are characterized by specific clusters of signs and symptoms occurring together (Kinoshita et al., 2008; Kohrman, 2007; Reynolds and Rodin, 2009). In fact, the ancient Babylonians, almost 3000 years ago, were the first to recognize that epilepsy was a heterogeneous disorder and provided accurate descriptors of what we recognize today as tonic–clonic, absence, Jacksonian, complex partial, and even gelastic seizures (Reynolds and Kinnier Wilson, 2008).

Epileptic seizures can be routinely classified into numerous schemes in order to help diagnosis and treatment. The International League against Epilepsy has derived one of the most useful classification systems for epilepsy diagnosis that combines clinical descriptions with electroencephalographic (EEG) findings (Table 1-1). Briefly, epileptic seizures may be classified according to whether the observed syndrome is *primary* (idiopathic, i.e., genetic or unknown origin) or *secondary* (i.e., symptomatic of an acquired cerebral injury or disorder producing a static or progressive lesional state) (Dreifuss, 1997; Engel, 1996a). In addition, the seizures may be further categorized by whether they are *partial* or *generalized* in origin. For example, generalized seizures may be preceded by an aura (i.e. symptoms that precede the onset of a seizure) and EEG recordings typically show a diffuse origin of aberrant electrical discharges in both hemispheres. These seizures are typified by the strong involvement of the descending motor system and involve the loss of consciousness often with bilateral tonic or clonic movements of the limbs, arrested respiration, and the sudden loss of muscle tone (atonia). The tonic phase usually lasts for less than a minute, and is followed by a clonic phase in which there is jerking of the body musculature that may last for an additional 1-2 minutes. During the seizure (ictus event), the tongue or lips may be bitten, urinary or

Table 1-1 International Classification of Seizures and Epilepsies

I. Partial (focal) Seizures
   - A.) Simple partial seizures (with motor, sensory, autonomic, or psychological symptoms)
   - B.) Complex partial seizures
   - C.) Complex partial seizures evolving to secondary generalized seizures

II. Generalized Seizures (convulsive or nonconvulsive)
   - A.) Absence
     1. Typical (Petit Mal)
     2. Atypical
   - B.) Myoclonic
   - C.) Clonic
   - D.) Tonic
   - E.) Tonic-Clonic (Grand Mal)
   - F.) Atonic

III. Unclassified Seizures

Adapted from: Commission on Classification and Terminology, International League Against Epilepsy (1985).

fecal incontinence may occur, and the patient may be injured. Immediately after the seizure (post-ictal period), the patient may either recover consciousness, drift into sleep, or have a further convulsion. If convulsive activity occurs without regaining consciousness for a period of 30 minutes or more, is called status epilepticus. Headache, disorientation, confusion, drowsiness, myalgia, hemiparesis (i.e., Todd's paralysis), and memory loss are all common post-ictal effects.

In contrast, the presence of partial seizures implies a highly focal, localized epileptogenic region. Clinically, these seizures manifest as alterations in motor, sensory, psychic, or autonomic symptoms. They may be either of the *simple partial* (without alterations in consciousness) or *complex partial* (with impairment in consciousness) types. With complex partial seizures, the patient may display automatisms (non-goal directed behaviours), periods of memory loss, or aberrations in behaviour, which can be confused with other non-epilepsy related disorders (e.g., syncope, night terrors, migraine, or benign paroxysmal vertigo). Complex partial seizures may also progress to a generalized seizure, which involves the spread of a focal seizure to both hemispheres. Motor manifestations are bilateral and there is a loss of consciousness. A partial seizure that becomes generalized is commonly referred to as a *secondarily generalized seizure*. Importantly, the clinical manifestations of the epileptic seizure will be largely determined by the region of initial epileptogenic lability and the circuit pathways involved with the spread of the seizure from its point of origin. For instance, seizures that include the corticomedial amygdala (often labeled as "uncinate fits") are often associated with reports of olfactosomatic or olfactogustatory sensations in human patients because this region receives direct input from the lateral olfactory tract and piriform cortices (Penfield

and Jasper, 1954), whereas seizures that propagate from the hippocampus into the indusium griseum are associated with distorted experiences of body image due to the close proximity of the sensory homunculus (Moodley and Fournier, 2009).

2.2. Temporal Lobe Epilepsy

Temporal lobe epilepsy (TLE) is the most common form of adult focal epilepsy and is also the most difficult of the epilepsies to treat (Engel, 1996b). Epidemiological studies have shown that TLE can develop following various types of injury or conditions, including head trauma, birth injury, infection, history of febrile illness, intermittent hypoxia, neoplasms, and vascular disease (French et al., 1993; Mathern et al., 1994a; Mihara et al., 1985; Porter et al., 2003; Tezer et al., 2008). Temporal lobe seizures are usually of the complex partial type and are generally accompanied by behavioral arrest, motionless staring, epigastric sensations (such as nausea, eructation, or "feelings of rising" or "floating sensations" within the alimentary canal), and orofacial or gestural automatisms (such as lip smacking, chewing, or repetitive facial wiping) (Kutlu et al., 2005). Approximately, 50% of patients have unilateral or bilateral secondary generalized tonic, clonic, or tonic-clonic seizures (Mikati and Holmes, 1993). Metabolic imaging have revealed hypoperfusion in cerebral blood flow within the hippocampus and surrounding structures during periods between seizures (interictal) compared to marked hyperperfusion during the actual seizure (ictal) events themselves (Berkovic, 2000).

The epileptic focus in TLE patients may be further divided into limbic or neocortical forms (Ojemann, 2000). Neocortical TLE is defined as an epileptogenic focus in the lateral temporal lobes, whereas limbic TLE generally involves a focus within mesial temporal lobe structures, such as the hippocampus, parahippocampal gyrus, or

amygdala. Limbic TLE is further characterized by a unique pattern of neuropathology involving the hippocampus and surrounding structures (often referred to as hippocampal scelorsis), in which gliosis and the loss of select hippocampal neurons and aberrant sprouting of axonal connections is believed to promote hyperexcitability and epileptogenesis in this structure (Houser, 1999; Isokawa et al., 1993; Isokawa, 2000; Mathern et al., 1994b; Mathern et al., 1997). Although it is not known whether this form of synaptic reorganization is adaptive or maladaptive to seizures, there is considerable evidence that the presence of hippocampal sclerosis increases the risk for developing refractory or pharmacoresistant forms of TLE later in life (Fuerst et al., 2003; Marques et al., 2007; Sloviter, 2008). Finally, for a significant number of TLE cases, the underlying source of the epileptogenic activity cannot be directly localized within the mesial limbic system or surrounding lateral temporal lobes, thus making treatment difficult if not impossible (Bertram, 2009). When taken together, these findings indicate that after a variety of neuropathological injuries or insults, selective modification of mesial limbic networks can increase the risk for developing recurrent seizures and epilepsy.

3. Animal Models of Temporal Lobe Epilepsy

A variety of experimental preparations have been developed to explore the cellular and molecular mechanisms responsible for seizure generation (Baraban, 2007; Pitkanen et al., 2007). These extend from the creation of acute or chronic epileptogenic foci *in vivo* to the induction of acute epileptiform discharges in *in vitro* preparations such as brain slices and CNS cultures. It is important to emphasize that each preparation and method used to induce epileptogenesis has its own particular set of advantages or disadvantages with respect to this goal. For example, in *in vitro* preparations, influences

from subcortical structures or cortical epileptogenic foci often cannot be critically assessed, nor can long-term factors associated with increased seizure development be examined over time. By contrast, chronic *in vivo* preparations offer several advantages in this respect, but suffer from the overwhelming complexity of CNS organization, which can make it difficult to obtain detailed information about the underlying pathophysiological processes.

Despite these difficulties, chronic *in vivo* animal models have fueled the exploration of neurobiological and clinical factors responsible for producing persistent interictal (i.e., between seizures) epileptogenic abnormalities that cannot be directly examined within clinical populations (Engel and Schwartzkroin, 2006). In addition, many chronic models have been developed to approximate specific forms of human epilepsy, particularly TLE (Coulter et al., 2002). In particular, many involve experimental manipulations in which the chronic epileptic condition results as a downstream consequence of multifocal brain damage produced by an acute episode of status epilepticus. The episode of status epilepticus generally lasts for no more than 1 hr and can be triggered through the use of chemoconvulsant drugs (such as kainic acid, pilocarpine, or lithium and pilocarpine) or by prolonged electrical stimulation (Fournier and Persinger, 2004; Sloviter, 1987; Tremblay and Ben-Ari, 1984; Turski et al., 1987). Following a 2 to 6 week period, the animal recovers and appears behaviorally “normal”. However, secondarily generalized seizures begin to occur after this point and the animals show considerable learning impairments and marked changes in emotional behaviour (Fournier and Persinger, 2004). In addition, pathological studies have reported extensive hippocampal damage that resembles the typical pattern of mesial temporal lobe sclerosis

found in many patients with refractory TLE (Pitkanen et al., 2007). Unfortunately, the extensive inflammatory changes and indirect injury associated with status models often makes it difficult to interpret which pathophysiological processes are crucial in the development of limbic epilepsy or simply an indirect consequence of neuroinflammatory changes in the brain after seizures. Therefore, it is important to consider the use of chronic *in vivo* animal models that promote seizure generation but limit the development of indirect or secondary sequelae that are not directly involved in the pathogenesis of epilepsy. This has been most elegantly done using repeated electrical stimulation models or electrical kindling. Kindling offers several advantages over status models, with the most notable advantage being that the process involved with the development of limbic epilepsy can be brought under vigorous experimental control and empirically studied in the absence of gross neuropathology (Morimoto et al., 2004).

3.1. The Amygdala Kindling Model of Temporal Lobe Epilepsy

During the early to mid-twentieth century, improvements in recording technology provided neuroscientists with the opportunity to finally "listen in on the private life of the neuron" (Morrell, 1961), and with the "Rosetta stone of the stimulating electrode" they were able to investigate the many secret functions of the CNS (Purpura, 1953). Consequently, the discovery of the convulsant power of electrical stimulation during the 1960s ushered in an exciting and remarkably rewarding era in experimental epilepsy research. By the late 1960s, Graham Goddard and his colleagues (Goddard et al., 1969) demonstrated that the repetitive application of initially sub-convulsant and invariant electrical stimulation to subcortical or cortical sites could trigger long-lasting, if not permanent, increases in seizure susceptibility. This phenomenon was later called *kindling*

(Goddard et al., 1969). Initially, the electrical stimulations produced little change in behavior. However, after repetition, stimulations began to trigger fully generalized motor convulsions that were readily evoked even after a large absence of stimulation.

Although it was known prior to Goddard's work that repeated electrical stimulation could evoke seizures (Delgado and Sevillano, 1961; Watanabe, 1936), Goddard was the first to recognize the significance of the progressive and permanent nature of kindling as a useful preparation for the study of epilepsy. With the use of the kindling model, investigators could imitate the slow developing process of epileptogenesis that is often reported to accompany progressive traumas such as head injury. Additionally, the discovery of kindling provided clear evidence for the proposal made by Gowers (1881) that "seizures beget seizures" and gave for the first time an experimental framework whereby the effects of repeated seizures could be dissociated and their consequence on neuronal function and behaviour evaluated.

3.2. Characteristics of Kindling

Electrical kindling typically involves the daily application of brief (1 or 2 s), low intensity trains of biphasic square wave pulses delivered at a frequency of 60 pulses per second through a chronically implanted bipolar electrode. Initially, stimulations evoke very little change in on-going behavior; however, EEG recordings reveal the presence of a focal response characterized by high amplitude, low frequency paroxysmal epileptiform activity at the site of stimulation. This brief focal seizure or after-discharge (AD) begins the process of epileptogenesis. The kindling process is critically dependent on the elicitation of the epileptogenic AD; stimulation intensities below the critical AD threshold are ultimately ineffective at kindling (Racine, 1972a). In addition, if the AD is

triggered by the appropriate stimulus configuration (e.g., frequency, current intensity, pattern of stimulation, site of stimulation, and inter-stimulus intervals), kindling will occur. This indicates the process of kindling is highly dependent on the specific details of the electrical stimulation protocol.

Early stimulations produce a short duration, low amplitude AD response with little propagation to other brain sites (Fig. 1-1). However, with repeated stimulation, the initially brief focal AD becomes altered, usually increasing in duration, amplitude, spike frequency, and spike morphology (Fig. 1-1) (Racine, 1972a). As the paroxysmal discharge and expanding epileptogenic network grows, seizure responses become more complex and generalized to the point of driving bilateral clonic seizures. This behavioural progression follows a series of well described behavioural stages of increasing severity in the rat (Racine, 1972b). For example, early stimulations produce orofacial automatisms and/or facial clonus (Class 1), followed by repetitive heading nodding and vibrissae twitching (Class 2), and finally progression into motor convulsions that begin initially as unilateral forelimb clonus (Class 3), progressing into bilateral clonus with rearing (Class 4), and ending with bilateral clonic seizures with rearing and loss of equilibrium (Class 5). An animal is termed “kindled” once it has had at least three consecutive class 5 convulsions. Interestingly, amygdaloid kindling typically requires 12 to 16 ADs to reach the fully kindled state (Fig. 1-1). Finally, once generalized motor seizures have been established, the brain alterations that accompany kindling appear to persist for months or even years and motor seizures can be easily provoked after only a few stimulations following a prolonged period of stimulation absence (Goddard et al., 1969; Hiyoshi and Wada, 1992).

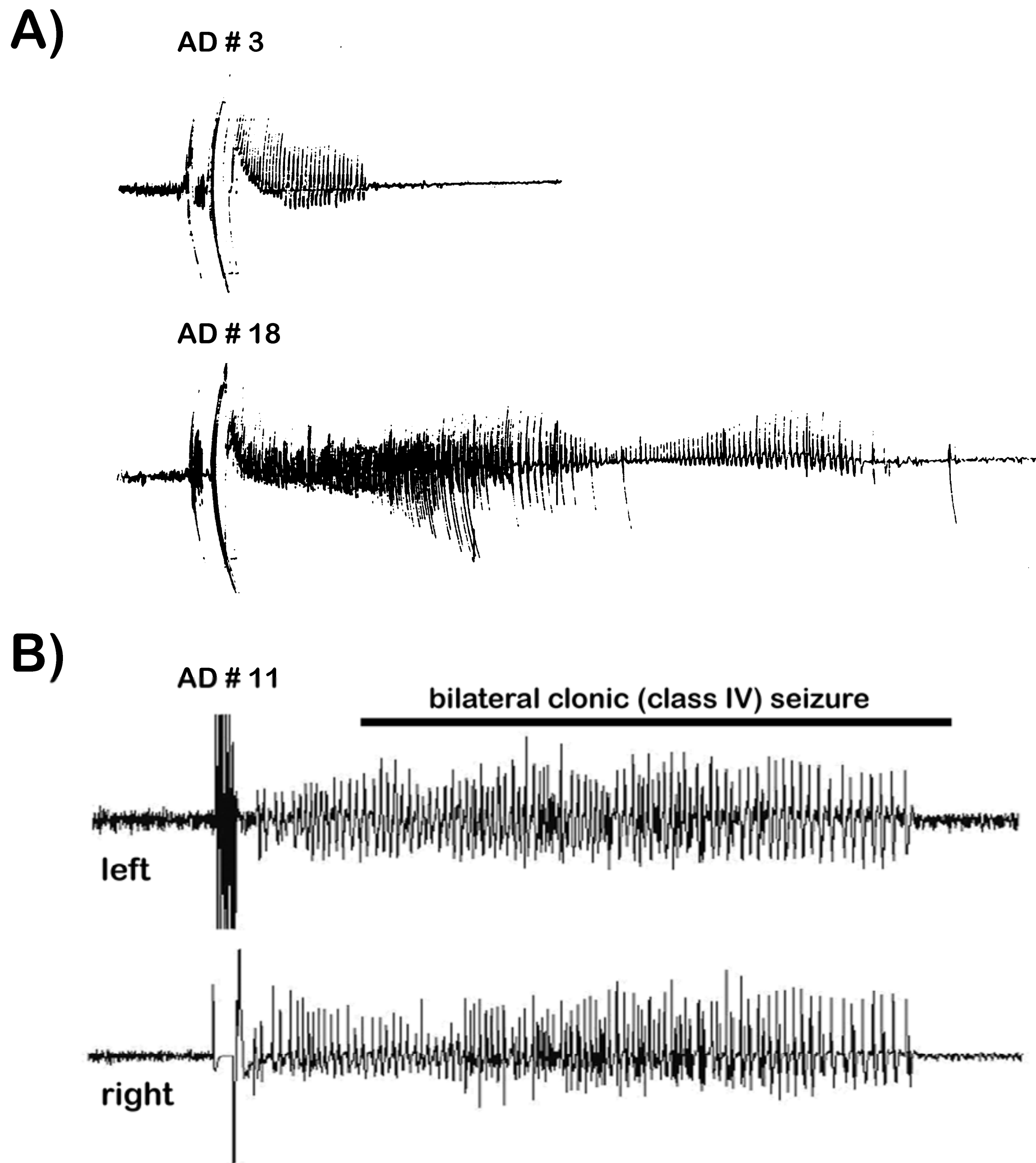


Figure 1-1 Electroencephalographic tracings showing the generation of an epileptiform afterdischarge following a kindling stimulation. A) Representative example of an afterdischarge evoked from a bipolar electrode implanted in the perforant path following three (AD#3) and eighteen (AD#18) kindling stimulations. Note that with repeated kindling stimulations, there is an increase in the AD amplitude, duration, frequency and spike complexity. B) Representative example of an afterdischarge evoked from the amygdala following eleven (AD#11) kindling stimulations to the left basolateral amygdala. Note the bilateral high amplitude, low frequency paroxysmal spikes within the structure during a class IV motor convulsion (bilateral clonic and rearing seizure).

Kindling can proceed from many limbic sites; however, not every brain region shows the same sensitivity. For instance, stimulation of the superior colliculus or cerebellum does not generally result in kindling (Paz et al., 1985; Racine, 1972a). Early studies pointed to the amygdala as a highly susceptible site to the effects of kindling and as a result this structure is often utilized in most kindling experiments (Goddard et al., 1969; Racine, 1972a). However, kindled seizures can also be rapidly achieved from several forebrain regions such as the olfactory bulb, piriform, perirhinal cortices, or claustrum often within a few stimulations (Cain and Corcoran, 1978; Loscher and Ebert, 1996; McIntyre et al., 1993; Mohapel et al., 2001). In addition, stimulation of several non-limbic brain regions such as thalamus (e.g., nucleus submedius, nucleus reuniens/rhomboid) or brain stem nuclei (mesencephalic reticular formation, reticularis pontis oralis) can also show kindling-like effects suggesting that the neural circuitry underlying kindling may be more widespread than originally suspected (Corcoran and Teskey, 2009).

3.3. Cognitive and Behavioural Changes Associated with Kindling

In spite of the high prevalence of cognitive disorders in chronically epileptic patients, very little is known about their underlying pathophysiological mechanisms. The development of animal models of epilepsy has helped in this regard by removing many of the inherent problems associated with human clinical research and allowing for controlled investigation into the cellular and molecular basis of interictal behavioural and cognitive disturbances (Engel et al., 1991; Kalynchuk and Fournier, 2009; Post, 2004). The advantages of the kindling model are clear in this respect: 1) precise focal activation of target brain sites is possible, thus allowing the researcher to determine the precise

anatomical circuitry responsible for the development of the behavioural impairment; 2) tight control over the window of stimulation also allows the researcher to determine the impact of interictal, ictal, and postictal factors on the behavioural impairment; and 3) because the development of epileptogenesis in kindling is progressive, the researcher can monitor the development of behavioural changes at different stages in epilepsy development (Coulter et al., 2002; Sato et al., 1990).

Although the relationship between kindling and behavioural alterations has received limited study, it is believed that the effects of kindling on behaviour are specific to the site of stimulation. For example, many researchers have found that amygdaloid kindling is associated with changes in anxiety-like behaviour, as well as an enhancement in the normal spectrum of fearful or defensive responses in rodents and felines (Adamec, 1990; Adamec, 1991; Adamec, 2001; Anisman et al., 2000; Barnes and Pinel, 2001; Hannesson et al., 2008; Helfer et al., 1996; Kalynchuk et al., 1997; Kellett and Kokkinidis, 2004; Nieminen et al., 1992; Peele and Gilbert, 1992; Pinel et al., 1977; Sutula et al., 1995). In contrast, dorsal hippocampal kindling is typically associated with a selective disruption of mnemonic functions important in spatial learning and short-term memory (de Toledo-Morrell et al., 1984; Feasey-Truger et al., 1993; Gilbert et al., 2000; Hannesson et al., 2001a; Hannesson et al., 2001b; Hannesson et al., 2004; Leung et al., 1994; Lopes da Silva et al., 1986; Sutherland et al., 1997; Sutula et al., 1995), whereas stimulation of the ventral hippocampus, perforant path, or perirhinal cortex do not normally affect spatial memory (Hannesson et al., 2005; Holmes et al., 1993; McNamara et al., 1992; Robinson et al., 1993). These findings argue that modifications at the site of stimulation rather than broader circuit changes associated with the propagation of

generalized seizures appear to play a role in the development of interictal behavioural deficits.

However, many of these studies have typically used partial (i.e., kindling that produces ADs but no generalized motor convulsions) or short-term (i.e., animals receive daily electrical stimulations until three to five consecutive generalized motor convulsions have been elicited or 30 electrical stimulations delivered) kindling protocols. It is now appreciated that after an extensive number of stimulations is delivered (i.e., long-term kindling), more pervasive and long-lasting changes in cognition and behaviour occurs (Kalynchuk, 2000). Indeed, experimental evidence has shown that after a considerable number of stimulations, propagation of seizure activity from remote sites, such as the amygdaloid complex, can alter hippocampal-mediated behaviour (Cammisuli et al., 1997; Kalynchuk et al., 2001; Kalynchuk and Fournier, 2009). From these studies, clearly the process of kindling, especially when administered over long periods (i.e., long-term kindling), can lead to widespread modifications in the function of various neural structures and circuits important in behaviour.

4. Neuroanatomy of the Limbic System

The hippocampal formation and amygdaloid complex have been the focus of extensive research by workers in the epilepsy field. A significant proportion of patients have seizure foci that involve the hippocampus or amygdala or both. In addition, a considerable number of studies have shown that repetitive seizure activity can trigger profound changes within the microcircuitry of the amygdala or hippocampus, including the reactive sprouting of new synaptic connections, astrogliosis, neuronal death, and reorganization of preexisting synapses (Cavazos and Sutula, 1990; Franck et al., 1995;

Zentner et al., 1999). In spite of their clinical importance, the functional properties of these changes are not well understood. However, it is believed that these complex changes could underlie the transition from normal to hyperexcitable networks and provide a mechanism for the development of cognitive deficits seen in both clinical populations and in animal models of epilepsy (Kalynchuk, 2000). I will now briefly discuss the general circuitry and function of both these brain structures.

4.1. The Amygdaloid Complex

The amygdaloid complex is an almond-shaped structure within the rostral pole of the mesial temporal lobe. It is a heterogeneous collection of nuclear groups that all differ in their cytoarchitecture, chemoarchitecture, connectivity, and functionality (Swanson and Petrovich, 1998). The amygdaloid complex can be partitioned into various nuclei and cortical areas that are homologous in different species, including the rat, cat, monkey, and human (Pitkanen et al., 2000a; Price et al., 1987; Price, 2003; Sorvari et al., 1995). Briefly, the deep nuclei are comprised of the lateral nucleus, basal nucleus, and accessory basal nucleus, whereas the superficial nuclei include the anterior cortical nucleus, bed nucleus of the accessory olfactory tract, medial nucleus, nucleus of the lateral olfactory tract, periamygdaloid cortex, and posterior cortical amygdala. Finally, the remaining nuclei include the anterior amygdaloid area, central nucleus, amygdalohippocampal transition area, and the intercalated nuclei. The location of several different amygdaloid regions in the rat brain is shown in Fig. 1-2. There are two subdivisions of the amygdala based on functional and connectional properties that are important in TLE: 1) the central-medial division (comprised of the medial and central amygdala nuclei) and 2) the

basolateral division (comprised of the lateral, basolateral, and basomedial amygdala nuclei).

4.2. The Hippocampal Formation

The hippocampal formation is easily identified on the mesial surface of the human brain, just caudal to the amygdala (Duvernoy, 2005). It extends along the floor of the temporal horn of the lateral ventricle. It comprises three major regions: the dentate gyrus, named because it grossly resembles teeth; the hippocampus proper or Cornu Ammonis (CA) a name derived from the Egyptian God Ammon Ra, who appeared as a ram; and adjacent to the hippocampus, the subicular complex, part of the parahippocampal gyrus continuous with the hippocampal fields (Fig. 1-3). Another closely related and interconnected structure is the entorhinal cortex, part of the parahippocampal gyrus that lies next to the parasubiculum.

The hippocampus proper is a cortical structure consisting of a rolled layer of excitatory glutamatergic pyramidal cells that is subdivided into the areas CA1, CA2 and CA3 (Lorente de Nó, 1933; 1934). In addition, the laminar organization of the hippocampus proper is generally similar in all subfields (Amaral and Witter, 1995). As a result, this clear organization has made the hippocampus particularly conducive for studies using *in vitro* brain slice preparations (Andersen et al., 2007). The principal cell layer is the stratum pyramidale, which contains the pyramidal neurons. Below the stratum pyramidale is a narrow and relatively cell-free layer called the stratum oriens and adjacent to stratum oriens is the alveus, which contains many myelinated efferent fibers from output neurons. For the CA3 subfield, a narrow acellular zone, called the stratum lucidum, is present and this region serves as the major site of mossy fiber innervation

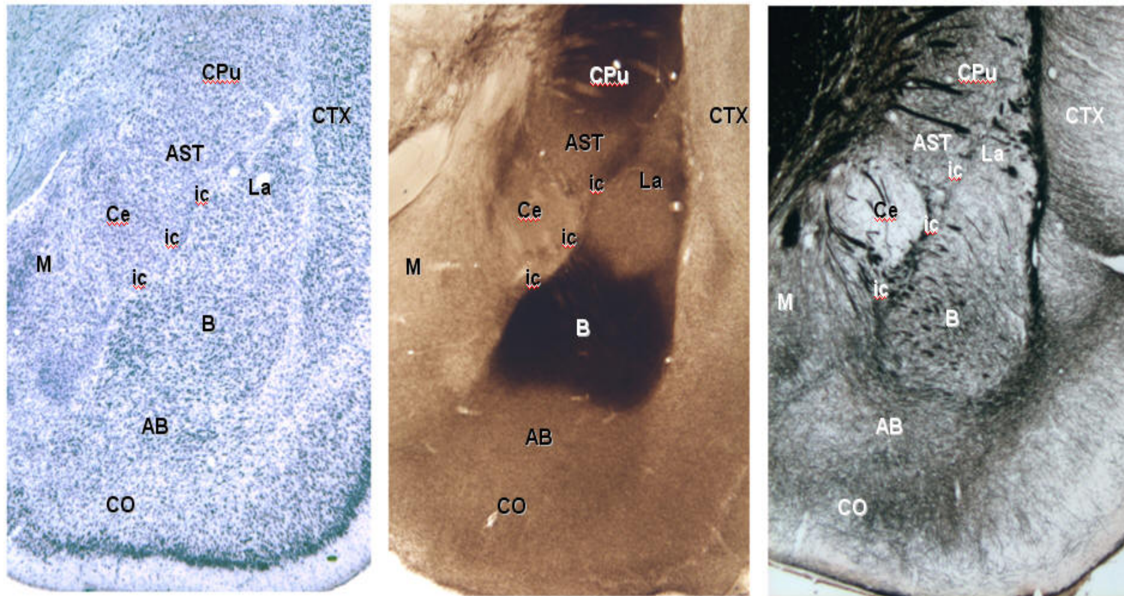


Figure 1-2 Brightfield photomicrographs of coronal sections of the rat amygdaloid complex showing the location of various amygdaloid nuclei and nuclear divisions. Different staining methods show amygdaloid nuclei from different perspectives. Left panel: Nissl cell body stain. Middle panel: acetylcholinesterase stain. Right panel: silver fiber stain. Abbreviations of amygdaloid areas: AB, accessory basal; B, basal nucleus; Ce, central nucleus; ic, intercalated cells; La: lateral nucleus; M, medial nucleus; CO, cortical nucleus. Non-amygdala areas: AST, amygdalo-striatal transition area; CPu, caudate/putamen; CTX, cortex. Image from: Joseph E. LeDoux (2008). http://www.scholarpedia.org/article/Amygdala

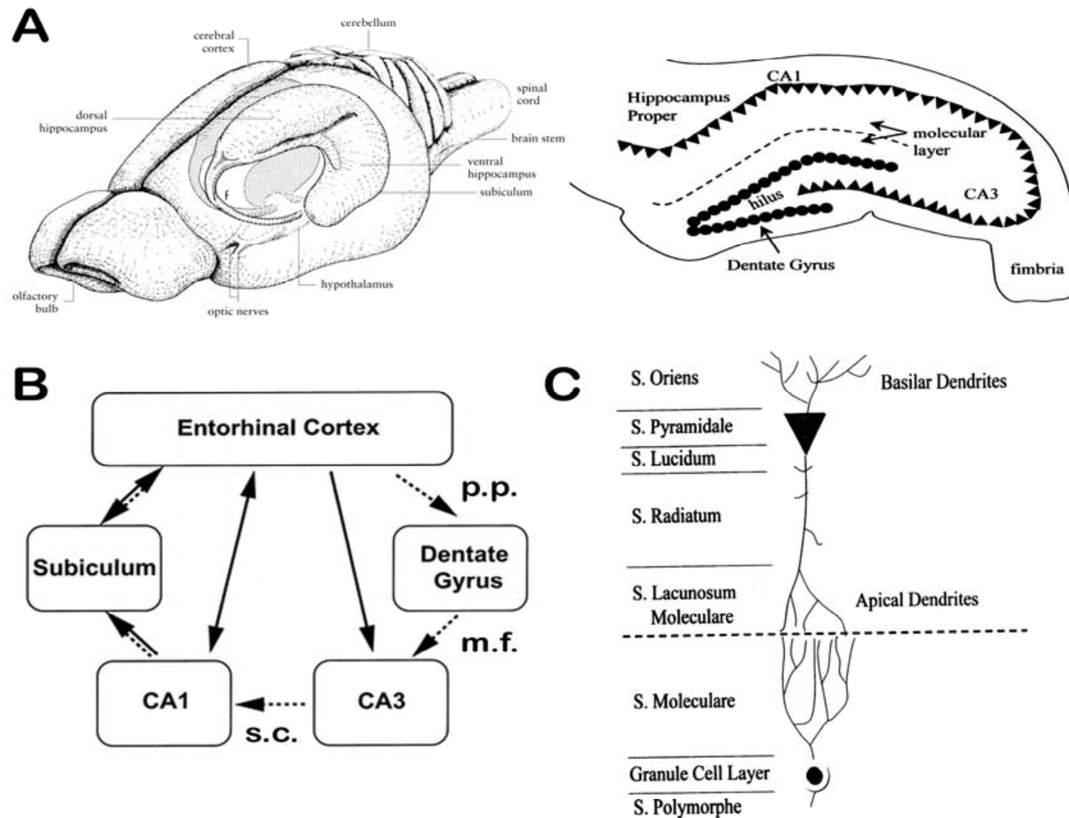


Figure 1-3 The rat hippocampus and related circuitry. A) Schematic of the rat hippocampus in the coronal plane. The hippocampal gyrus is divided into three subfields: the CAl, CA2 and CA3. The superior portion of the hippocampus corresponds to CA1, whereas area CA3 is partly bounded by the dentate gyrus and represents the inferior portion of the hippocampus. Area CA2 (not shown) serves as the transition between CA3 and CA1 (Amaral & Witter, 1995). B) Depicts the intrahippocampal circuitry and classic tri-synaptic circuit. p.p.: perforant path, m.f.: mossy fibers, s.c.: Schaefer collaterals. C) Schematic of the laminar organization of the hippocampus: the stratum oriens, the stratum pyramidale, the strata radiatum and the stratum lacunosum moleculare. The principle ce11 type in the hippocampal gyrus is the pyramidal cell and the ce11 bodies of these neurons form the stratum pyramidale. These pyramidal cells have large apical dendrites, along with several shorter basal dendrites and a single axon located at the opposite pole. In CA3**,** there is an additional stratum referred to as the stratum lucidum, which is located between the pyramidal ce11 layer and the stratum radiatum. (Below the dotted line). The DG can be divided into three layers: the stratum molecular layer, dentate granule ce11 layer, and the polymorph layer. The molecular layer consists of the granule cell dendrites. The dentate granule cells constitute the next layer, which consists of a narrow band of densely packed cells. The axons of the dentate granule cells are the mossy fibers. The polymorphic layer lies between the upper and lower blades of the granule ce11 layer and is sometimes referred to as the hilus. This layer contains a range of different ce11 types, including intemeurons, stellate cells, mossy cells, and basket cells. Figure adapted from (Amaral and Witter, 1995)

from the dentate gyrus. Adjacent to the stratum lucidum, and above the pyramidal layers of CA2 and CA1, is the stratum radiatum and stratum lacunosum-moleculare. The hippocampal pyramidal cells typically have large apical dendrites, along with several shorter basal dendrites and a single axon located at the opposite pole. These basal and apical dendrites terminate in different strata on either side of the pyramidal cell layer (i.e., basal dendrites ramify in the stratum oriens, whereas the apical dendrites branch extensively throughout the remaining strata).

Inhibitory γ-aminobutyric acid (GABA)-containing interneurons are mainly distributed basal and apical to the pyramidal cell layer in stratum oriens, stratum radiatum and stratum lacunosum-moleculare (Freund and Buzsaki, 1996). The CA3 area of the hippocampus is capped by the dentate gyrus (DG), which consists of the densely packed glutamatergic granule cell layer (GCL), the hilus or polymorphic layer, and the molecular layer. The somata of most DG GABAergic interneurons are located in the latter two regions and their processes ramify in very distinctive laminar patterns depending on the interneuron subtype (Freund and Buzsaki, 1996; Ramón y Cajal, 1911).

The principal cells of the hippocampus and the DG are linked via the trisynaptic circuit: Briefly, dentate granule cells, which are the primary target for afferent input from the entorhinal cortex, synapse via their axons, the *mossy fibers*, onto the somata of CA3 pyramidal cells (Lomo, 1971). The axons of CA3 pyramidal cells form the *Schaffer collateral* pathway to the CA1 area, where they contact the proximal dendrites of pyramidal cells via excitatory synapses (Andersen et al., 1971). CA1 pyramidal cell axons, in turn, project to the entorhinal cortex and thus convey the processed information back to the cortex (Witter and Groenewegen, 1990). The function of this principal cell

network is modified by local interneurons that shape the activity of pyramidal and granule cells through a complex array of feedback and feedforward inhibitory mechanisms (Andersen et al., 1963; Buzsaki, 1984; Buzsaki and Eidelberg, 1981).

A great deal of our understanding of the function of the hippocampal formation has come from of the study of patients with TLE and from individuals that underwent hippocampal resections to treat drug resistant seizures (Leritz et al., 2006; Scoville and Milner, 1957). These studies unambiguously support that this brain region displays a remarkable level of functional plasticity and is important for a number of behaviours, including exploration, anxiety, learning, and memory (Altman et al., 1973; Baxendale, 1995; Eichenbaum et al., 1990; Eichenbaum, 1997; Good and Honey, 1997; Post et al., 1998; Shors et al., 2000; Squire, 1987). First, the importance of the hippocampus in exploration and spatial navigation was initially demonstrated in rats by O'Keefe and colleagues, who showed the existence of place cells in behaving rats by *in vivo* recordings (O'Keefe and Nadel, 1978). Place cells were shown to be preferentially active when an animal moves through a specific location in the environment (O'Keefe and Dostrovsky, 1971). Second, a key role of the hippocampus and related structures in mediating anxiety-like behaviour has been described (Gray, 1978). For example, during novel situations, a conflict emerges between active approach behaviours (addressing a state of curiosity) and active avoidance behaviours (addressing the fear of a potential threat). This conflict is mediated in part by septohippocampal circuits (McNaughton and Gray, 2000). Third, the formation of spatial and contextual representations is highly dependent on the integrity of the DG-CA3 network. Due to the low probability that two CA3 pyramidal cells will receive input from identical subsets of DG granule cells

(Amaral and Witter, 1995), the DG can function as a pattern separator for incoming spatial information and thus facilitate the resolution of spatially related cues (Gilbert et al., 2001; Kesner, 2007; Leutgeb et al., 2007; O'Reilly and McClelland, 1994). And finally, the hippocampus, or more specifically the DG, is unique among brain structures because it contains one of the rare neurogenic niches of the adult brain where new neurons can be continually generated throughout life (Altman, 1962). When taken together, these findings highlight an important role for the hippocampus and related structures in mediating a variety of complex behavioural processes.

The DG is situated in a critical position to modulate the amount of incoming information that gets through to the hippocampus. Consequently, dysfunction of the DG can significantly impact network and computational activity for the entire hippocampus. In the chronically epileptic brain, there is substantial evidence that DG function is compromised. This important observation has provided researchers with clues into how epileptiform generation and propagation occur within hippocampal circuits. However, it is presently not understood which specific aspects of DG function that are compromised in the epileptic brain directly contribute to the generation of seizure activity. It has been speculated that the uncanny capacity for the DG to create new synaptic circuits through the sprouting of preexisting synaptic terminals or through the birth and integration of new neurons may play a key role in the development of epilepsy. I will now review the general anatomical structure of the DG and the contribution of neurogenesis to dentate function.

4.3. Dentate Gyrus: Structure and Connectivity

The dentate gyrus (DG) is the primary target of cortical input to the hippocampal formation. From an inside-out perspective, the trilaminar organization of the DG is: the polymorphic layer (or hilus), the stratum granulosum, and stratum moleculare (Lopes da Silva et al., 1990). The stratum moleculare lies closest to the hippocampal fissure and is devoid of dentate granule cells, but is occupied by the dendrites of granule cells. The granule cell layer (GCL) is made up of densely packed granule cells (with a somatic diameter of ~10 μm) that show a clear, bipolar orientation with their apical dendrites extending into the molecular layer and axons invading the hilar region. The thickness of the GCL ranges from 4 to 8 neurons or ~60 μm (Amaral et al., 2007). Estimates of the total number of granule cells in the rat are between 0.6 to $2.2 * 10^6$ cells/per dentate gyrus (Amaral et al., 2007; Amaral and Witter, 1989; Seress and Pokorny, 1981). The principal cell layer of the DG, the stratum granulosum or GCL, is comprised of numerous granule cells. In addition, the polymorphic (hilar) layer is home to a variety of cells that include the excitatory (glutamatergic) mossy cells (the primary source of dentate commissural and associational connections) and the inhibitory basket cells (Amaral, 1978). In adult rodents, basal dendrites on dentate granule cells are largely absent although in young rats of 5-10 days of age such basal dendrites have been described (Ribak et al., 2000; Seress and Pokorny, 1981; Spigelman et al., 1998). In monkeys and in humans, a substantial number of granule cells display basal dendrites, which extend into the hilus (Seress and Mrzljak, 1987). It is believed that these basal dendrites may be involved in a mossy cell mediated excitatory circuit that regulates excitability of the dentate gyrus (Frotscher et al., 1991).

Input: The major afferent input to the DG arises from layer II pyramidal cells of the entorhinal cortex (EC), which in turn receives projections from various cortical regions, including olfactory, visual, and somatosensory cortices. Thus, the DG can be seen as a "gateway" into the hippocampus proper because it directly receives 90% of the EC input to the hippocampus (Goldowitz et al., 1975; Hjorth-Simonsen and Jeune, 1972; Steward, 1976). This massive input is transmitted via synapses onto the distal dendrites of granule cells (and on interneurons) in the outer two-thirds of the dentate stratum moleculare. The DG does not receive any extra-hippocampal projections (but see (Scharfman, 2007) for an interesting discussion on the possibility of DG modulation by CA3 backprojections), nor does it receive any direct input from neocortical structures other than from the EC. The entorhinal input to the DG is topographically organized and subdivided into two main divisions: fibers from the medial portion of the EC synapse in the middle third of the molecular layer (e.g., medial perforant pathway), whereas fibers from the lateral EC synapse in the outer third (e.g., lateral perforant pathway) (Wyss, 1981). These subdivisions mediate different forms of synaptic plasticity. For instance, the medial division of the perforant path utilizes glutamate and evokes excitatory postsynaptic potentials (EPSPs) with both an N-methyl-D-asparate (NMDA) and non-NMDA component (Christie and Abraham, 1992), whereas the lateral division provides an opioid (enkephalin)-dependent form of long-term potentiation (Bramham, 1992; Xie and Lewis, 1991). Because of the abundance of mu and delta opioid receptors on the somatodendritic compartment of dentate granule cells, the latter pathway is thought to mediate "disinhibition" of these cells (Drake et al., 2007)—a process that might be important for dentate seizure generation and susceptibility (Lothman et al., 1992). In addition, dentate

granule cells also receive inputs from contralateral (commissural) and ipsilateral (associational) hilar neurons that terminate in the inner third of the molecular layer that are distinct from the entorhinal afferents described previously (Scharfman et al., 1990; van and Wyss, 1988; Voneida et al., 1981). Finally, the DG receives sparse cholinergic and GABAergic projections from the medial septum/diagonal band of Broca, as well as various serotonergic, dopaminergic, and noradrenergic projections from several distinct brainstem nuclei (Leranth and Hajszan, 2007). Thus, the complex network of intrinsic afferent projections to the DG is likely to exert powerful control over the electrical activity of the hippocampus (Leranth and Hajszan, 2007).

Output: Bundles of unmyelinated axons from dentate granule cells are commonly referred to as mossy fibers. These projections pass through the hilus on their way to their target, the CA3 pyramidal cells, and in the hilus they send off collaterals that either synapse directly onto mossy cells (Claiborne et al., 1986) or form recurrent collaterals into the deepest portion of the molecular layer, where they target basket cells (Ribak and Peterson, 1991). These fibers form giant, spatially complex synaptic contacts onto the proximal dendrites of CA3 pyramidal cells. As the primary connection between the DG and the CA3 region of the hippocampus, the mossy fiber network provides a critical pathway linking cortical activity to intrinsic hippocampal circuits. In contrast to many CNS axons, mossy fiber axons make only a small number (~15) of bouton connections with CA3 principal cell targets (Amaral and Witter, 1995). Yet, despite the sparse innervation that principal CA3 cells receive from dentate granule cells (i.e., only about 50 synapses per CA3 cell in rodents (Amaral and Witter, 1995), these connections appear to be extremely efficacious. For instance, activation of even a single mossy fiber synapse

may be sufficient to fire a CA3 pyramidal cell (Brown and Zador, 1990) resulting in the moniker of a “detonator synapse” (McNaughton and Morris, 1987).

5. Neurogenesis and Functional Integration of New Neurons in the Adult Dentate Gyrus

5.1. History of Neurogenesis

At the beginning of the twentieth century, an overwhelming assumption held by many prominent neuroanatomists was that after birth, no neurons could be added to the mammalian CNS. This rigidity of the brain was first advocated by Albert Kölliker (1896) and then by Wilhelm His (1904) whose elegant and thorough work on the developmental anatomy of the mammalian brain suggested that there was a general constancy in its overall architecture throughout maturation. Although His observed the possibility of two morphologically distinct subtypes of proliferative “indifferent” cells within the neurogenic zone of the ventricular epithelium, he believed that these cells were largely absent in adult nervous tissue. This view was upheld for most of the century, despite early evidence against it. For instance, based upon anatomical morphology, Alfred Schaper (1897) observed that these indifferent cells (now speculated to be radial glia) might be maintained, albeit at low numbers, within the adult brain and that the differences in the morphology of the two subtypes might reflect different phases in the cell cycle of the same cell. This finding was later confirmed by Sauer (1935). In a bold step, Schaper speculated that these undifferentiated cells might give rise to neurons or glial cells. However, in these studies, it was impossible to unequivocally prove that the

cells undergoing mitosis subsequently became neurons or glia (Ramón y Cajal, 1928), so the belief that neurogenesis did not occur in the adult brain prevailed.

One major advance in the methodological study of neurogenesis came from Sidman, Miale, and Feder (1959) and their use of [H3]-thymidine autoradiography to study the development of the nervous system. With the introduction of [H3]-thymidine autoradiography, it became possible to label dividing cells and determine the precise time and place of their birth. When incorporated into newly synthesized DNA during the S-phase of the cell cycle, thymidine can be detected several weeks or months later using autoradiographic analysis of prepared tissue. This method has been extremely useful for charting the temporal and spatial developmental of the CNS (e.g., (Sidman et al., 1959)). One of the first studies set out to examine if neurogenesis occurred in the adult brain was by Ian Smart (1961). Unfortunately, he could only detect newly generated cells in three-day old mice and not in adult mice (Smart, 1961). However, Joseph Altman, a young postdoctoral fellow working independently in the Department of Psychology at the Massachusetts Institute of Technology provided the first direct experimental evidence that continuous generation of new neurons took place in various brain structures, including the neocortex, olfactory bulb, and dentate gyrus of young and adult rats (Altman, 1962; Altman, 1966; Altman, 1969b).

These findings provoked new thoughts on the adult brain and adaptation; however, they were relatively ignored by the general research community. Independent support for Altman's findings came in a series of in-depth and elegant electron microscopy studies by Michael Kaplan's group where they found that tritiated thymidine-labeled cells showed pronounced ultrastructural characteristics reminiscent of neurons,

but not astrocytes or oligodendrocytes (Kaplan, 1985; Kaplan et al., 1985; Kaplan and Bell, 1983; Kaplan and Bell, 1984; Kaplan and Hinds, 1977). (Unfortunately, the extensive refusal and often combative criticism from the broader research community to the possibility of neurogenesis in the adult brain discouraged Kaplan from continuing neuroscience research, and he subsequently pursued a career in clinical medicine, see (Kaplan, 2001) for an interesting discussion on the history of neurogenesis).

A few years later it was established that neurogenesis occurred in the vocal control nucleus of adult canaries where it was critical for song-learning behaviour (Goldman and Nottebohm, 1983; Nottebohm, 1980; Nottebohm, 1981; Nottebohm, 1985) and through a series of experiments, Fernando Nottebohm and his colleagues demonstrated: 1) the production of thousands of newly labeled cells using tritiated thymidine in the caudal forebrain during song learning season in adult males, 2) that newly labeled cells showed ultrastructural characteristics reminiscent of neurons, and 3) that these cells appeared to be functionally incorporated into the complex neurocircuitry of the adult brain where they made functional connections and exhibited synaptic potentials (Burd and Nottebohm, 1985; DeVoogd et al., 1985; Kirn et al., 1994; Kirn et al., 1999; Kirn and Nottebohm, 1993; Nottebohm, 1985; Nottebohm, 1989). However, these findings were considered to be of little consequence for the mammalian brain and simply the result of evolutionary processes that provided advantages for avian species but not mammals (Gross, 2000). In addition, Rakic was unable to find any compelling evidence for neuronal addition or replacement after an extensive series of autoradiographic analyses of the CNS of juvenile and adult monkeys (Rakic, 1985a; 1985b). He concluded that "…all neurons of the primate central nervous system are

generated during restricted developmental periods, mostly before birth and not after infancy. It is not surprising that a stable population of neurons in mature primates, including man, may be essential for the retention of memory and learned behavior" (Rakic, 1985a).

With the introduction of 5-bromo-2'-deoxyuridine (BrdU) as a tool to study the generation of new cells, the larger scientific community slowly began to accept that functional neurogenesis could take place in the adult brain (Miller and Nowakowski, 1988). BrdU is a halogenated thymidine analogue, which is incorporated in replicating DNA, and in contrast to [3H]-thymidine, BrdU can be visualized with immunocytochemistry (Fig. 1-4). The use of BrdU as a tool to label dividing cells made it possible to investigate the phenotype of newly formed cells with double-labeling immunohistochemistry. With these techniques, several laboratories confirmed that new neurons were indeed added to the granule cell layer of the dentate gyrus (Boonstra et al., 2001; Cameron and Gould, 1994; Gage et al., 1998; Kempermann et al., 1997; Kempermann et al., 1998; Kronenberg et al., 2003; Kronenberg et al., 2006; Kuhn et al., 1997) and olfactory bulbs (Fasolo et al., 2002; Fukushima et al., 2005; Kato et al., 2001; Kuhn et al., 2005; Lledo and Gheusi, 2003; Mirich and Brunjes, 2001; Nissant et al., 2009; Suhonen et al., 1996; Whitman and Greer, 2009) of adult rodents. New olfactory bulb neurons originate from neural precursor cells that are located along the wall of the lateral ventricles in a region commonly referred to as the subventricular zone (SVZ). These cells migrate by "chain migration" along the rostral migratory stream into the olfactory bulbs where they differentiate into granule or periglomerular inhibitory interneurons (Bedard et al., 2002; Winner et al., 2002). In the hippocampus, precursor

cells reside in the small layer between the GCL and the hilus in an area commonly referred to as the subgranular zone (SGZ). After a few cell divisions, the proliferating cells become postmitotic, migrate into the adjacent granule cell layer, differentiate into excitatory granule cells and become firmly integrated into the existing hippocampal circuitry (Kempermann and Gage, 2000; van Praag et al., 2002). In addition, adult neurogenesis has also been found in the dentate gyrus of adult tree shrews, marmoset monkeys, and macaque monkeys (Gould et al., 1997; Gould et al., 1999b; Gould et al., 1999c; Gould et al., 2001; Kornack and Rakic, 1999; Leuner et al., 2007). Indeed, it was the demonstration of neurogenesis in the adult human hippocampus by Eriksson and colleagues (1998) that spurred a realization that neural stem cell based treatments may one day be used to possibly cure the progression of various neurodegenerative diseases, including epilepsy.

5.2. Stages of Neurogenesis in the DG

Adult neurogenesis involves the production of new neurons in the adult brain. The term comprises a complex multifactorial process that begins with the proliferation of progenitor cells, followed by commitment to a neuronal phenotype, and the development of morphological and functional properties (Kempermann and Gage, 2000) (Fig. 1-4, 1-5). This process is completed with the integration of these cells into pre-existing circuits followed by the gradual increase in connectivity and the development of physiological properties that resemble mature granule cells (Abrous et al., 2005).

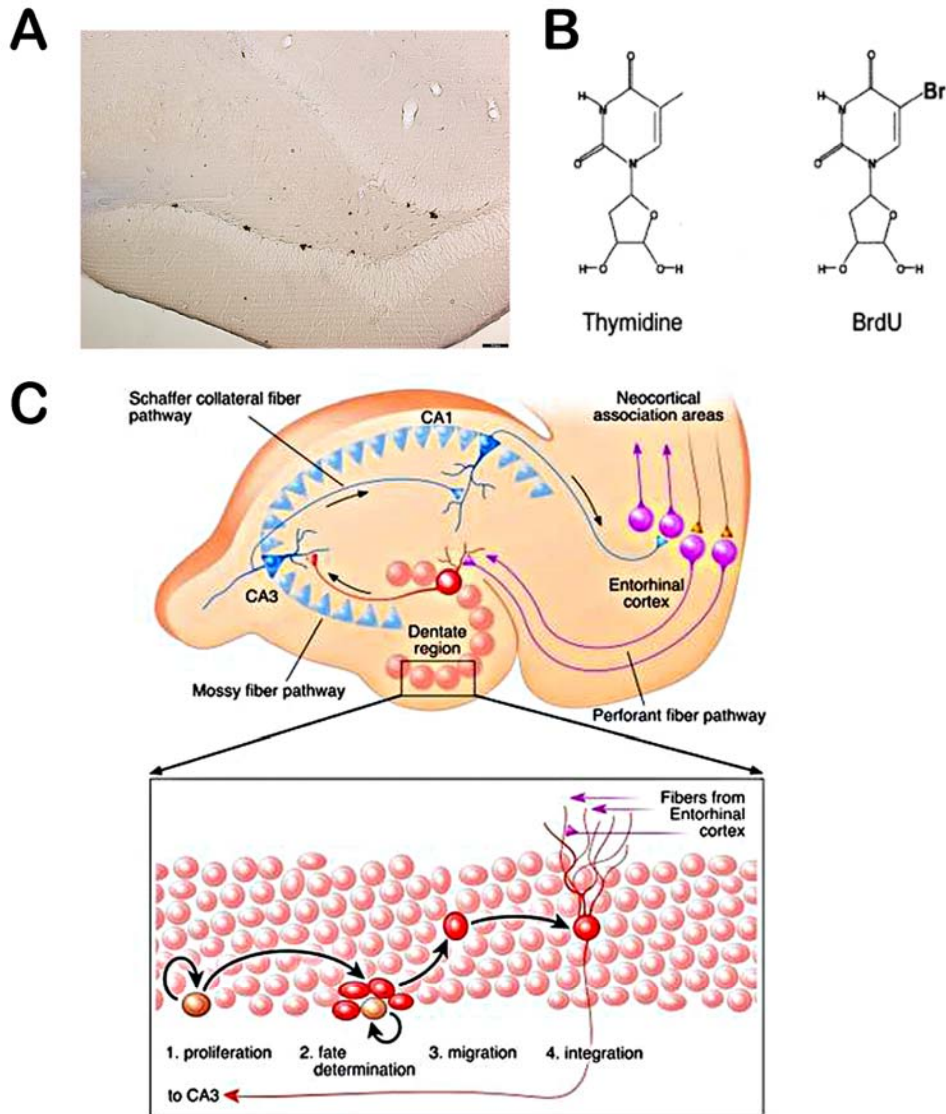
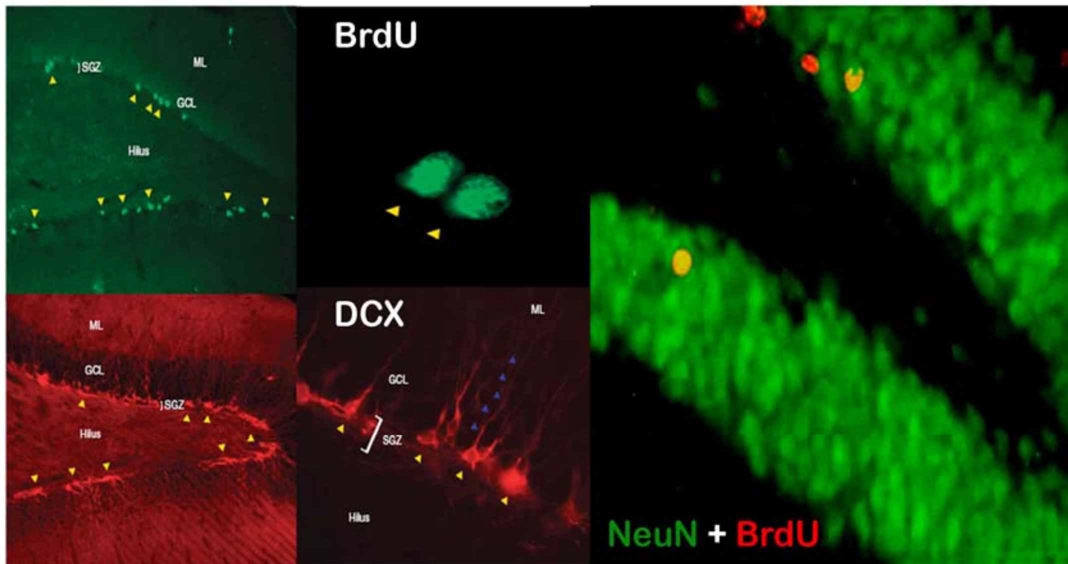


Figure 1-4 Adult Hippocampal Neurogenesis. A) An example of BrdU-immunolabeled cells in the adult dentate gyrus of a rat injected with bromodeoxyuridine (BrdU) at 200 mg/kg and sacrificed 24 hrs later (Fournier unpublished observations, 2008). B) Chemical ring structures for the nucleoside thymidine (left) and the analogue 5-bromo-2'-deoxyuridine (right). C) Model of neurogenesis in the adult hippocampal dentate gyrus. 1. Proliferation and fate determination: Stem cells (beige) in the subgranular zone of the dentate gyrus give rise to transit amplifying cells that differentiate into immature neurons. 2. Migration: Immature neurons migrate into the granule cell layer of the dentate gyrus. 3. Integration: Immature neurons mature into new granule neurons, receive input from the entorhinal cortex and extend projections into CA3. Adapted from (Lie et al., 2004).

A



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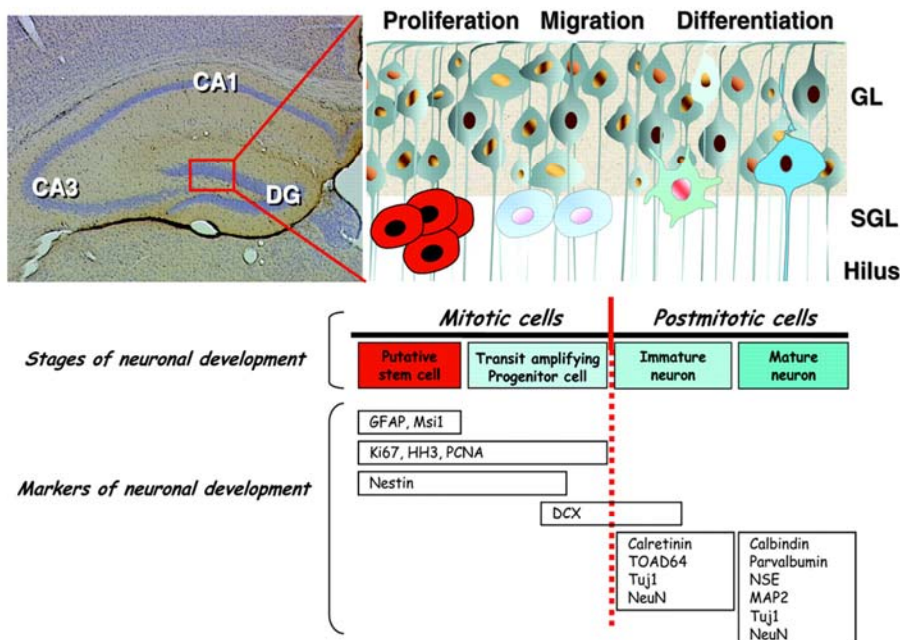


Figure 1-5 Expression of various neuronal markers at different stages of maturation. A) Photomicrograph examples of newly generated neurons. Rats were treated with a single injection of BrdU at 200 mg/kg and 30 days later. A significant proportion of BrdU-labeled shows NeuN (neuronal nuclei specific) markers 1 month after prelabeling with BrdU. In addition, a large number of proliferating cells display the immature neuronal marker doublecortin (DCX). B) A diagram showing the sequence of cellular marker expression associated with neuronal differentiation and maturation.

5.2.1. Proliferation

Adult CNS stem cells or precursor cells are characterized by their proliferative capacity to continue to undergo mitosis and their multipotency, that is, the ability to generate a multitude of different neuronal and glial lineages (Sohur et al., 2006). In the adult hippocampus, the precursor cells reside in a narrow band of tissue, the SGZ, a layer about two to three nuclei wide that begins at the base of inner GCL and extends into the hilus. The SGZ contains a heterogeneous population of precursor cells. The first type of precursor cell, the quiescent neural precursor cells (or Type-1 cells), are considered to be the putative stem cell residing in the hippocampus (Kempermann et al., 2004). This was shown in an elegant series of experiments from Arturo Alvarez-Buylla's group in which they ablated proliferating hippocampal cells with an antimitotic agent and showed that the first cell cohort to re-appear was the putative Type 1 precursor cell (Seri et al., 2001; Seri et al., 2004).

Type 1 cells are multipotent and show a characteristic morphology that resembles radial glial cells, including a triangular soma with thick apical processes that reach into the granule cell layer and then branches extensively (Seri et al., 2001). Radial glial cells are known to play an important role during development, because they provide a scaffold that aids in the migration of newly generated cells (Caviness, Jr., 1973; Frotscher et al., 2003; Stanfield and Cowan, 1979). These cells express the astrocytic marker glial fibrillary acidic protein (GFAP), but not S100β, and share several features including vascular end-feet and electrophysiological properties suggestive of astrocytes, such as passive membrane properties and potassium currents (Filippov et al., 2003; Fukuda et al., 2003). They also express nestin (class IV intermediate filament) and make up about two-

thirds of the nestin expressing cells in the SGZ of the adult mouse, however, they account for only 5% of cell divisions among these cells (Filippov et al., 2003; Kronenberg et al., 2003). This surprising finding suggests that type-1 cells most likely divide symmetrically, giving rise to one identical type-1/precursor cell and one neuronal lineage restricted progenitor daughter cell (Kempermann et al., 2004). Finally, consistent with the view that this population of cells reflects a stable foundation of precursor cells, various neurogenic stimuli, such as wheel running, environmental complexity, and seizure activity, do not appear to influence the proliferative activity of type-1 cells (Kronenberg et al., 2003).

5.2.2. Differentiation and migration

The quiescent neural precursors give rise to transiently amplifying progenitor cells (type 2 and type 3 cells). Type-2 cells express the markers nestin and Sox2 but not GFAP (Encinas et al., 2006). They have a plump short process oriented parallel to the SGZ and have an irregularly shaped dense nucleus. These cells are often seen in clusters along the SGZ and label with BrdU at a high frequency suggesting that they are highly proliferative (Filippov et al., 2003; Kronenberg et al., 2003). Type 2 cells can also be subdivided into two subtypes: type 2a cells, which are nestin-positive but negative for the immature neuronal marker doublecortin (DCX); and type2b cells, which are both nestin- and DCX-positive. The expression of DCX is associated with the initiation of neuronal differentiation and migration (Francis et al., 1999). In addition, the polysialylated form of the neural adhesion molecule (PSA-NCAM) is also widely expressed and shows significant overlap with DCX (Nacher et al., 2001; von Bohlen Und, 2007). These markers are expressed immediately after exit from the cell cycle and continue to be expressed up to 3 weeks after postmitotic development.

The type 2b are the first progenitor cells to begin showing antigenic signals suggestive of neuronal lineage commitment, most notably expressing granule cell-specific markers such as Prospero-homeobox 1 transcription factor (Prox1) and Neuron-specific nuclear (NeuN) protein. In addition, many type 2 cells (presumably type-2b cells) begin displaying complex membrane features, such as sodium currents (Filippov et al., 2003). It is at this time that these neuronal progenitor cells receive active neural input from the hippocampal circuitry. The proliferation of type 2 cells is also positively regulated by a variety of different stimuli, including physical exercise and environmental enrichment (Kronenberg et al., 2003).

By contrast, type-3 cells are DCX positive, but nestin negative. Complete exit from the cell cycle occurs at the type-3 cell stage and coincides with the transient expression of the calcium-binding protein calretinin (Brandt et al., 2003) Thus, the type 3 cell stage represents an important transition from a potentially proliferative state to a postmitotic immature neuron (Kaneko and Sawamoto, 2009; Kempermann, 2002; Suh et al., 2009). During this stage, there is also considerable variability in dendritic morphology and growth of type-3 cells reflecting this developmental transition (Plumpe et al., 2006). This is also the point in which newly generated immature neurons begin migrating towards the dentate GCL suggesting that the loss of nestin immunoreactivity and terminal exit of the cell cycle is associated with active migration. The distance of radial migration into the GCL is limited. Most new cells remain in the SGZ and the inner third of the GCL; few reach the outer third (Kempermann et al., 2003). It is likely that these differences in migration simply reflect differences in the spatial and temporal gradients associated with granule cell neurogenesis (the “outside-in pattern”) during

development, where neurons that are born first are located closer to the molecular layer, whereas younger neurons are located in the bottom portion of the GCL near the hilar border (Caviness, Jr., 1973). Finally, under normal conditions, these cells show limited proliferative activity (Kronenberg et al., 2003); however, epileptic seizures robustly increase the proliferation of type-3 cells (Jessberger et al., 2005).

Approximately, two to three weeks after becoming postmitotic and expressing calretinin, the new cells switch the expression of calretinin to calbindin (Kempermann et al., 2004). Calbindin is present in all mature dentate granule cells and represents an important point in the maturation of newly generated neurons. However, it will take an additional 4 to 7-weeks for the new cells to become functionally and morphologically indistinguishable from older granule cells (Jessberger and Kempermann, 2003; van Praag et al., 2002).

5.2.3. Neuronal Maturation and Integration

Postmitotic differentiation and maturation is characterized by the initial expression DCX and calretinin, and by the subsequent expression of more mature neuronal markers such as NeuN and calbindin. The speed of maturation is likely dependent on a variety of factors, such as experience, and will ultimately vary between neurons. The newborn cells begin integrating into the GCL 4–10 days after their generation. As they form dendrites, they rapidly extend axons into the CA3 pyramidal cell layer 4 to 10 days later, suggesting that they make synapses long before being fully mature (Ambrogini et al., 2004; Hastings and Gould, 1999; Toni et al., 2008). Indeed, using viral-based transsynaptic neuronal tracing techniques, the neurons generated in the DG have been shown to be synaptically integrated by 4 to 8 weeks after their birth

(Carlen et al., 2002; van Praag et al., 2002), whereas they reach a mature morphology (soma size, total dendritic length, dendritic branching, and spine density) only 4 months after birth (Song et al., 2002). Stimulation of the perforant pathway, the main excitatory afferent to the DG, can elicit responses in newborn GFP-labeled cells, indicating that they receive functional synaptic inputs and are therefore functionally integrated into the preexisting network (van Praag et al., 2002).

When still located in the SGZ, newly born cells exhibit electrophysiological properties characteristic of immature neurons: they are completely unaffected by $GABA_A$ receptor inhibition, they exhibit paired-pulse facilitation, and they have a lower threshold for the induction of long-term potentiation (LTP) (Wang et al., 2000), a cellular correlate of learning (Bliss and Collingridge, 1993). With the use of nestin-promoter green fluorescent protein transgenic mice, type 1 cells (nestin-positive) were found to have low input resistance values, whereas type 2b cells (expressing PSA-NCAM and nestin) exhibited higher input resistance and voltage-dependent sodium currents (Fig. 1-6) (Fukuda et al., 2003). Interestingly, recent work has also shown that PSA-NCAM expressing cells differ from mature neurons in their passive (input resistance) and active membrane properties (such as T-type Ca2+-mediated spikes that boost fast Na+ action potentials) and in their enhanced ability to develop associative LTP (Schmidt-Hieber et al., 2004; Stocca et al., 2008). These exciting findings suggest that newly generated neuron express unique intrinsic mechanisms that can facilitate synaptic plasticity within the dentate. Indeed, the functionality of the adult-born granule cells has been further demonstrated using the immediate gene marker *c-fos*. Studies have shown that at least 40% of 1-month old newborn granule neurons are able to respond to various

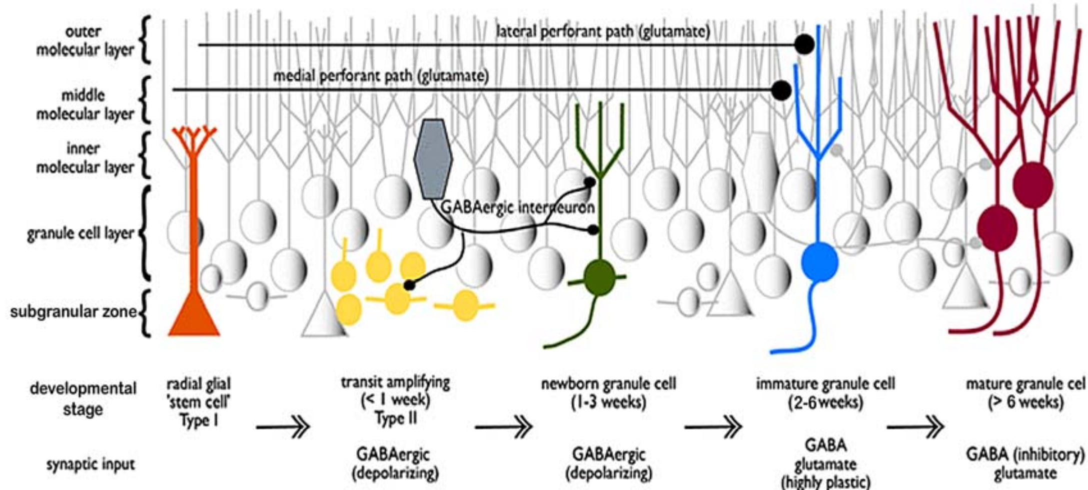


Figure 1-6 The timing of functional integration of adult-born dentate granule cells. Newly born cells of the adult DG follow a precise sequence for the maturation of neuronal function and connectivity that requires about 6 weeks and exhibits a striking similarity to the events observed during hippocampal development. The neuronal phenotype is acquired within the first few days. Those early immature neurons show small action potentials, express immature neuronal markers, and are spatially restricted to the subgranular zone. They lack afferent synaptic contacts and display a high membrane resistance that reflects a low density of ion channels, but show tonic activation of $GABA_A$ receptors. One week later, neurons are localized in the inner GCL, exhibit spineless dendrite trees that reach the inner molecular layer, and receive depolarizing GABAergic inputs of dendritic origin. By the third week, newborn neurons begin to receive functional glutamatergic afferents and display repetitive action potentials with high frequency adaptation. Detailed morphological analysis of retrovirally labeled neurons resulted in a starting point for dendritic spine formation of 16 days, indicative of glutamatergic synaptogenesis (Zhao et al., 2006). Consistent with these observations, kainate-induced seizures elicited activity-dependent expression of the immediate early genes c-fos, zif268, and Homer1A in BrdU-labeled neurons of greater than 15 days of age (Jessberger and Kempermann, 2003). Neuronal maturation becomes complete by the fourth week with the onset of perisomatic GABAergic contacts and the presence of spiny dendrites reaching the outer molecular layer (Espósito et al., 2005).

chemoconvulsants (i.e., $BrdU^+/c\text{-}fos^+$) (Jessberger and Kempermann, 2003; Jiang et al., 2004a; Scharfman et al., 2000). In contrast, learning that specifically targeted the hippocampus (e.g., contextual fear conditioning) only resulted in ~3% of cells being activated (Kee et al., 2007; Trouche et al., 2009).

5.2.4. Cell death and cell numbers

In the rodent DG, the vast majority of new born cells are postmitotic and at least partially differentiated between 3 and 7 days (Dayer et al., 2003). The cells that do not terminally differentiate within this period, typically die within 1 week of their generation, a process affecting 60% of the newborn cells (Dayer et al., 2003; Hastings and Gould, 1999). Interestingly, the DG harbors a much lower number of proliferating cells compared with the SVZ. In a study performed in 9- to 10-week old rats, ~9000 newborn cells were found to be generated per day (Cameron and McKay, 2001). In a more recent study, only ~4000 new cells, out of which 3000 were found to be new neurons, were shown to be added daily in the DG of 4-month old rats (Rao and Shetty, 2004); this discrepancy in the number of newborn cells is certainly linked to the difference in the age of the animals used in the studies, as the production of new neurons in the DG diminishes with age (Kuhn et al., 1996).

5.3. Factors Regulating Adult Neurogenesis

A considerable number of studies have shown that adult neurogenesis can be dynamically regulated and modulated at different stages, including proliferation, fate specification (glial vs. neuronal), migration, integration, and survival (Duman et al., 2001; Gould et al., 2000; Kempermann et al., 2000; Lennington et al., 2003). This

regulation occurs through an immense network of interactions involving a variety of intrinsic and extrinsic factors that range from alterations in progenitor microenvironment to the direct effect of secreted growth and neurotrophic factors on the development and/or maintenance of newly generated cells. In addition, environmental stimuli, such as housing conditions or physical exercise, can also influence the proliferation and survival of new neurons. It is through this dynamic and selective process that it becomes clear that neurogenesis is not merely restorative in nature but reflects an adaptive response to challenges imposed by an animal's environment and/or its internal state (Lledo et al., 2006). Therefore, by understanding how this regulation occurs, we will gain significant insight not only on normal adult neurogenesis, but also on how this process contributes to the etiology and pathophysiology of various clinical disorders, including epilepsy.

5.4. Role of Hippocampal Neurogenesis in Memory and Learning

Although there has been no direct evidence demonstrating that adult generated neurons are necessary for normal learning, numerous studies have suggested a strong link between the rate of neurogenesis and learning capabilities or proficiency. Altman (1969a) was one of the first to suggest that the preservation of neurogenesis within the hippocampal region might serve as way to facilitate the continuous formation of new memories. He speculated that the continuous generation of new neurons might serve as a means to encourage novel associations between previous learning episodes and on-going experience. However, more direct evidence linking neurogenesis and learning came from Nottebohm and coworkers in relation to song-learning and seed-caching behavior (Barnea and Nottebohm, 1996; Goldman and Nottebohm, 1983; Nottebohm, 1985). In addition, several studies since then have shown that hippocampal-dependent learning

tasks impact neurogenesis in rodents. For instance, training in a trace eyeblink conditioning task, conditioned food preference task, or in the water maze results in an increase in the number of newly generated neurons (Ambrogini et al., 2000; Dupret et al., 2007; Gould et al., 1999a; Hairston et al., 2005; Leuner et al., 2004). Importantly, these learning-induced changes in neurogenesis are specifically attributed to hippocampal functioning because tasks that do not require the hippocampus, but nonetheless activate it, such as delayed-eye blink conditioning or training on a cued test version of the water maze task do not alter the rate of proliferation (Van der Borght et al., 2005).

Alternatively, if neurons born in adulthood are necessary for certain hippocampus-dependent tasks, then reduced neurogenesis should be associated with impaired learning, whereas enhanced neurogenesis should improve learning. Support for this comes from observations that physical activity and environmental enrichment increase both neurogenesis and performance on hippocampus-dependent learning tasks (Bruel-Jungerman et al., 2005; Rossi et al., 2006; Snyder et al., 2009; Steiner et al., 2008; van Praag, 2009), whereas aging, prolonged exposure to stressogenic stimuli or stress-related hormones, or cholinergic lesions are all associated with deficits in both of these phenomena (Eisch et al., 2008; Falconer and Galea, 2003; Gould, 1994; Ho et al., 2009; Kempermann et al., 1998; McDonald and Wojtowicz, 2005; Tanapat et al., 1998; Tanapat et al., 2001) Previous work has revealed that two week treatment with the non-specific mitotic inhibitor methylazoxymethanol acetate (MAM), which reduces neurogenesis by approximately 80%, significantly impairs trace eye-blink conditioning and trace fear conditioning in rats (Shors et al., 2001; Shors et al., 2002). However, it appears that ablation of hippocampal neurogenesis does not aversely affect all forms of hippocampal

learning. For example, MAM treatment or focal cranial irradiation, which also dramatically reduces hippocampal neurogenesis (Monje et al., 2002), does not adversely affect water maze learning (Madsen et al., 2003; Shors et al., 2002). Although the acquisition of many hippocampus-dependent tasks does not appear to be affected by inhibition of neurogenesis, recent studies suggest that neurogenesis may be crucial for the long-lasting consolidation or retention of spatial memories. In support of this, several studies have found that almost total ablation of hippocampal neurogenesis impairs long-term memory retention in the water maze (Rola et al., 2004; Snyder et al., 2005) and prevents environmental enrichment-induced improvement in long-term recognition memory (Bruel-Jungerman et al., 2005). Finally, the use of conditional transgenic strategies that permit specific ablation of newborn hippocampal neurons in adult animals have shown impairments in complex spatial memory, whereas simpler forms of spatial knowledge and memory that are independent of the hippocampus are spared (Clelland et al., 2009). When taken together, these finding suggest that the functional loss of newly generated neurons in the adult hippocampus can adversely impact normal dentate functioning leading to the emergence of pervasive learning and memory-related impairments.

6. The Role of the Dentate Gyrus in Epilepsy

The DG, sitting between the EC and area CA3, is in a critical position to modulate the amount of incoming information that gets through to the hippocampus. Several studies have shown that adult granule cells are rather “unexcitable”, mainly because of their limited recurrent connectivity and also from the fact that they have a very negative resting membrane potential and receive powerful inhibitory modulation from local

interneurons (Fricke and Prince, 1984; Lothman, 1994; Scharfman, 1992; Staley et al., 1992; Teyler and Alger, 1976). Prolonged stimulation under normal conditions fails to evoke rapid, repetitive firing because these cells exhibit a high degree of spike accommodation to maintained depolarization (Alger and Teyler, 1976; Staley et al., 1992). Because of these fundamental properties, the DG has often been viewed as a "gate" or a "filter" at the entrance to the hippocampus that blocks or sifts through incoming excitation from the EC (Hsu, 2007).

This "gating" property of the DG is thought to actively impede the propagation of epileptiform activity into the hippocampus proper. As a result, the DG was relatively neglected by most epilepsy researchers mainly because of the resistance of dentate granule cells to generate epileptiform activity (Lothman, 1994). However, the seminal work by Eric Lothman's group revealed that the "gating" function of the DG may operate in an "all-or-none" fashion, in which maximal dentate activation results in the normal function of the dentate changing from a restrictive to a more supportive mode (Stringer et al., 1991). Electrophysiologically, this transition reflects the sudden appearance of bursts of large amplitude (20-40 mV) population spikes associated with a negative shift in the DC potential and a secondary rise in extracellular potassium levels (Lothman et al., 1992). Maximal dentate activation has been shown to be a critical step in the production of hippocampal AD and to be important for the lengthening of ADs and kindling of the motor response that occurs with repeated stimulation (Stringer et al., 1991; Stringer and Lothman, 1989). In the absence of maximal dentate activation, seizure activity does not normally propagate into the hippocampal formation, but after the initiation of maximal dentate activation, the "gate" function of the dentate is compromised leading to an

amplification and propagation of seizures to other limbic and extra-limbic areas (Heinemann et al., 1992; Stringer and Lothman, 1989; Stringer and Lothman, 1992).

The findings presented above suggest that the high seizure threshold of the normal DG can dramatically be overcome after a few electrical induced ADs to this area (Heinemann et al., 1992; Lothman et al., 1992; Stringer and Lothman, 1989). There is evidence that the induction of long-term potentiation or even a single AD in the DG can increase synaptic transmission in this area for periods as long as 3 months (Sayin et al., 1999; Sutula and Steward, 1987). These findings are consistent with the idea that long-term cellular alterations and circuit remodelling in DG occurs in response to repeated seizure activity. Thus, the extent of filtering or modulating of some forms electrical activity will be reduced in the DG by the activity-dependent enhancement of synaptic transmission in dentate granule cells (Dudek and Sutula, 2007).

It is presently unclear which specific cellular or circuit changes within the dentate contribute to alteration in its "gating" capacity. However, there are three experimental findings that may contribute to this process. First, the selective loss of specific populations of hilar neurons after seizure activity might alter the electrophysiological properties of the dentate network. Second, dentate granule cell axons have been shown to display an extensive degree of plasticity (i.e., sprouting) in response to epileptogenic stimulation, thus providing a basis for increased recurrent excitation in the dentate after seizures. And finally, dentate granule cells show constant turnover throughout adulthood. This latter finding has generated considerable interest among researchers in the epilepsy community because it has been shown that seizures in both animals and human epileptic patients can stimulate the birth of new neurons in the adult DG (Mohapel et al., 2004;

Parent et al., 1998; Scharfman and Gray, 2007; Scott et al., 1998; Siebzehnrubl and Blumcke, 2008). Interestingly, these new neurons show many abnormal morphological characteristics that could serve to promote excitation within dentate networks (Scharfman et al., 2000).

6.1. The Creation of New Circuits Following Kindling

It is clear that the brain can reorganize itself in response to excessive neural stimulation, such as seizures. A number of studies have shown that kindling can produce an increase in the amplitude of evoked synaptic responses in various limbic system pathways (Corcoran and Teskey, 2009). For example, when stimulation pulses are applied to the amygdala after the completion of kindling, an increase in the amplitude of the evoked response can be recorded in target structures, such as the hippocampus (Racine et al., 1975; Racine et al., 1972; Racine et al., 1983). The long-lasting nature of these effects (Goddard and Douglas, 1975; Maru and Goddard, 1987a; Maru and Goddard, 1987b; Scharfman, 2002) most likely reflects functional changes involving pre-synaptic and post-synaptic properties (reviewed by Morimoto et al., 2004).

Although enhancement of existing excitatory connections remains one way to alter the functional properties of neural circuits after seizures, it is also possible that new connections are formed. Reactive synaptogenesis is a classic response to tissue damage (Murray, 1986; Raisman, 1969) and there is a strong association between cellular damage in patients with chronic TLE and axonal sprouting (Proper et al., 2001). However, it is now clear that axonal growth and synaptogenesis can also be induced by high levels of neural activity in the absence of neuronal damage (Tominaga-Yoshino et al., 2008; Urakubo et al., 2006).

6.1.1. Mossy fiber sprouting and epileptogenesis

One of the most remarkable examples of activity-driven structural plasticity in the epileptic brain involves the dentate-CA3 mossy fiber network. Mossy fiber sprouting has been found in resected samples of hippocampus from patients with TLE (Babb, 1999; Isokawa et al., 1993; Mathern et al., 1994b; Mathern et al., 1995; Pitkanen et al., 2000b). In addition, animal models, ranging from pilocarpine-treated rats to the kindling model, have all demonstrated a time-dependent increase in mossy fiber terminal sprouting after seizures (Cavazos and Sutula, 1990; Danzer et al., 2009; Frotscher et al., 2006; Lynch and Sutula, 2000; Nadler, 2003; Scharfman et al., 2002b; Sutula et al., 1989). The targets of these sprouting axons and their terminals are the inner molecular of the DG and stratum oriens of CA3, regions that only sparsely receive innervation in the normal brain. Electrophysiological studies and ultrastructural data have confirmed that most if not all of these new synapses are excitatory (Sutula and Dudek, 2007). There is also a strong correlation between the extent of synaptic reorganization of the mossy fiber pathway and the degree of hyperexcitability of granule cells (Cavazos and Cross, 2006) or kindling progression (Sutula et al., 1989).

The precise molecular mechanisms that regulate mossy fiber sprouting are not presently known (McNamara, 1999). One hypothesis suggests that hilar neuron loss (especially mossy cells) vacate postsynaptic sites on dentate granule cell dendrites, thereby triggering the formation of recurrent sprouting into the inner molecular layer of the DG (Sloviter et al., 2003). In support of this, a negative correlation between the density of mossy fiber sprouting in the inner molecular layer and hilus cell counts has been found in resected human hippocampal tissue and in kainate-treated rats (Buckmaster and Dudek, 1999; Masukawa et al., 1995). However, it is important to note that axonal

sprouting of the mossy fiber system does not necessarily depend on neuronal damage or loss for its expression. For example, Racine and colleagues have found a moderate increase in mossy fiber sprouting with no detectable cell loss in response to amygdaloid or perforant path kindling (Adams et al., 1997; Adams et al., 1998). Interestingly, the sprouting proceeds at similar rates, despite the fact that the kindling rates for the amygdala and perforant path are significantly different.

Seizures may also trigger a cascade of gene changes that facilitate axonal sprouting. It has been well established that seizures rapidly increase the expression of various immediate early genes (e.g., c-fos, c-jun, erg1), followed by expression of genes encoding neurotrophic factors (e.g., BDNF) and axonal growth (e.g., growth associated protein 43 kDa, GAP-43) (Bendotti et al., 1993; Dalby et al., 1995; Dragunow et al., 1988; Dragunow and Robertson, 1987; Ernfors et al., 1991; Isackson et al., 1991; Madsen et al., 2006; Van der Zee et al., 1995). Both perforant path and partial hippocampal kindling increased expression of GAP-43 mRNA in the CA3 region and the hilus (Bendotti et al., 1993). Other synaptic proteins, such as the synapsins, which are associated with axonal terminal growth, are also changed following kindling. For instance, amygdaloid kindling transiently increases synapsin I (specific to terminals) but not synapsin II (wide subcellular localization) mRNA in the dentate gyrus ((Morimoto et al., 1998). In addition, synapsin I immunoreactivity is also increased in the DG and CA3 but not CA1 after short-term kindling (Suemaru et al., 2000).

In summary, the morphological change in the mossy fiber system after intense seizure stimulation reflects the creation of novel recurrent and feedforward excitatory circuits (Morimoto et al., 2004). However, it is unlikely that mossy fiber sprouting on its

own plays a major role in the development of epileptogenesis. In amygdala kindling, the onset of Class 5 motor convulsions occurs well before the presence of mossy fiber sprouting (Ebert et al., 1995; Elmer et al., 1997; Mohapel et al., 2000). In addition, intra-hippocampal infusions of BDNF, a manipulation that inhibits perforant path kindling, does not influence mossy fiber sprouting (Xu et al., 2004). An intriguing possibility is that mossy fiber sprouting may serve to increase inhibition by synapsing directly on local inhibitory interneurons in the inner molecular layer or hilus (Franck et al., 1995) or by releasing GABA from synaptic terminals onto CA3 principle cells during periods of hyperexcitability (Gutierrez, 2003). Finally, administration of the protein synthesis inhibitor cycloheximide prevented the development of mossy fiber sprouting after status epilepticus-induced seizures but did not influence the development of spontaneous recurrent seizures (Longo and Mello, 1997; Longo and Mello, 1998). Although these studies argue that mossy fiber sprouting does not play a role in the development of epileptogenesis, it is possible that such reactive sprouting is crucial for maintaining the chronic epileptic state once it has been established (Masukawa et al., 1995).

7. Adult Neurogenesis in the Epileptic Hippocampus

Although structural changes among pre-existing cells and/or cell loss in the hippocampus represents one way to alter excitability, seizures and seizure-induced injury also influences the process of cell birth. These findings have led to two distinct areas of inquiry with respect to epileptogenic brain injuries. The first question is whether the neural stem cells that persist in the adult hippocampus are capable of repairing this region after seizure-induced injury. For instance, early postmortem studies showed that hippocampal pathology was frequently observed in patients with epilepsy (Bouchet and

Cazauvielh, 1825; Bratz, 1899). Hippocampal sclerosis or Ammon's horn sclerosis is found in approximately 50-75% of temporal lobe resections made for intractable limbic epilepsy (Bote et al., 2008; Fisher et al., 1998; Thom et al., 2009) and its presence is often considered a good predictor of seizure relief following surgery (Mintzer and Sperling, 2008). Initial microscopic work by Wilhelm Sommer (1880) revealed a characteristic pattern of neuronal loss and astrogliosis within the hippocampus, particularly within the CA1 subfield (Lorente de Nó, 1934) and varying degrees of neuronal damage in the CA3 subfield, polymorphic zone, and DG, with selective sparing of CA2 often occurring (Bote et al., 2008; Sano and Kirino, 1990; Thom, 2008). In the context of epilepsy, new neurons could conceivably reverse the neuronal loss associated with hippocampal sclerosis (Fujikawa, 2005). As tempting as this hypothesis may be, it now appears that neurogenesis in the adult epileptic brain may be more pathological than beneficial.

The growing evidence of the modulation of neurogenesis by seizure activity (Parent et al., 1998; Scott et al., 1998; Smith et al., 2006) has raised several important questions. Does the proliferation of new neurons after seizures contribute to aberrant plasticity and alterations in excitability? And if new neurons integrate abnormally into existing neuronal circuits after an epileptogenic insult, do they contribute to the development of epilepsy or associated cognitive/behavioural co-morbidities such as memory dysfunction? At present, the molecular mechanisms and functional consequences of seizure-induced neurogenesis remain largely unknown. However, this thesis will attempt to shed some light on the biological role of persistent neurogenesis in the setting of epilepsy.

7.1. Modulation of Dentate Gyrus Neurogenesis by Seizure Activity

Studies of adult rodent models of limbic epileptogenesis have shown that prolonged seizures stimulate hippocampal neurogenesis (Bengzon et al., 1997; Gray and Sundstrom, 1998; Parent et al., 1997; Parent et al., 2006; Scott et al., 2000). In the kainate and pilocarpine models of TLE, chemoconvulsant-induced status epilepticus results in a dramatic increase in dentate cell proliferation, approximately five- to ten-fold, with over 90% of the newly generated cells differentiating into dentate granule cells (Gray and Sundstrom, 1998; Parent et al., 1997). This increase is observed after a latent period of a few days after the induction of seizures and persists during the initial two to three weeks after seizure, before levels of neurogenesis return to baseline over the following weeks (Nakagawa et al., 2000; Parent et al., 1997). Importantly, these newly generated neurons have been shown to survive for at least 53 days after pilocarpine seizures (Parent et al., 1997) and at least 6 months after rats were subjected to 2 hrs of electrically-induced self-sustaining status epilepticus (Bonde et al., 2006). These findings suggest the long-lasting presence and possible functional integration of seizure-generated neurons in the chronically epileptic brain. Interestingly, although the severe seizures that accompany status epilepticus enhance neurogenesis to a greater extent than other models of epilepsy (i.e., kindling models), the survival of newborn dentate granule cells tends to decrease with increasing seizure severity indicating that the long-term outcome of neurogenesis can be influenced by the degree of injury to the DG microenvironment (Mohapel et al., 2004).

As suggested above, hippocampal neurogenesis can also be enhanced by kindling-induced epileptogenesis suggesting that the induction of severe seizures and/or neurodegeneration is not a prerequisite for this phenomenon. Kindling of the amygdala

acutely increases hippocampal cell proliferation (Auvergne et al., 2002; Parent et al., 1998; Sato et al., 2002a; Sato et al., 2002b; Scott et al., 1998; Smith et al., 2005; Smith et al., 2006). Similar neurogenic effects have been observed after intermittent kindling of the perforant path or via direct kindling of the ventral hippocampal CA1 subfield (Bengzon et al., 1997; Nakagawa et al., 2000). This robust neurogenic effect following the induction of epileptiform activity is further evidenced by the observation that even a single AD induced by ventral CA1 stimulation can lead to an increased number of newly differentiated dentate granule cells (Bengzon et al., 1997).

There are also regional hippocampal differences in progenitor mitotic activity in response to kindling stimulation. For instance, although progenitor mitotic activity appears to be greater in the dorsal hippocampus compared to the ventral hippocampus at baseline, the relative change in mitotic activity is higher in the ventral hippocampus than in the dorsal hippocampus following fluorthyl kindling (Ferland et al., 2002). Interestingly, the threshold to evoke epileptiform activity differs between the dorsal and ventral hippocampus in that the dorsal hippocampus tends to have a slightly lower AD threshold than the ventral hippocampus (Racine et al., 1977). However, the progression or rate of kindling to generalized motor convulsions (i.e., the number of stimulations required to elicit a motor seizure) is significantly faster for the ventral hippocampus (~22 stimulations on average) in comparison to the dorsal hippocampus (~40 stimulations on average). These kindling differences may account for the regional differences in baseline progenitor activity as well as the changes in their activity following kindling.

Although the molecular mechanisms responsible for mediating increased cell proliferation in response to seizure activity is largely unknown, the $5HT_{1A}$, CB1

cannabinoid, and galanin type 2 receptors have all been shown to be important (Aguado et al., 2007; Mazarati et al., 2004; Radley and Jacobs, 2003). In addition, growth factors such as neuropeptide Y, BDNF, and basic fibroblast growth factor are known to contribute to seizure-induced cell proliferation and to encourage adoption of a neuronal fate after cell division (Altar et al., 2004; Howell et al., 2007; Laskowski et al., 2007; Newton et al., 2003; Paradiso et al., 2009; Scharfman et al., 2005; Yoshimura et al., 2001; Zucchini et al., 2008). Unfortunately, many of these studies utilized methods (e.g., chemoconvulsants) that also produce significant damage and inflammatory changes in the brain. Thus, it is presently unclear if the cellular injury and inflammation that accompany seizure activity are critical factors that stimulate progenitor activation and proliferation. Studies in organotypic hippocampal slices appear to support the view that degeneration is an important prerequisite by showing that kainate acid-induced injury generally precedes increased progenitor proliferation (Sadgrove et al., 2005). However, cellular injury and inflammation are also associated with upregulation of neurotrophic and growth factor secretion, which in turn might mediate direct mitogenic effects on dentate progenitor cells (Jankowsky and Patterson, 2001; Racine et al., 2002).

Perhaps the strongest evidence that the proliferative response of progenitor cells after seizures can occur independently of cellular injury comes from studies showing that rapid kindling increases neurogenesis without producing seizure-induced cell death (Smith et al., 2005; Smith et al., 2006). These important studies suggest that seizure-induced neurogenesis may not be a direct consequence of neuronal degeneration but may be the result of aberrant stimulation of dentate progenitor cells. Interestingly, these pro-mitogenic effects may not be completely mediated by growth factor secretion from

neuronal cells but might be triggered from altered responses of surrounding supportive cells, like glial cells. For instance, the expression of the cysteine protease cystatin C is upregulated in activated astrocytes and microglia cells following injury (Pirttila and Pitkanen, 2006) and cystC$^{-/-}$ knockout mice show reduced progenitor division after pilocarpine seizures (Pirttila et al., 2005). Cystatin C can trigger the release of nitric oxide (NO) from macrophages via the induction of tumor-necrosis and interleukin-10 signaling cascades (Verdot et al., 1999) and interestingly, proliferation of dentate progenitor cells after pentylenetetrazol-induced seizures has been found to be mediated by a NO-dependent mechanism (Jiang et al., 2004b). Because glial cells are known to contribute to the pathophysiology of epilepsy (Jabs et al., 2008; Wetherington et al., 2008), it is possible that they may play a role in mediating seizure-induced neurogenesis.

It is also possible that the sustained increase in neurogenesis following seizures might be explained by the effects of the epileptic activity directly on the process of progenitor cell differentiation (Scharfman and Gray, 2007). In other words, do seizures impact the differentiation process or transition of type-1 cells (i.e., radial glial-like precursor cells) to type-2a and b (i.e., transiently amplifying progenitor cells, or type-3 cells (i.e., late progenitor cells)? To address this question, Huttmann and colleagues (2003) examined type-1 cells using transgenic mice expressing GFP in the GFAP promoter. They found a greater number of proliferating astrocytes with radial glial characteristics (type-1 cells) in the dentate SGZ 72 hrs after kainate-induced seizures. These findings were later replicated by Zhu and colleagues (2005) using ribonucleotide reductase probes to label proliferating progenitor cells in the DG and SVZ after kainate seizures again suggesting that more type-1 cells were recruited into the cell-cycle after

seizures (Zhu et al., 2005). In contrast, Jessberger et al., (2005) was unable to detect differences in type-1 GFAP+/radial glial-like cell proliferation following kainate seizures, a finding that supported past work indicating that the division of type-1 neuroblast precursors is generally unaffected by physiological stimuli, such as physical activity, which typically affect the division of transiently amplifying progenitor type-2a and b cells (Kronenberg et al., 2003). Instead, Jessberger and colleagues reported that seizures stimulated the division of late DCX-positive type-3 cells, a stage associated with early stages of neuronal maturation. It was speculated that when progenitor cells are in a migratory state and beginning to initiate terminal differentiation, they may be particularly sensitive to the effects of seizures (Jessberger et al., 2005). However, these authors evaluated their animals 9 days after kainate treatment, so it is conceivable that both undifferentiated GFAP-expressing precursors and more committed neuronal-like progenitors may increase proliferation after seizure activity.

7.2. Morphological Development and Functional Integration of New Neurons in the Epileptic Brain

Apart from the stimulatory effects on progenitor division, seizures can also impact the structural and functional development of new neurons. In epileptic animals, new neurons have elongated dendritic trees with a greater number of branch points and larger number of spines capable of receiving synaptic inputs in comparisons to new neurons from non-epileptic animals (Jakubs et al., 2008; Jessberger et al., 2007; Overstreet-Wadiche et al., 2006; Walter et al., 2007; Zhao and Overstreet-Wadiche, 2008). Importantly, these observations are not found in animals that receive only brief seizures suggesting that processes critical in epileptogenesis are responsible for altering the

dendritic development of new neurons (Overstreet-Wadiche et al., 2006). In addition, the accelerated dendritic maturation of new neurons after seizures also corresponds with early circuit integration. For example, Ovestreet-Wadiche and colleagues (2006) showed that medial perforant path stimulation could readily evoke excitatory postsynaptic currents (EPSCs) in newborn granule cells from slices of epileptic animals, whereas EPSCs were never evoked in slices from controls. They also demonstrated that newborn granule cells with synaptic input had lower input resistance after seizures (i.e., input resistance is inversely correlated with granule cell maturity ((Schmidt-Hieber et al., 2004) compared with newborn cells from control mice suggesting that the appearance of medial perforant path-evoked EPSCs, along with the reduction in input resistance, were associated with accelerated maturation and functional integration of adult-generated granule cells in the epileptic DG (Overstreet-Wadiche et al., 2006). Although the precise molecular mechanisms responsible for the accelerated integration are unknown, one likely candidate is the direct depolarizing action from GABAergic inputs (presumably from inhibitory interneurons) onto newborn neurons, which might provide trophic support that drives dendrite development and synaptogenesis (Ge et al., 2006; Represa and Ben-Ari, 2005).

Using a variety different immunohistochemical or retroviral approaches, several studies have shown that seizures also change the morphology of newly generated granule cells. For example, after seizures, a greater proportion of newborn granule cells displayed hilar basal dendrites in comparison to mature granule cells (Dashtipour et al., 2001; Jessberger et al., 2007; Ribak et al., 2000; Shapiro and Ribak, 2006). Although newly generated neurons in the rodent adult hippocampus normally display a prominent basal

dendrite during specific developmental phases, the absence of synaptic contacts has strongly suggested these dendrites are transient structures (Seress, 2007). However, for reasons that are unclear, basal dendrites appear to be a persistent feature of adult granule cells in human and non-human primates (Seress and Mrzljak, 1987) and they are increased in patients suffering from TLE (von Campe et al., 1997). These morphological changes also appear to reflect a stable modification in dendritic structure as basal dendrites have been found to persist for long durations after seizures (Ribak et al., 2000; Walter et al., 2007) and develop mature synapses on dendritic spines (Shapiro et al., 2008).

The critical question is whether these aberrant modifications contribute to recurrent excitation among granule cells in the epileptic DG. Indeed electrophysiological studies have confirmed that basal dendrites promote a hyperexcitable state in the hippocampus (Austin and Buckmaster, 2004). Interestingly, Shapiro and colleagues (2007) found both immature and developing synapses on DCX-labeled basal dendrites can be observed as early as 4 days after pilocarpine seizures. These hilar basal dendrites were shown to grow along an ectopic glial scaffold suggesting that glial hypertrophy associated with seizure-induced inflammation and hilar interneuronal degeneration may serve as a critical factor for the development of hilar basal dendrites (Shapiro et al., 2005). It would be interesting to determine if an animal model of epilepsy that is not typically associated with extreme hilar cell loss, such as amygdaloid kindling, also promotes the formation of basal dendrites. If these conditions can demonstrate that new neurons display basal dendrites after kindled seizures, then they will provide further

support that newly born neurons display high levels of plastic responsiveness that can be associated with significant changes in their morphology.

7.3. Abnormal Migration in the Epileptic Brain

An increasingly recognized abnormality of hippocampal neurogenesis observed in many experimental models of TLE concerns the ectopic migration of adult born granule cells. Studies have shown that although most new neurons born after seizures migrate appropriately into the inner granule cell layer, a significant proportion seem to migrate aberrantly from the dentate subgranular proliferative zone to the hilus (Parent et al., 1997; Parent et al., 2006; Scharfman et al., 2000). Given that a fairly large population of displaced granule cells develop after the induction of seizures, it is possible that seizure-induced neurogenesis might lead to disorganization of the DG. In fact, the dentate granule cell layer is frequently unusual in human TLE due to cell dispersion and there is evidence of ectopic granule-like neurons in the hilus and inner molecular layer (Dashtipour et al., 2001; Houser et al., 1992; Parent et al., 2006). This may cause a problem for normal information processing in the hippocampal formation. Seminal studies led by Helen Scharfman and her group have shown that a large number of hilar ectopic granule-like cells are innervated by mossy fiber terminals and in turn send their axonal collaterals back into the inner molecular layer (Pierce et al., 2005; Scharfman et al., 2000). Furthermore, although the intrinsic properties of ectopic granule cells confirm their identity as granule cells, functional electrophysiological studies in hippocampal slices have shown that these ectopic granule cells are hyperexcitable and discharge spontaneous bursts of action potentials that are synchronized with CA3 pyramidal cells (Scharfman et al., 2000). Epileptiform burst discharges are not normally recorded in

granule cells that are located in the dentate granule cell layer, even after exposure to chemoconvulsants (Scharfman, 1994). Finally, expression of the activation-induced immediate early gene c-fos by ectopic hilar granule cells has also been found during spontaneous seizures (Scharfman et al., 2002a) and interestingly, the number of ectopic cells is correlated with seizure frequency (McCloskey et al., 2006) suggesting that the maturation and integration of ectopic granule cells may contribute to the development recurrent seizures. When taken together, these findings suggest that the expanded population of functionally integrated ectopic granule cells in the epileptic hippocampus promotes hyperexcitability by establishing an excitatory loop between CA3 pyramidal cells and the DG.

At present, the molecular mechanisms underlying the formation of ectopic dentate granule cell or the pathological integration of new neurons after seizure activity are not well understood. However, there are two molecular systems that might be instrumental in this process. First, it has been shown that the glycoprotein reelin is necessary for the normal lamination of the brain during embryonic and perinatal development (D'Arcangelo et al., 1997; D'Arcangelo, 2006). Importantly, the loss of reelin contributes to abnormal migration of newly generated neurons in both the developing and adult hippocampus (Fatemi, 2005; Gong et al., 2007). Second, recent data have also suggested that disrupted-in-schizophrenia (DISC1), a gene which confers increased susceptibility for schizophrenia (Ishizuka et al., 2006), plays an important role regulating the integration of newly born granule cells in the adult hippocampus (Duan et al., 2007). These important observations provide a starting point to begin an investigation into the

potential molecular mechanisms that may be dysfunctional in the epileptic brain and contribute to aberrant neurogenesis.

7.4. Role of Reelin in Temporal Lobe Epilepsy

The glycoprotein reelin is a major secretatory protein with important roles in embryogenesis and adult life. The protein product of the reelin (*Reln*) gene has been identified as a major determinant of neuronal migration that also plays a significant role in cellular maturation and synaptic function. Although the cellular function of reelin in the adult brain is not well understood, a number of reports implicate reelin dysfunction in the etiology of several clinical disorders, including schizophrenia, bipolar disorder, major depression, and autism. Animals with a spontaneous mutation of the reelin gene show significant disruption in cortical lamination that includes abnormal positioning of neurons and aberrant orientation of neuronal cell bodies and fibers (Badea et al., 2007; Caviness, Jr., 1976; Caviness, Jr., 1982; Caviness, Jr. and Sidman, 1973; D'Arcangelo, 2005; Hamburgh, 1963; Landrieu and Goffinet, 1981; Mikoshiba et al., 1980). These mutants, called *reeler* mice, were originally described over 50 years ago (Falconer, 1951) and appear at a frequency expected for a recessive trait according to classical Mendelian inheritance patterns. They display behavioural abnormalities, such as ataxia, tremor, and reeler gaits, that can be explained in part by the maldevelopment of the inferior olivary complex and cerebellum (Blatt and Eisenman, 1985; Goffinet, 1983). Importantly, anatomical studies indicated that all major cortical structures of the brain were present, but appeared disorganized in the *reeler* mutant. By 1995, the gene responsible for producing the *reeler* phenotype was identified, and it was found that large deletions of

the distal region of murine chromosome 5 results in the loss of the extracellular reelin protein product (D'Arcangelo et al., 1995).

The murine *Reln* gene is composed of 65 exons spanning a region of approximately 450 kb (Fig. 1-7D). The encoded reelin protein is a large secreted protein of approximately 385 kDa (D'Arcangelo et al., 1995) that consists of an N-terminal region followed by eight internal repeats with each repeat containing a cysteine-rich sequence similar to epidermal growth-like motif (DeBergeyck et al., 1998), a feature that is common to extracellular proteins. The N-terminal contains the signal peptide and a small domain with modest similarity to F-spondin, a protein secreted by floor plate cells and promotes cell adhesion and neurite growth (Klar et al., 1992). The C-terminal region contains a stretch of positively charged amino acids (D'Arcangelo et al., 1997). The protein is glycosylated, increasing its molecular weight from the predicted 385 kDa to approximately 400 kDa, and it is secreted in the extracellular environment (D'Arcangelo et al., 1997). Secretion occurs through a constitutive pathway (Lacor et al., 2000), and requires the C-terminal region (D'Arcangelo et al., 1997). Thus, deletion of the C-terminus resulting from retroviral insertion is responsible for the lack of secreted, functional reelin and the appearance of the reelin null mutant *Reeler* (D'Arcangelo et al., 1997). Reelin is secreted as a full-length protein, but it is then subjected to proteolytic cleavage, which produces three major fragments: an N-terminal fragment of approximately 180 kDa (N terminus to repeats 1 and 2), a central fragment of approximately 120 kDa (repeats 3 to 6), and a C-terminal fragment of approximately 100 kDa (repeat 7 and 8 to C terminus) (Jossin et al., 2003). Functional studies have demonstrated that the central fragment of reelin is sufficient to confer biological and

biochemical activity (D'Arcangelo, 2006). However, the full-length protein appears to be more active than the central fragment alone.

The reelin protein is present in all vertebrates, but absent in invertebrates and plants (Lambert de Rouvroit et al., 1999). To date, homologous reelin sequences have been identified and their expression has been characterized in the CNS of developing and adult rodents and primates (Martinez-Cerdeno and Clasca, 2002; Rodriguez et al., 2002). In addition, *reelin* expression has also been documented in the CNS of a number of other vertebrate species including adult ferret, zebra fish, sea lamprey, and embryonic chicken, crocodile, lizard, and turtle (Bernier et al., 1999; Bernier et al., 2000; Candal et al., 2005; Costagli et al., 2002; Martinez-Cerdeno et al., 2003; Perez-Costas et al., 2004; Perez-Garcia et al., 2001; Tissir et al., 2003). Soon after identification of the murine gene, the human *Reln* gene was isolated and shown to reside on chromosome 7q22 (DeSilva et al., 1997). The human gene shares an 87.2% sequence homology with the *Reln* mouse gene, and encodes a protein that is 94.2% identical to the mouse protein at the amino acid level, strongly suggesting a conserved function (DeSilva et al., 1997). A point mutation of this gene in humans results in lissencephaly, a condition characterized by diffuse pachygyria, hippocampal dysplasia, and a profoundly hypoplastic cerebellum and brainstem, with a nearly complete absence of cerebellar folia (Chang et al., 2007). The condition is marked by significant developmental delay and mental retardation, severe ataxia, and pharmacoresistant seizures.

Reelin signal transduction involves binding to the apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR), followed by intracellular activation of the cytoplasmic adapter protein disabled-1 (Dab1) (Fig. 1-7E). Other

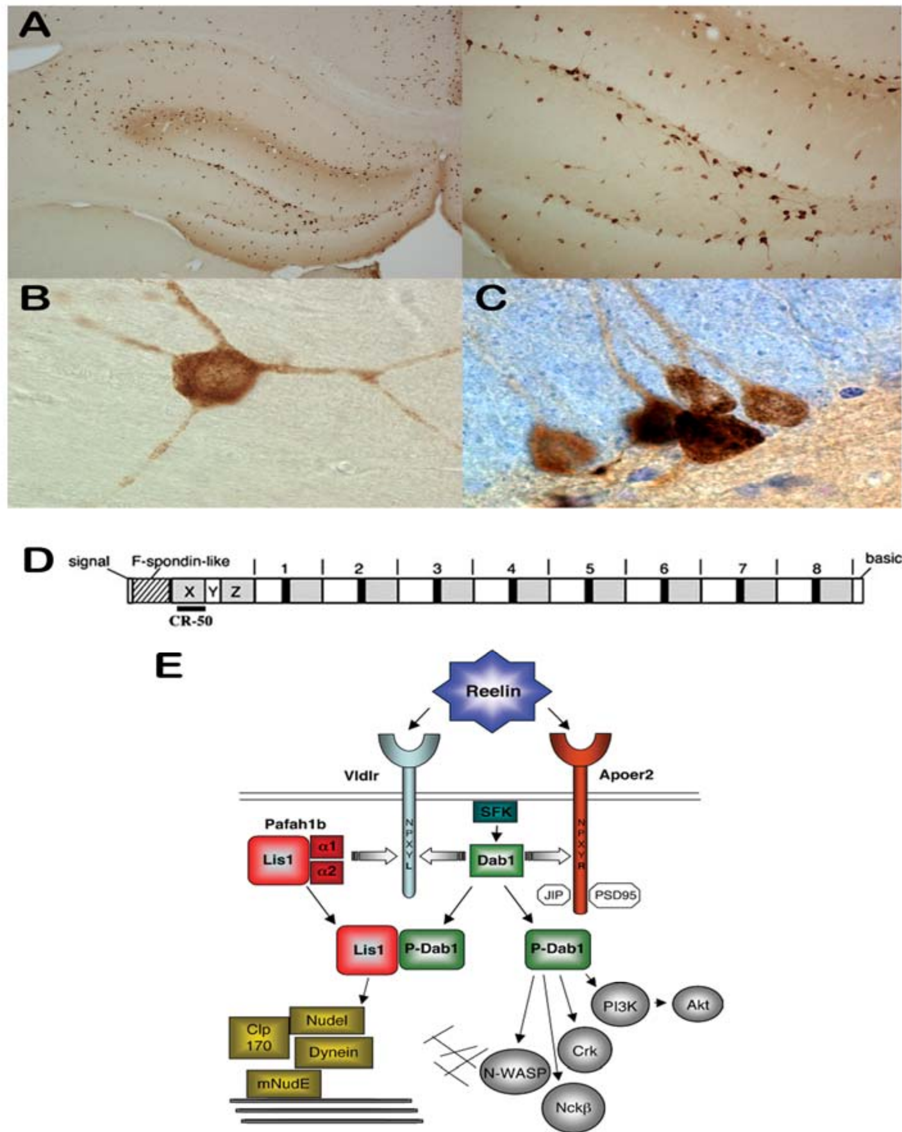


Figure 1-7 Reelin Expression in the Adult Hippocampus. A) Photomicrographs showing the distribution of reelin neurons in the adult hippocampus. B) and C) are examples of reelin neurons in the hilus and dentate SGZ. Note: the reelin cells in the dentate SGZ (C) often form clusters with a visibly stained apical dendrite protruding through granule cell layer and terminating in the inner molecular layer. D) Schematic representation of the domain arrange in reelin primary structure. (see text for further description). E) Integrated model of Reelin and Lis1 signaling. Reelin binds to VLDLR and ApoER2 and causes src-family kinase (SFK) activation and Dab1 phosphorylation. Dab1 binds to the NPxY motif of both, VLDLR and ApoER2. Upon Reelin stimulation, phosphoDab1 (P-Dab1) interacts with Lis1 and with other signal transduction molecules (grey circles). Lis1 also binds the catalytic subunits of the Pafah1b complex (α1 and α2) as well as components of the cytoplasmic dynein complex (yellow rectangle). α1 and α2 also bind VLDLR at the NPxYL motif and compete with Dab1 for receptor occupancy. A unique domain of ApoER2 enables unique interactions with synaptic and trafficking proteins (white octagons). The binding of catalytic Pafah1b subunits to VLDLR displaces P-Dab1 and promote its interaction with Lis1. Signaling molecules downstream of Lis1 and Dab1 affect cytoskeleton dynamics by acting on microtubules (thick lines) or actin filaments (thin lines), thereby controlling neuronal migration and layer formation.

possible reelin signal transduction pathways involve interaction with the $\alpha_3\beta_1$ integrin and cadherin-related neuronal receptors. The binding of reelin to its receptor, specifically ApoER2 and VLDLR, induces clustering of these receptors and oligomerization of Dab1. Receptor clustering is necessary for activating the Src family tyrosine kinases (SFKs) family/Fyn-kinase which in turn leads to phosphorylation of Dab1. A considerable number of biochemical studies have demonstrated that phospho- Dab1 interacts with a variety of signaling proteins, some potentially important for modulating cytoskeletal dynamics, thereby inducing changes in migration, synaptogenesis, and synaptic plasticity.

Reelin has pleiotropic actions in the brain: that is, the reelin gene controls critical aspects of brain development by orchestrating neuronal positioning during migration, but it also plays a role in regulating GABAergic transmission, dendritic spine morphology, and synaptic plasticity in postmigratory neurons of the mature brain (Costa et al., 2002; Costa et al., 2004; Guidotti et al., 2005). During embryonic development, reelin is selectively expressed by early-generated Cajal-Retzius (CR) cells populating the marginal zone of the cortex and hippocampus, and in the cerebellum by cells in the external granule cell layer (Del Rio et al., 1997; Derer et al., 2001; Frotscher et al., 2001; Senzaki et al., 1999). Importantly, during early postnatal stages and after neuronal migration has been completed, the vast majority of CR cells disappear by cell death, although a subpopulation of CR-like cells that are calretinin positive may persist in the stratum lacunosum-moleculare throughout adulthood (Coulin et al., 2001). For most adult mammalian species, reelin is synthesized and secreted predominately by specific GABAergic interneurons in the cortex and hippocampus of adult rodents and primates (Guidotti et al., 2000; Pesold et al., 1998). Dab1 is consistently expressed by the main

excitatory neurons that are primary targets of reelin-positive interneurons. Detailed cellular and ultrastructural studies have shown reelin immunolabeling in neuronal somata, axons, and dendritic spines, as well as extracellular matrix labeling. In addition, high resolution electron microscopy has demonstrated that reelin can also be present in glial cells, albeit at very low levels compared with its presence in neurons (Roberts et al., 2005).

Reelin is highly expressed in both the developing and adult hippocampus and EC. In the adult EC, reelin is present in stellate cells that form clusters in layer II (Alcantara et al., 1998). These neurons are important, insofar as they give rise to the major source of excitatory synaptic input to the DG. In the hippocampus, high levels of reelin persist into adulthood, and several reelin immunopositive neurons can be identified in all hippocampal subregions (Fig. 1-7A-B). However, pyramidal cells of the CA1-CA3 regions are always devoid of reelin labeling. Several reelin immunoreactive multipolar and bipolar interneurons are found along the border of the stratum radiatum and stratum lacunosum-moleculare, as well as in the deep part of the stratum oriens. Additional cells are scattered in the stratum lucidum and radiatum and sometimes within the stratum pyramidal but often at a lower number. In the DG, several populations of interneurons are immunostained; however, there is no labeling of dentate granule cells. A large population of basket cells is heavily stained along the dentate SGZ and hilar region. These cells typically have solid staining throughout the soma with primary and secondary dendrites clearly labeled and ramifying throughout the dentate GCL into the molecular layer. Double immunolabeling studies have revealed that over 95% of reelin expressing neurons

in the hippocampus and DG co-localize with the inhibitory neurotransmitter GABA (Alcantara et al., 1998).

Given that neurodevelopmental anomalies are frequently found in patients with TLE, many researchers have suggested that alterations in reelin expression may contribute to these pathogenic conditions. For instance, many patients with mesial TLE show dispersion of the dentate GCL with dentate granule cells extending into the molecular layer (Fig. 1-8) (Houser, 1990; Lurton et al., 1998). As a result, the outer border of the GCL is less sharply defined in specimens with hippocampal sclerosis. Granule cell dispersion (GCD) is typically observed in about half of hippocampal tissue specimens from patients surgically treated for intractable TLE (Houser, 1990) and has been shown to be associated with an early seizure onset (Houser et al., 1992; Sagar and Oxbury, 1987). It has been recently proposed that reelin deficiency might contribute to the development of altered granule cell lamination and the cytoarchitectural abnormalities contributing to GCD (Haas et al., 2002; Heinrich et al., 2006).

7.5. Role of DISC1 in Temporal Lobe Epilepsy

The disrupted-in-schizophrenia 1 (DISC1) gene is a 414.3 kb gene located on the chromosomal region 1q42.2, and consists of 13 exons (Austin et al., 2003; Ishizuka et al., 2006; Millar et al., 2004). DISC1 was originally identified as a candidate gene for schizophrenia in a large Scottish family, in which a balanced translocation involving chromosome 1 and 11 t(1;11) was strongly linked with schizophrenia, bipolar disorder, and recurrent depression (Millar et al., 2000; Millar et al., 2001). The majority of family members with the translocation have one of these diagnoses, whereas family members without the translocation do not show evidence of psychiatric illness. The Scottish

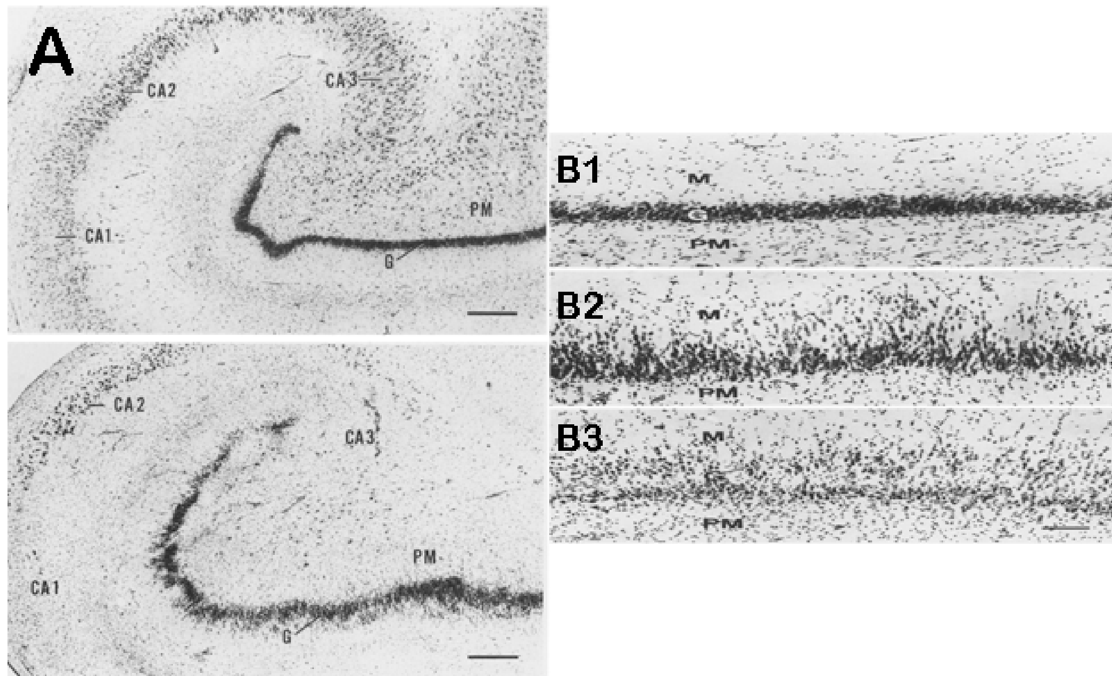


Figure 1-8 Dentate granule cell layer dispersion. A) Cresyl violet sections from the hippocampal formation of control and temporal lobe epileptic patients. In the control specimen (top) the granule cell layer (G) is relatively narrow, and the cell bodies are closely approximated. In the epilepsy specimen (bottom), the granule cell layer (G) is wider and appears disorganized due to dispersion of many of the granule cells. Marked cell loss is evident in the polymorph region (PM), CA1, and CA3 fields. B) The granule cell somata form a highly organized lamina that has distinct borders with the molecular and polymorphic region (B1; control specimen). In contrast, an example from a patient with refractory temporal lobe epilepsy shows that granule cell somata are dispersed, and many extend into the molecular layer (M). Thus, the outer border of the granule cell layer (G) is quite irregular (B2: epilepsy specimen). Interestingly, dispersed granule cells can form a bilaminar pattern (G) with a relatively neuron-free zone between the two layers (B3: epilepsy specimen). Adapted from: (Houser, 1990)

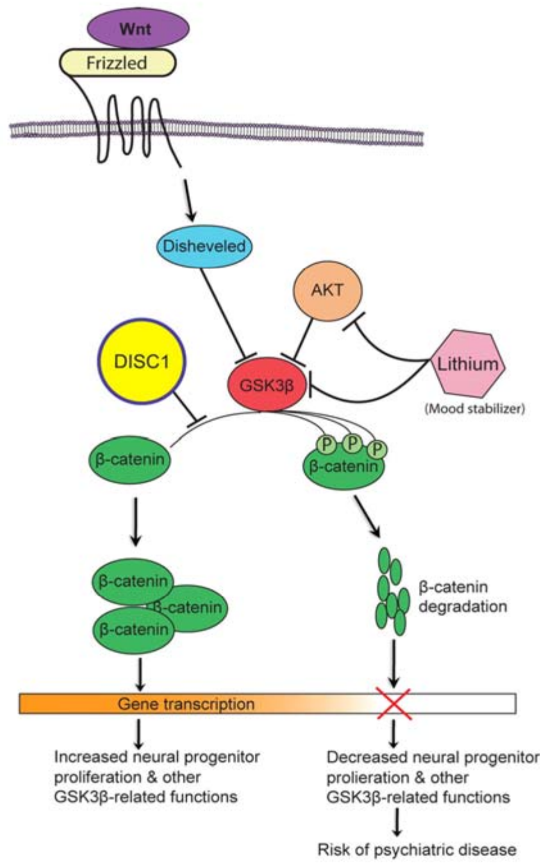
translocation occurs between exons 8 and 9, interrupting the coding sequence of the DISC1 gene, which leads to loss of the C-terminal 257 amino acids of the protein. A second gene, disrupted-in-schizophrenia 2 (DISC2), was also associated with the breakpoint. However, it appears that DISC1 is the only disrupted gene that has an open reading frame or protein coding potential; DISC2 is an untranslated gene with no ostensible function. Subsequent association studies identified numerous polymorphisms in the DISC1 gene associated with schizophrenia and affective disorders, although different polymorphisms/haplotypes in various regions of the gene were implicated in these studies (Hashimoto et al., 2006; Hodgkinson et al., 2004; Nakata et al., 2009; Takahashi et al., 2009).

DISC1 transcript(s) are expressed in many adult and fetal tissues including the brain, placenta, testis, heart, and kidneys (Ma et al., 2002; Millar et al., 2000). In both the embryonic and adult mouse brain, immunohistochemistry and *in situ* hybridization studies have detected widespread DISC1 expression in multiple regions with the highest expression occurring in the DG, accompanied by lower expression in CA1-CA3 hippocampal subfields, cerebral cortex, cerebellum, and olfactory bulbs (Austin et al., 2003; Meyer and Morris, 2008; Rastogi et al., 2009; Schurov et al., 2004). Very low or absent expression has been reported in the paraventricular and arcuate nuclei of the hypothalamus, as well as in the amygdala. Finally, subceullar distribution of DISC1 has been analyzed in great detail (Brandon et al., 2004; James et al., 2004; Miyoshi et al., 2004; Morris et al., 2003; Ozeki et al., 2003; Sawamura et al., 2005). Ultrastructural DISC1 expression is localized to the nucleus, dendritic spines and postsynaptic density, as well as axonal terminals of neurons. DISC1 markers have also been colocalized with

markers of the mitochondria and centrosome. In addition, cytoplasmic localization of DISC1 closely overlaps with that of F-actin, α-tubulin, microtubule associated protein 2, and gelsolin supporting the interaction between DISC1 and the cytoskeleton (Ishizuka et al., 2006; Jaaro-Peled et al., 2009; Kamiya et al., 2006; Morris et al., 2003). It is clear from these studies that DISC1 is localized to many subcellular compartments suggesting that it may have multiple roles in various locations throughout the cell.

Functional studies have shown that DISC1 is critically involved in several important cellular processes, including 1) regulating intracellular signaling by interacting with several key protein partners (such as phosophodisesterase 4B) (Hashimoto et al., 2006; Millar et al., 2005; Morris et al., 2003; Schosser et al., 2009), 2) directing axon guidance and neurite extension (Miyoshi et al., 2003; Ozeki et al., 2003), 3) forming a complex with nuclear distribution gene E homolog-like 1 (Nudel/NDEL1) and lissencephaly 1 (Lis1) to regulate nuclear attachment to the centrosome and promotion of radial migration in the developing brain (Brandon et al., 2004; Kamiya et al., 2006; Morris et al., 2003; Ozeki et al., 2003), and 4) regulating neural progenitor proliferation through modulation of β-catenin and GSK-3β (Fig. 1-9) (Mao et al., 2009). During embryonic development, DISC1 disruption greatly limits radial migration of neuroblasts toward their cortical destinations and decreases the complexity of dendritic branching and neuritic sprouting (Brandon et al., 2009; Hashimoto et al., 2006; Jaaro-Peled et al., 2009; Kamiya et al., 2006; Kvajo et al., 2008; Meyer and Morris, 2009). Consistent with these findings, DISC1 disruption has been linked to impaired hippocampal structure and function in both schizophrenic patients and healthy carriers (Arnold, 1999; Callicott et al., 2005; Hodgkinson et al., 2004; Ni Dhuill et al., 1999), and transgenic mice expressing

A



B

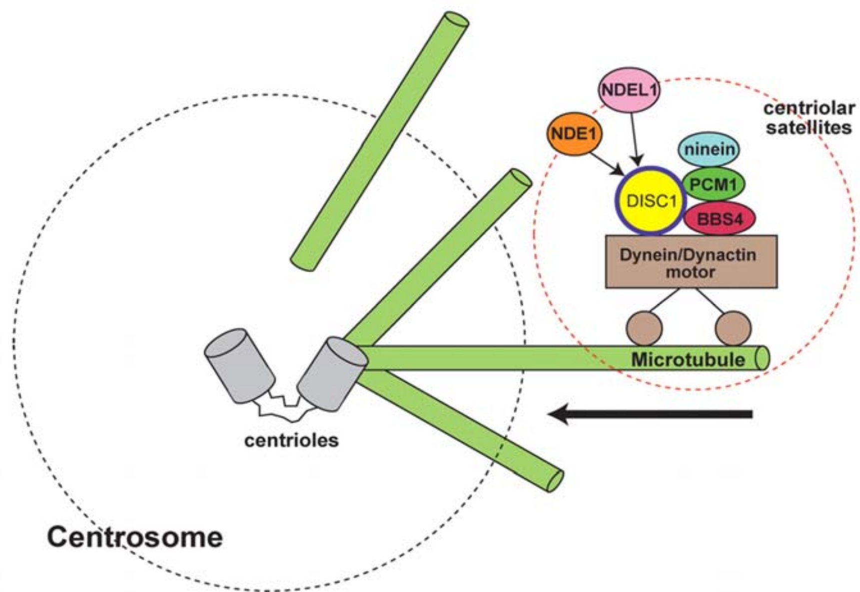


Figure 1-9 Proposed mechanism illustrating how DISC1 regulates the proliferation and migration of adult-born dentate granule cells. A) DISC1 regulates neurogenesis via Wnt/β-catenin signaling. During canonical Wnt signaling, β-catenin levels are kept low in the cytosol due to GSK3β-mediated phosphorylation which targets β-catenin for degradation. In the presence of Wnt ligands, the receptor complex and downstream signaling machinery is engaged, leading to increased accumulation of β-catenin and transcription of Wnt-dependent genes. In embryonic and adult neuronal progenitor cells, DISC1 directly binds and inhibits the function of GSK3β, thereby increasing cytosolic β-catenin concentration and functioning as a positive regulator of Wnt signaling. B) DISC1 at the centrosome. DISC1 binds to a number of proteins localized to the centrosome including nudE nuclear distribution gene E homolog-like 1 (NDEL1), pericentriolar material 1 (PCM1), and Bardet-Biedl syndrome proteins. DISC1 play a role in anchoring these molecules in association with the dynein motor complex and centrosome, and hence regulating microtubule organization. Through influence microtubule networks, DISC1 can play a significant role in orchestrating neuronal migration as well as dendritic and axonal development. Adapted from: (Brandon et al., 2009)

a mutated form of DISC1 display functional and anatomical hippocampal abnormalities (Shen et al., 2008). Interestingly, DISC1 expression appears to be developmentally regulated in the hippocampus with peaks in protein expression occurring at embryonic day 13.5 and postnatal day 35, when active neurogenesis occurs in the mouse brain (Schurov et al., 2004).

The functional role of DISC1 in the adult brain has received only limited study. Using single-cell RNA interference strategies to selectively knockdown DISC1 expression in differentiating hippocampal precursor cells of the adult DG *in vivo*, Duan and colleagues (Duan et al., 2007) showed that DISC1 regulates the development and integration of adult-born neurons. For example, downregulation of DISC1 accelerated the morphological development of new neurons, resulting in somatic hypertrophy and enhanced dendritic outgrowth. However, these cells also showed pathological features, such as the maintained appearance of basal dendrites. Moreover, inhibition of DISC1 functioning leads to ectopic migration of new neurons suggesting that DISC1 serves as an interpreter that relays positional signals to the intracellular migratory machinery of the cell. And last, new neurons with DISC1 knockdown exhibit more mature neuronal firing patterns and accelerated synapse formation suggesting that DISC1 deficiency is associated with enhanced integration of adult-born neurons.

The findings described above support the idea that DISC1 serves as a key regulator of adult neurogenesis. In addition, these findings are particularly exciting because they are remarkably similar to the effects of seizures on new neuron development and integration. For example, prolonged seizures lead to inappropriate migration, development, and integration of new neurons in the adult hippocampus.

Therefore, activity-driven changes DISC1 expression may serve as a critical molecular mechanism underlying aberrant neurogenesis in the epileptic brain. However, this specific question has not been directly tested or studied experimentally.

7.6. Role of Aberrant Neurogenesis in the Memory Deficits Associated With Temporal Lobe Epilepsy?

Previous studies have shown that although the rate of neurogenesis is higher during the early period of epilepsy development, this rate declines substantially in animals exhibiting chronic TLE (Hattiangady et al., 2004; Heinrich et al., 2006). Part of the decline in the neuronal differentiation of newly born cells could be attributed to inference in neuronal fate choice with a greater proportion of new cells becoming glia (Kralic et al., 2005; Kuruba et al., 2008). These findings have also been observed in hippocampal tissues resected from human TLE patients (Fahrner et al., 2007; Siebzehnrubl and Blumcke, 2008).

It has been hypothesized that maintenance of hippocampal-dependent learning and the formation of long-term episodic memories requires the continual addition of new and functional dentate granule cells in the GCL circuitry (Jessberger et al., 2007; Kempermann, 2002; Shors et al., 2002; Toni et al., 2008). Indeed, clinical studies have shown that the most frequently reported cognitive complaint in adults with epilepsy is memory difficulty. A community-based survey of over 1000 patients in the United States revealed that poor memory was listed among the top cognitive problems of potential concern for people with epilepsy (Fisher, 2000). In another study, 54% of epileptic patients regarded memory problems as a moderate to severe nuisance (Corcoran and

Thompson, 1992). In addition to diminished neurogenesis, aberrantly integrated neurons, such as ectopically positioned granule cells, may also affect hippocampal network activity and thus impact normal learning and memory function (Scharfman and Gray, 2007). Therefore, it is possible that the cognitive impairments observed in some patients with refractory epilepsy may be at least partially linked to aberrant or diminished hippocampal neurogenesis.

8. Specific Aims and Goals

The specific aim of this dissertation is to examine the effect of amygdaloid kindling on hippocampal structural plasticity and functional neurogenesis. To address this issue, several questions were asked:

Question 1: What effect does amygdaloid kindling have on structural plasticity and synaptic reorganization within the hippocampus and beyond? To examine this question, we evaluated the effect of kindling on synapsin I expression, a marker of structural plasticity, in a variety of brain regions (e.g., hippocampus proper, dentate gyrus, amygdala, striatum, piriform, and sensorimotor cortices) after kindling. Because there has been limited study of the effects of repeated seizure activity on synaptic growth and plasticity in male and female rats, I decided to determine whether kindling would differentially affect synapsin I expression in male and female rats. In order to determine, if kindling-induced changes in synaptic growth are associated with alterations in emotional behaviour, the level of synapsin I for a variety of different brain regions was correlated with several different measures of fearful behavior in the open-field.

Hypothesis: I expect to find that kindling increases synapsin I expression in the amygdala and hippocampus in both male and female rats. In addition, because circulating ovarian hormones are known to exert neuroprotective effects, we expect that the female kindled rats would show greater variability in the effects of kindling on synapsin I expression in these regions. Finally, based on previous work, we expected that alterations in synapsin I expression specifically within the hippocampus and dentate gyrus would be associated with the largest change in emotional behaviour after kindling. **(Chapter 2)**.

Question 2: Does long-term kindling produce changes in hippocampal neurogenesis that are similar to those observed in other animal models associated with more severe seizures? If so, what are the potential molecular changes that could mediate aberrant neurogenesis after kindling? To begin addressing these questions, I evaluated the effects of both short-term and long-term amygdala kindling on hippocampal neurogenesis. Because reelin is important in regulating migration in both the developing and adult brain, we also examined whether kindling directly affected reelin expression in the DG.

Hypothesis: I predict that long-term kindling would be associated with a significantly greater number of mispositioned granule cells in the dentate hilus in comparison to non-kindled controls. In addition, I expectthat reelin expression will be decreased in the dentate GCL and SGZ after long-term kindling. Finally, I expect that many newly generated granule cells will show aberrant dendritic outgrowth and structural abnormalities, such as basal dendrites, after long-term kindling **(Chapter 3)**.

Question 3: Because of the abundant expression of DISC1 within dentate granule cells and the evidence that decreases in DISC1 is associated with aberrant structural changes in new neurons that resemble the effects of seizures, I then asked the question: Does the repeated induction of seizure activity alter DISC1 expression? In addition, do deficiencies in DISC1 expression contribute to cytopathological changes in the dentate, such as dentate granule cell layer dispersion or ectopic cell migration, which are commonly found in patients with TLE?

Hypothesis: I predict that long-term kindling will be associated with decreased DISC1 expression in the dentate gyrus. The corresponding decrease in DISC1 expression will be associated with the presence of hilar ectopic granule-like cells given that DISC1 is important in mediating intact cell migration **(Chapters 3 and 4)**.

Question 4: Are new neurons born after epileptic seizures capable of functionally integrating into behavioural circuits that are important for learning and memory? To address this, long-term kindled rats were injected with BrdU at a point when kindled seizures significantly up-regulate hippocampal cell proliferation (i.e., around 30 electrical stimulations). Importantly, kindling stimulations were continued after labeling until the delivery of 99 stimulations. Animals were then trained on a hippocampal dependent learning task (trace fear conditioning) and sacrificed two hours after behavioural testing (i.e., context testing) in order to examine the expression of the immediate early gene product c-fos, which is a marker of neuronal activation. I then set out to examine whether new cells generated earlier in the process of kindling would show functional activation during the testing of memory recall.

Hypothesis: Because new neurons display a critical period of enhanced plasticity for approximately 4 to 6 weeks after birth, these young neurons would be at a point in their maturational development when they should respond to behavioural experiences more effectively in comparison to mature granule cells. However, because seizures also influence the morphological and functional development of new neurons, these cells may be intrinsically abnormal and unfit to properly integrate into the hippocampal circuits. Consequently, I predict that new cells born after seizures will not respond properly during their critical period due to the continual bombardment of epileptiform stimulation and thus produce faulty pathways that prevent their activation during behavioural learning. The consequence of this will be impaired learning of a trace fear conditioning paradigm after long-term kindling **(Chapter 5)**.

CHAPTER 2

Altered Synapsin I Immunoreactivity and Fear Behavior in Male and Female Rats Subjected To Long-term Amygdala Kindling

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1. Introduction

Patients with temporal lobe epilepsy often experience behavioral co-morbidities such as fear, anxiety, and depression (Gilliam et al., 2004; Gloor et al., 1982; Strauss et al., 1982). These behavioral co-morbidities tend to occur during the interictal gaps between individual seizures, so they are not linked to seizure activity per se. However, they do appear to worsen in patients with increased seizure frequency, poorer seizure control, and a longer duration of epilepsy (Johnson et al., 2004). Gaining an understanding of the neural mechanisms by which temporal lobe seizures can produce these problematic changes in emotional behavior has been difficult, largely due to disease and treatment heterogeneity within patient populations. To overcome these problems, many researchers have turned to animal models.

Kindling is an animal model that is particularly useful for studying the fear behavior that is co-morbid with temporal lobe epilepsy (Adamec, 1990b; Kalynchuk, 2000). Kindling refers to the gradual development and intensification of elicited motor seizures that result from daily administration of electrical brain stimulation (Goddard, 1967). In addition to its epileptogenic effect, kindling also produces changes in behavior that are similar in many respects to the fear and anxiety observed in patients with temporal lobe epilepsy (Kalynchuk, 2000). For example, kindled rats show increased fear on several different behavioral tasks, including the open field, the elevated plus maze, the social interaction test, and the resident-intruder paradigm (Helfer et al., 1996; Kalynchuk et al., 1997; Murphy and Burnham, 2003). Several aspects of this kindling-induced fear are notable. First, the fear behavior produced by kindling is most pronounced with amygdala kindling, but it is also produced by hippocampal and perirhinal cortex kindling (Hannesson et al., 2005; Kalynchuk et al., 1998a). Second, the fear behavior produced by

kindling is greatest in the first few days after the final stimulation, and although it does dissipate somewhat over time, it also remains significantly elevated above control levels for several weeks (Hannesson et al., 2005; Kalynchuk et al., 1998b). Third, the fear behavior produced by kindling occurs in both male and female rats, but female rats tend to show more active fear responses and male rats tend to show more passive fear responses (Wintink et al., 2003). And finally, the fear behavior produced by kindling is more reliable and robust after long-term kindling to about 100 stimulations than it is after partial or short-term kindling (i.e., between 5-25 stimulations) (Helfer et al., 1996; Kalynchuk et al., 1997). Therefore, kindling produces the greatest increases in fear after long-term kindling of the amygdala in male and female rats tested within a few days of the final stimulation. Our most recent work has focused on identifying the neural correlates of these kindling and behavioral testing parameters in order to increase our understanding of the neural mechanisms by which temporal lobe seizures can increase fear behavior.

The specific purpose of this experiment was to determine whether kindled fear in male and female rats is related to synapsin I levels within various temporal lobe brain regions. Synapsin I belongs to a family of phosphoproteins that cross-links synaptic vesicles to cytoskeletal elements within the presynaptic terminal (Greengard et al., 1993). It plays an important role in synaptic development (Sanchez-Islas and Leon-Olea, 2004), synaptogenesis (Lu et al., 1996), and the regulation of neurotransmitter release (Chi et al., 2003). These observations suggest that synapsin I could act as a molecular marker of synaptic growth and plasticity in neuronal networks that maintain high levels of activity, such as those undergoing epileptogenesis (Chi et al., 2003). It is well known that

recurrent temporal lobe seizures evoke profound hippocampal synaptic reorganization (Sutula and Dudek, 2007). The continued increase in synaptic reorganization with an extended number of seizures could help to explain the association between seizure frequency/duration of epilepsy and the severity of behavioral co-morbidities. Therefore, we would expect to see a positive relationship between increased synapsin I expression and fear behavior in long-term kindled rats. Previous experiments have shown that short-term amygdala kindling increases hippocampal synapsin I mRNA and protein expression in male rats (Morimoto et al., 1998a; Suemaru et al., 2000). However, it is not known whether long-term amygdala kindling similarly increases hippocampal synapsin I expression in either male and female rats or whether this effect is related to kindled fear. There are also no data on whether kindling-induced alterations in synapsin I extend beyond the hippocampus. Accordingly, we examined the effect of long-term kindling of the left amygdala on fearful behavior and synapsin I levels in the hippocampus, amygdala, piriform cortex, sensorimotor cortex and caudate/putamen located both ipsilateral and contralateral to the site of stimulation in both male and female rats.

2. Materials and Methods

2.1. Subjects

A total of 36 adult (18 male and 18 female) Long-Evans rats were obtained from Charles River (Quebec, Canada). They were individually housed in rectangular polypropylene cages with wood shavings as bedding. Food and water were freely available throughout the duration of the experiment. The rats were maintained on a 12:12 h light:dark cycle with lights on at 0800 h. Ambient temperature was maintained

between 20 and 21ºC. All animals were treated in accordance with the guidelines of the Canadian Council on Animal Care.

2.2. Surgery

Rats were handled once daily for approximately 1 week prior to surgery. At the time of surgery, all rats weighed between 275 and 300 g. Each rat was anesthetized with sodium pentobarbital (Somnotol; 65 mg/kg; i.p.) and an incision was made down the scalp so that the overlying skin could be retracted. The fascia was then incised to expose the skull. A single bipolar electrode (MS-303-2, Plastics One, Roanoke, VA) was implanted stereotaxically into the left basolateral amygdala at the following coordinates: -2.8 mm posterior to bregma, 5.0 mm medial/lateral, and 8.5 mm ventral from the surface of the cranium, with the incisor bar set at -3.3 mm (Paxinos and Watson, 1998). The electrode was secured to the skull with four stainless steel screws and dental acrylic. A topical antibacterial ointment (Flamazine, 1% silver sulfadiozine) was applied around the incision site in order to reduce the risk for infection. After surgery, animals were kept warm until they were ambulatory.

2.3. Kindling

After a 10-day postoperative recovery period, male and female rats were randomly assigned to either a kindled group or a sham-stimulated group. The groups were: male-kindled (n = 9), male-sham (n = 9), female-kindled (n = 9), and female-sham (n = 9). All kindled rats received a total of 99 stimulations. Three stimulations were delivered each day (5 days per week) with a minimum of 3 hours between consecutive stimulations. Prior to each stimulation, the rat was placed in a plastic container containing

a thin layer of commercial bedding, the wire lead was attached and the stimulation was delivered. Each stimulation comprised a 1 s, 60 Hz train of square-wave pulses. Each pulse had a biphasic amplitude of 800 μA peak-to-peak and a duration of 1 ms. This stimulation protocol has been shown to be well above the threshold for evoking epileptiform afterdischarges in the amygdala (Racine, 1972a). After all convulsive activity had ceased, the rat was returned to its home cage. Rats receiving sham stimulations were treated in exactly the same manner except that no current was delivered.

The measure of seizure severity was the convulsion class elicited by each stimulation. Convulsion class was scored according to Pinel and Rover's (1978) extension of Racine's (1972b) widely used five-class scale. Class 0 convulsions were classified as an arrest in behavioral mobility. Class 1 convulsions were characterized by orofacial automatisms. Class 2 convulsions were defined as orofacial automatisms and repetitive head nodding. Class 3 convulsions comprised unilateral forelimb clonus and mastication with salivation. Class 4 convulsions involved generalized convulsions with rearing and forelimb clonus. Class 5 convulsions involved rearing with rapid bilateral forelimb clonus followed by a loss of equilibrium. Class 6 convulsions were associated with bouts of multiple class 5 convulsions. Class 7 convulsions were comprised of running fits and jumping. And finally, Class 8 convulsions were associated with any of the preceding symptoms with intermittent periods of tonus.

2.4. Behavioral testing

All behavioral testing took place in a small, brightly lit testing room. An open field was located in the center of the room. The open field was a 70 cm long x 70 cm

wide x 40 cm high wooden box with no top and 36 identical squares defined by tape on the Plexiglas floor. For the purpose of measuring thigmotaxis, the squares were considered to belong to two groups: the 20 squares adjacent to the walls of the box (i.e., the peripheral squares) and the 16 squares in the central region of the box (i.e., the central squares). One day after the final kindling stimulation, each rat was placed individually into the unfamiliar open field for 5 min. To analyze locomotor activity and thigmotaxis, the number of lines crossed during each successive minute (five in total) in the open field was recorded and the percentage of time spent in the periphery of the open field was calculated. A rat received credit for 1 line cross when the center of its back crossed over from one square and into an adjacent square. The open field was thoroughly cleaned between rats in order to minimize potential olfactory cues.

At the end of the 5 min, each rat's duration of fleeing and resistance to being captured from the open field was recorded. An experimenter, who was naïve to the experimental history of each rat, attempted to pick up the rat from above while wearing a leather glove. The rat's resistance to being picked up was scored according to the following scale (Albert and Richmond, 1976): 0 = easy to pick up, 1 = vocalizes or shies away from experimenter's hand, 2 = shies away from hand and vocalizes, 3 = runs away from hand, 4 = runs away and vocalizes, 5 = bites or attempts to bite, and 6 = launches a jump attack.

2.5. Histology and synapsin I immunohistochemistry

Four hours after the behavioral testing, each rat was given an overdose of sodium pentobarbital and transcardially perfused with normal saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS: 0.1 M, pH 7.4). The brain was

quickly removed, post-fixed in the same fixative for 48 h, and then cryoprotected in 30% sucrose (pH 7.4). Once the brains lost buoyancy in the sucrose solution (i.e., after about 3 days), they were sectioned coronally on a cryostat at 40 μm. For long-term maintenance, the brain sections were stored in 0.1 M PBS/0.1% sodium azide at 4 °C until needed.

Free-floating tissue sections (1 in 12 series) were processed for synapsin I immunoreactivity using the aviditin-biotin-peroxidase method (protocol modified from (Suemaru et al., 2000). In order to minimize variability in immunoreactivity, tissue from all animals was stained simultaneously. All washes and incubations were carried out under gentle agitation. To minimize endogenous peroxidase activity, the sections were washed in 0.1 M PBS (pH 7.4) and then incubated in 0.06% hydrogen peroxide for 10 min. The sections were then washed several times in 0.1 M PBSX (with 0.3% Triton X-100; pH 7.4) and finally incubated for 7 days at 4 °C with a primary rabbit polyclonal antibody raised against the synapsin I protein (1:10000 in PBSX; Calbiochem, USA). Following incubation, the sections were incubated for 2 h with a biotinylated anti-rabbit IgG antibody (1:1000 in PBSX; Vector Labs, USA) and finally incubated in an aviditin-biotin complex (1:500; PK-6101; Vectastain ABC kit, Vector Labs, USA) for an additional 2 h at room temperature. Peroxidase activity was visualized using 3,3'-diaminobenzidine and hydrogen peroxide in PBS. After sufficient coloration, the reaction was halted by washing in PBS several times. The sections were then mounted onto glass slides and left to air dry overnight. Slides were dehydrated through a series of alcohols, cleared in xylene, and coverslipped with Entellan mounting medium. The specificity of the antibody was determined by running the immunostaining protocol in the absence of the primary antibody. No staining was observed for any of these sections.

To determine if the electrode placement in each animal was correct, a second adjacent series of tissue was counterstained with 0.1% cresyl violet. For each animal, the electrode placement was verified using the Paxinos and Watson (1998) stereotaxic atlas. Any animal with an incorrectly positioned electrode was removed from the study.

2.6. Densitometric analyses of synapsin I immunoreactivity

Synapsin I immunoreactivity was quantified under gray-scale using a computer-based image-analysis system (ImageJ 1.37, National Institute of Health, Bethesda, MD). Sections were captured using a digital camera (Photometrics Coolsnap) that was attached to a light microscope (Nikon Eclipse E800, 10X objective). We quantified synapsin I immunoreactivity within demarcated laminar boundaries of the CA3 (stratum oriens, so; stratum radiatum, sr; stratum lucidum, sl; stratum pyramidal, sp) and the CA1 (so, sr, sp) subfields of the hippocampus, as well as the molecular layer of the dentate gyrus (stratum moleculare) and the hilus. We chose these regions based on previous studies reporting differences in hippocampal synapsin I expression after seizure activity (Morimoto et al., 1998a; Suemaru et al., 2000). In addition, we also quantified synapsin I levels in the piriform cortex, basolateral amygdala, central amygdala, sensorimotor cortex, and caudate/putamen based on previous reports of altered neuroplasticity in these brain regions after kindling (Burazin and Gundlach, 1996; McIntyre and Plant, 1989; Okada et al., 1993; Uno and Ozawa, 1991; van Rooyen et al., 2006). All brain regions were analyzed bilaterally, with separate measures taken for the ipsilateral and contralateral sides. The mean relative optical density for each quantified brain region was calculated from at least three adjacent coronal sections (~480 μm apart) and standardized between

white (0) and black (255). Background staining was controlled by calculating the average optical density levels from the corpus callosum and subtracting these values from the areas of interest.

To ensure that our measurement of synapsin I optical density reflected the number of synapsin I-positive terminals found within that brain region, we used an analysis previously used by Li and colleagues (2002). The number of synapsin I immunoreactive terminals was manually counted within a rectangular open cursor of a 500 μm2 area for CA3 stratum oriens and a rectangular open cursor of a 400 μm2 area for CA3 stratum lucidum/radiatum in 6 randomly chosen animals (3 sham-stimulated and 3 amygdala-kindled rats). The calculated optical density measurements for these areas were then correlated with the number of synapsin I immunoreactive terminals obtained for each of the areas. In both cases, the obtained optical density was strongly correlated with the number of synapsin I terminals ($R = 0.98$; $R2 = 0.96$) suggesting that our optical density measurements closely reflected the quantity of synapsin I terminals found within these regions.

2.7. Statistical Analyses

The statistical significance of the data was assessed in several ways. First, group differences in exploratory behavior during each successive minute in the open field were analyzed using a one-way ANOVA, followed by post hoc analyses when appropriate using the Fisher's least significant differences test. Second, group differences in open field line crosses and thigmotaxis during the entire open-field session and in the duration of fleeing at the end of the open-field session were each analyzed using a two-way

ANOVA with sex and stimulation as the between subject factors. This was followed by post-hoc one-way ANOVAs and the Fisher's least significant differences test. Third, group differences in resistance to capture from the open field were assessed using Mann-Whitney U tests. Fourth, group differences in synapsin I immunoreactivity in each quantified brain region were analyzed using a two-way ANOVA, with sex and stimulation as the between subject factors. Finally, correlations between behavioral changes and synapsin I immunoreactivity were evaluated using a series of Pearson (R) product moment correlations for male and female rats. All statistical analyses were done using SPSS version 13.0. The criterion for statistical significance was set at $P < 0.05$.

3. Results

3.1. Electrode placements

All electrodes for male kindled rats were correctly implanted in the left basolateral amygdala (final total $n = 9$); however, there were two female kindled rats whose electrodes terminated outside of the amygdala and as a result they were removed from the study (final total $n = 7$).

3.2. Kindling

The kindling stimulations produced the typical progression of behavioral convulsions in all rats (data not shown). The initial stimulations elicited a short period of behavioral arrest accompanied by some facial automatisms. However, within a few days, almost every stimulation elicited a class 5 generalized convulsion characterized by forelimb clonus, rearing, and loss of equilibrium. There were no statistically significant sex differences in the number of electrical stimulations required to evoke the first class 5

motor convulsion [male: 11.2 ± 2.34, female: 14.8 ± 1.02, $P > 0.10$]. Similarly, there were no statistically significant sex differences in the total number of generalized convulsions (class 5 or higher) elicited during the course of kindling [male: 62.2 ± 8.06 vs. female: 68.6 ± 4.37, $P > 0.50$]. We did not observe spontaneous seizures in any rat at any time during the experiment.

3.3. Open-Field Behavior

Kindling significantly altered exploratory behavior in the open field and resistance to capture from the open field. This is shown in Fig. 2-1.

Exploratory Behavior: We first analyzed the effect of kindling on exploratory behavior during each successive minute of the open-field test (see Fig. 2-1A). There was a significant difference among the groups for line crosses during the each minute of the open-field test [$F(3,30) = 3.62$, $P < 0.03$ for min 1; $F(3,30) = 4.62$, $P < 0.01$ for min 2; $F(3,30) = 9.28$, $P < 0.001$ for min 3; $F(3,30) = 6.22$, $P < 0.002$ for min 4; $F(3,30) = 9.19$, $P < 0.0001$ for min 5]. Post hoc tests revealed that during the first min, the kindled males crossed fewer lines than did the sham-stimulated males and females and kindled females [$P < 0.05$]. The kindled females did not differ statistically from the sham-stimulated males and females [$Ps > 0.11$]. During the second min, the kindled females crossed significantly more lines than the kindled males [$P = 0.02$] and sham males [$P = 0.001$].

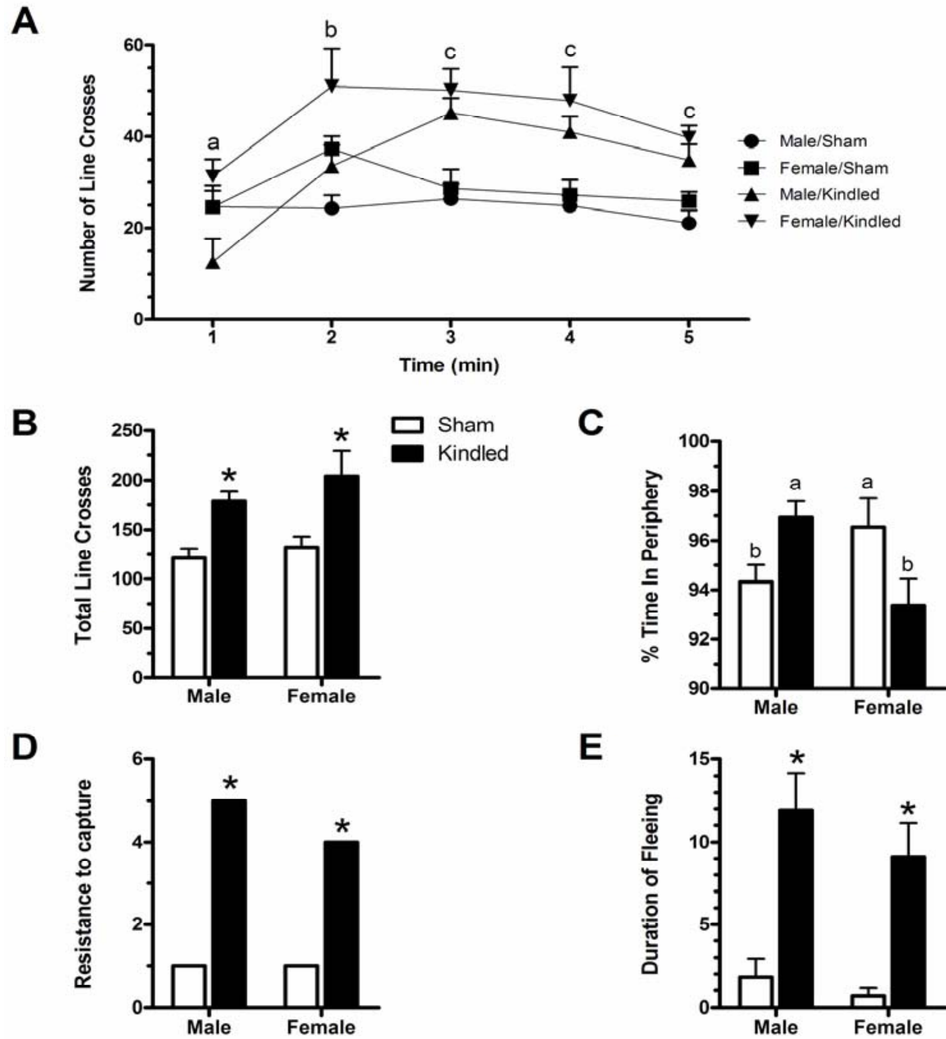


Figure 2-1 The effect of long-term amygdala kindling on open-field behavior. Panel A shows the mean number of lines crossed by the rats in each group during each successive minute of open-field testing. The letter a indicates a significant difference between the kindled males and all other groups ($P < 0.05$); the letter b indicates a significant difference between the kindled females and kindled males and sham-stimulated males ($P < 0.02$); the letter c indicates a significant difference between the kindled males and the sham-stimulated male and females and between the kindled females and the sham-stimulated males and females ($P < 0.01$). Panel B shows the total lines crossed during the 5-minute open-field session for the rats in each group. The letter a indicates a significant difference between kindled and sham-stimulated rats ($P < 0.006$). Panel C shows the percentage of time spent in the peripheral and center regions of the open-field during the 5-minute open-field session. Panel D shows the median resistance to capture displayed by the rats in each group. The letter a indicates a significant difference between kindled and sham-stimulated rats ($P < 0.001$). Panel E shows the duration of fleeing displayed by the rats in each group. Error bars denote the S.E.M.

They also showed a non-significant trend to cross more lines than the sham females [$P = 0.065$]. During the third, fourth, and fifth min of open-field testing, both kindled females and males crossed significantly more lines than the sham-stimulated males and females [all Ps < 0.003]. There were no significant differences between the kindled males and females in lines crossed during the third, fourth, or fifth min [All Ps > 0.11].

We also analyzed the effect of kindling on overall exploration during the entire 5 min open-field session. There were significant main effects of stimulation [$F(1,30) = 12.49$, $P < 0.001$; see Fig. 2-1B] and sex [$F(1,30) = 7.67$, $P < 0.01$], but no significant interaction between stimulation and sex [$F < 1.00$, $P = 0.60$]. Post hoc analyses revealed that the kindled males and females crossed more lines in the open field compared to the sham-stimulated males and females [all Ps < 0.006]. These analyses also showed that the female rats crossed more lines in the open field than did the male rats [$P = 0.036$].

To further explore the effect of kindling on open-field behavior, we analyzed group differences in the amount of time spent in the periphery of the open field (i.e., thigmotaxis). There was no significant main effect of stimulation [All Fs < 1.51] or sex [All Fs < 1.00] on time spent in the periphery but there was a significant interaction between stimulation and sex [$F(1,30) = 10.06$, $P < 0.003$; see Fig. 2-1C]. Post hoc analyses revealed that the kindled males spent more time in the peripheral regions of the open-field than did the kindled females [$P = 0.012$] and the sham-stimulated males [$P = 0.045$], but not the sham-stimulated females [$P = 0.76$]. In addition, the kindled females spent significantly less time in the peripheral regions of the open-field than did the sham-stimulated females [$P = 0.023$] and the kindled males [$P = 0.012$], but not the sham-stimulated males [$P = 0.47$].

Resistance to Capture and Duration of Fleeing: At the end of the open-field test, both the kindled males and females showed significantly higher resistance to capture than did the sham-stimulated males and females [Mann-Whitney $U = 4.64$, $P < 0.001$, see Fig. 2-1D]. There were no significant sex differences in resistance to capture [Mann-Whitney $U = 1.00$, $P = 0.33$]. In addition, the kindled males and females had a significantly longer duration of fleeing compared to the sham-stimulated males and females [all $Ps < 0.005$; see Fig. 2.1E]. There were also no significant sex differences in duration of fleeing [Mann-Whitney $U = 0.93$, $P = 0.38$]

3.4. Synapsin I immunoreactivity

To ensure that our correction procedure for controlling the influence of non-specific background staining was valid, individual analyses on synapsin I immunoreactivity in the corpus callosum were performed. The results revealed no significant difference in background staining from the corpus callosum between kindled and sham-stimulated rats [$P = 0.42$], or between male and female rats [$P = 0.76$].

Kindling significantly altered synapsin I immunoreactivity in several brain regions. The effect of kindling on synapsin I in hippocampal regions is shown in Fig. 2-2 and the effect of kindling on synapsin I in amygdalar regions is shown in Fig. 2-3. Quantified values of these effects are shown in Fig. 2-4.

In general, the results from a series of separate two-way ANOVAs revealed a significant main effect of kindling [All $Fs \geq 6.04$, $Ps < 0.02$], but no significant main effect of sex [All $Fs < 1.72$, $Ps > 0.18$] on synapsin I immunoreactivity. Subsequent post hoc analyses revealed that in the hippocampus, kindling significantly increased synapsin I

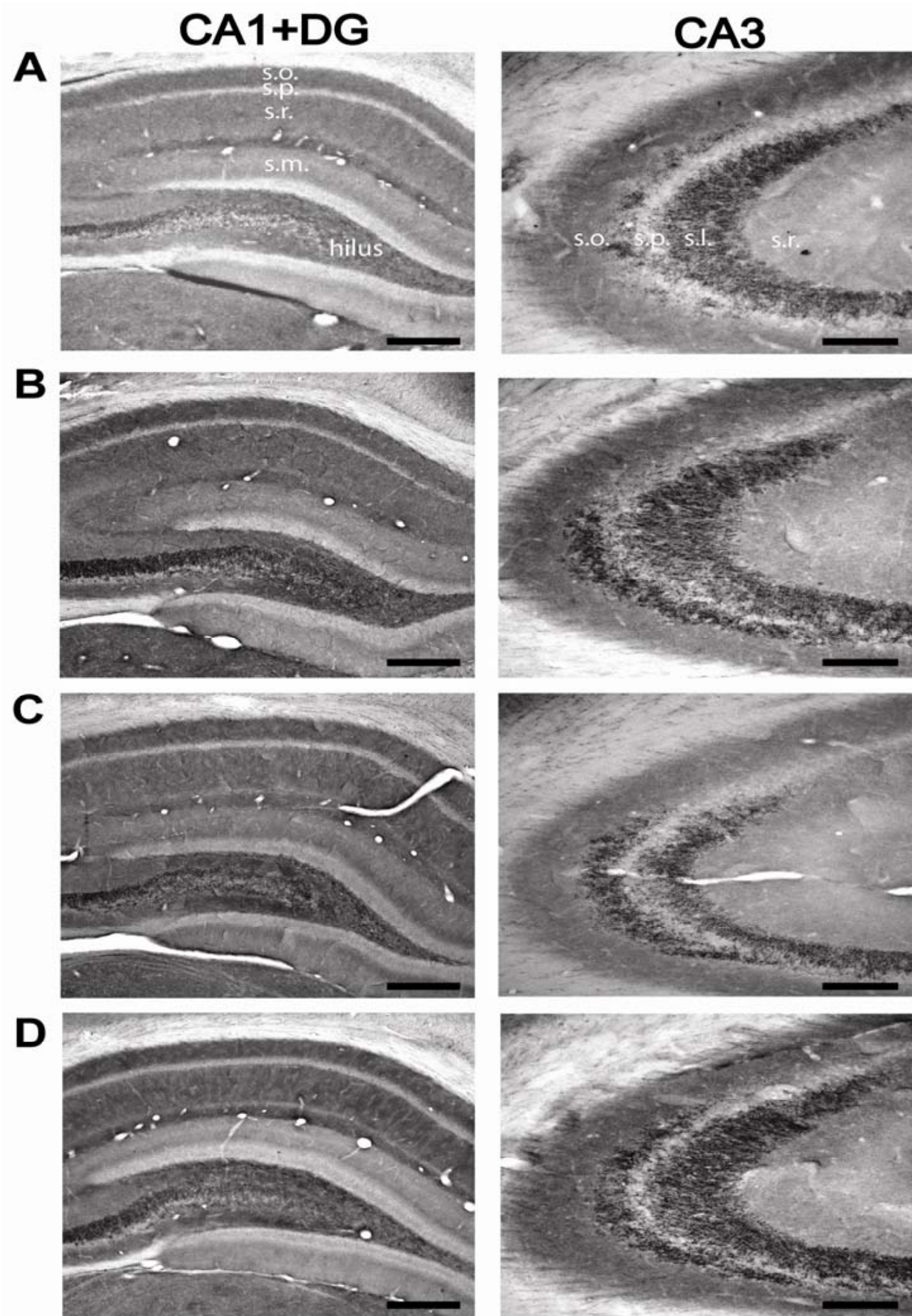


Figure 2-2 Representative photomicrographs of coronal sections showing synapsin I immunoreactivity in the ipsilateral hippocampus of rats from each group. Panel A, sham-stimulated males; Panel B, kindled males; Panel C, sham-stimulated females: and Panel D, kindled females. Scale Bar: 500 μm (CA1+DG), 200 um (CA3). Anatomical abbreviations: DG (dentate gyrus), s.o. (stratum oriens), s.l. (stratum lucidum), s.r. (stratum radiatum), s.p. (stratum pyramidale), s.m. (stratum moleculare).

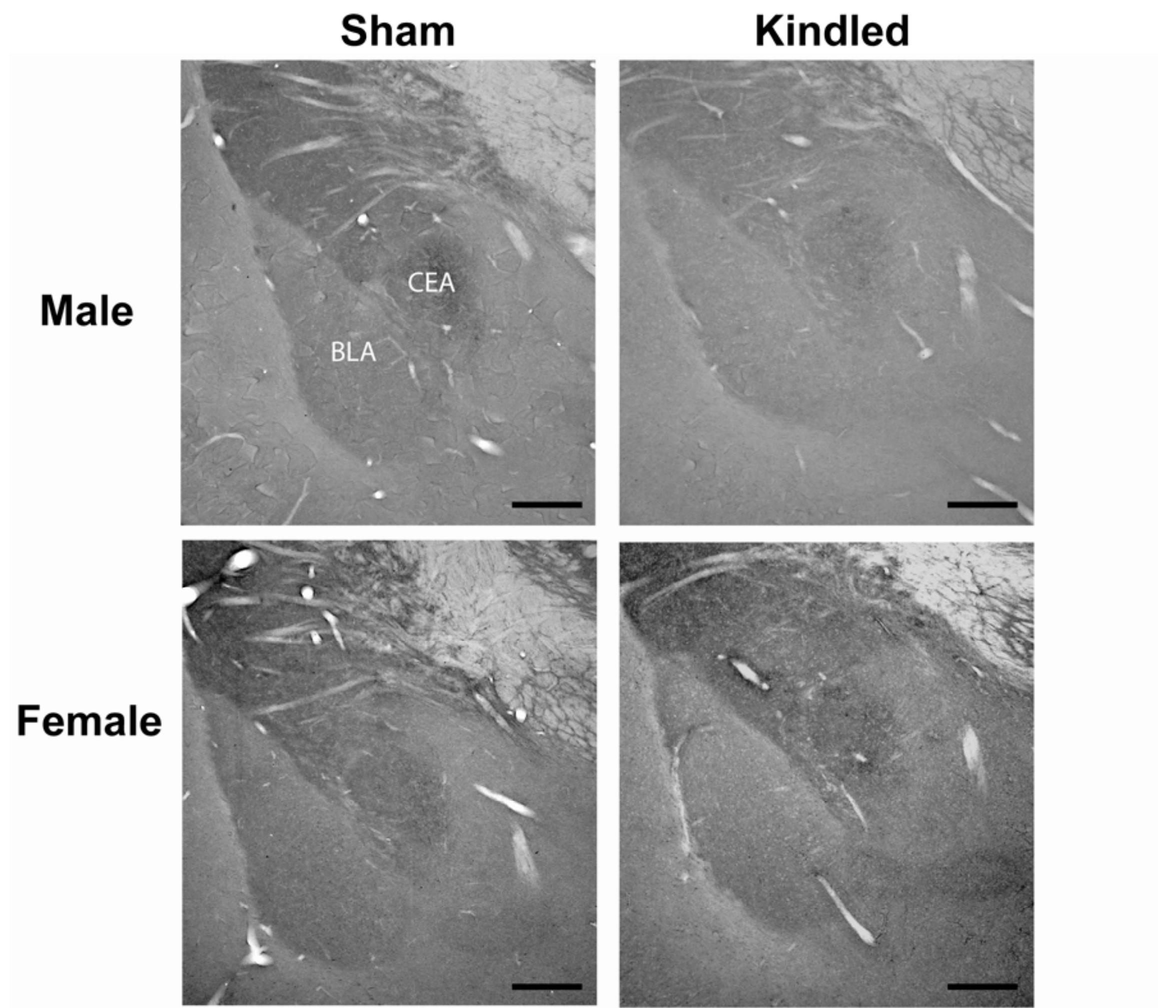
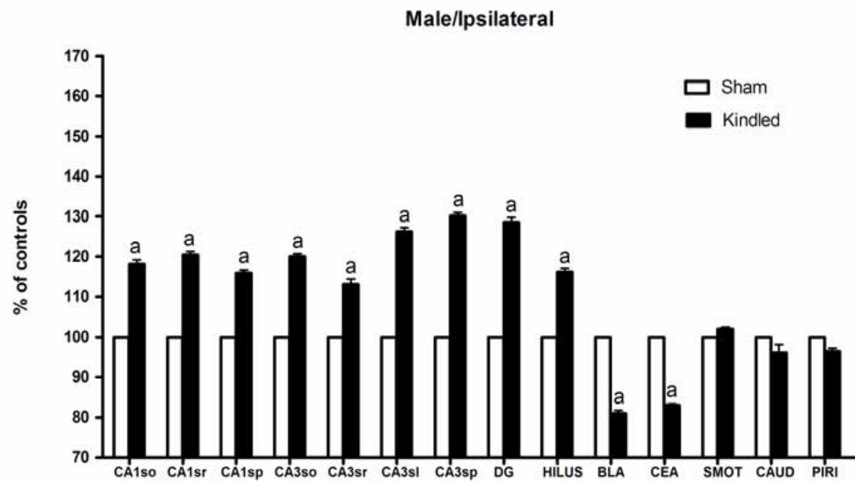


Figure 2-3 Representative photomicrographs of coronal sections showing synapsin I immunoreactivity in the ipsilateral basolateral and central amygdala of rats from each group. Scale Bar: 500 μm. Anatomical abbreviations: CEA (central amygdala), BLA (basolateral amygdala).


Male/Ipsilateral
% of controls
170
160
150
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120
110
100
90
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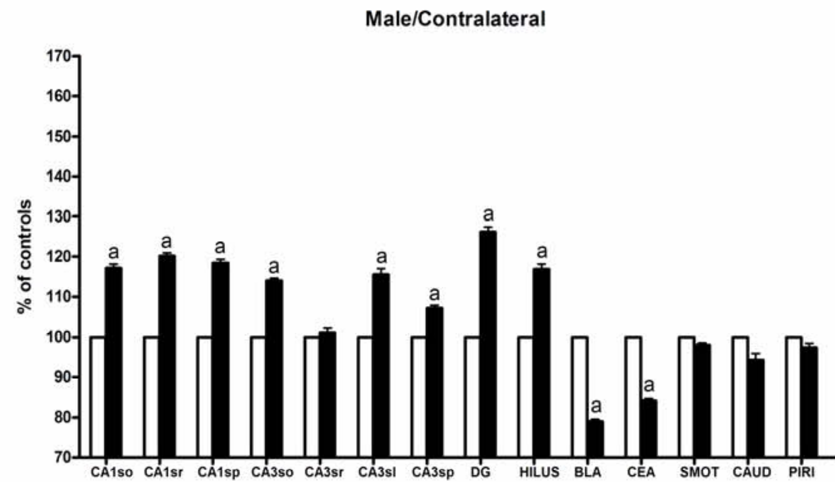
Sham
Kindled
a
CA1so CA1sr CA1sp CA3so CA3sr CA3sl CA3sp DG HILUS BLA CEA SMOT CAUD PIRI


Male/Contralateral
% of controls
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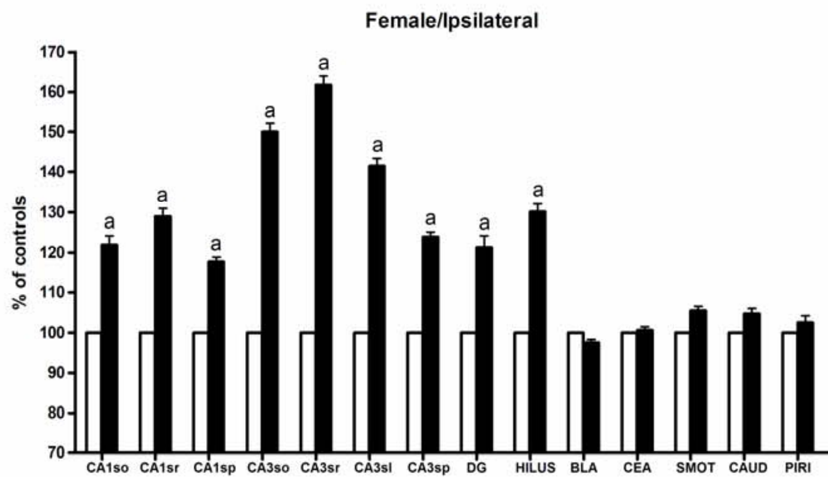
Female/Ipsilateral
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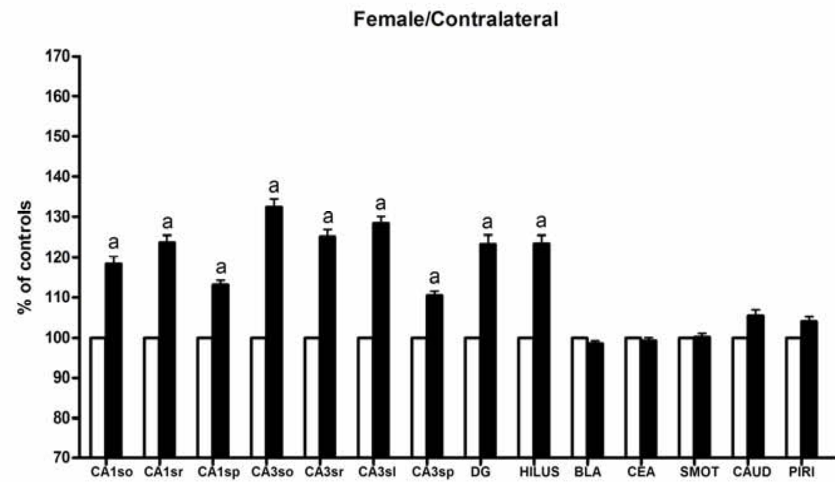
Female/Contralateral
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CA1so CA1sr CA1sp CA3so CA3sr CA3sl CA3sp DG HILUS BLA CEA SMOT CAUD PIRI

Figure 2-4 The effect of long-term amygdala kindling on synapsin I immunoreactivity in several brain regions for male and female rats. All data are normalized to control values and expressed as the mean percentage change from controls. The letter a indicates brain regions in which kindled rats differ significantly from sham-stimulated rats ($P<0.05$). Error bars denote the S.E.M. Abbreviations: DG (dentate gyrus), BLA (basolateral amygdala), CEA (central amygdala), SMOT (sensorimotor cortices), CAUD (striatum), PIRI (piriform cortices).

immunoreactivity in male and female rats in all quantified regions of CA1, CA3, and the dentate gyrus [All Ps < 0.037], except for the contralateral (to the stimulating hemisphere) CA3sr in the kindled males [$P = 0.15$]. There was a significant interaction between kindling and sex in the ipsilateral basolateral amygdala [$F(1,29) = 10.37$, $P = 0.003$], contralateral BLA [$F(1,29) = 9.67$, $P = 0.004$], ipsilateral CEA [$F(1,29) = 6.39$, $P = 0.017$], and contralateral CEA [$F(1,29) = 6.42$, $P = 0.018$]. The major source of this interaction was a significant decrease in synapsin I immunoreactivity in the kindled males compared to all other groups [All Ps < 0.012].

3.5. Correlations between behavior and synapsin I immunoreactivity

Kindling-induced alterations in behavior were significantly correlated with synapsin I immunoreactivity in some brain regions for males and females as shown in Table 2-1. Correlational analyses were only done for brain regions that showed significant changes in synapsin I immunoreactivity after kindling. To facilitate the interpretation of these analyses, averages were compiled for each of the hippocampal subfields (laminar subdivisions: sl, so, sp, sr) so that these structures could be organized according to traditional aggregates (i.e., CA1 and CA3, respectfully).

In male rats, there were a number of significant correlations between behavioral changes and synapsin I immunoreactivity, as described below. First, there were positive correlations between total line crosses and synapsin I levels in the ipsilateral CA1, CA3 and hilus, as well as in the bilateral dentate gyrus [all Ps < 0.05]. Second, there were negative correlations between total line crosses and synapsin I levels in the bilateral BLA and CEA [all Ps < 0.001]. Third, there were negative correlation between time in the periphery of the open field and synapsin I levels in the bilateral BLA and CEA [all Ps <

0.03]. Fourth, there were negative correlations between resistance to capture and synapsin I levels in the ipsilateral CEA and bilateral BLA [all Ps < 0.05]. And finally, there were negative correlations between duration of fleeing and synapsin I levels in the bilateral CEA [all Ps < 0.05].

In contrast to the male rats, there were relatively few significant correlations between behavioral changes and synapsin I levels in female rats. We found positive correlations between total line crosses and synapsin I levels in the ipsilateral CA1 and CA3 [P=0.049], between resistance to capture and synapsin I levels in the ipsilateral CA1 and CA3 [P = 0.001], and between duration of fleeing and synapsin I levels in the ipsilateral CA3 [P = 0.006].

4. Discussion

This experiment examined the relation between sex, fear, and synapsin I immunoreactivity in several brain regions of amygdala-kindled rats. As expected based on previous work (Hannesson et al., 2005; Wintink et al., 2003), long-term amygdala kindling increased fearful behavior in both male and female rats, although there were some sex differences in the pattern of these changes in behavior. Kindling also increased synapsin I immunoreactivity bilaterally in the hilus and molecular layer of the dentate gyrus, and throughout the CA1 and CA3 hippocampal subfields in both male and female rats (with the single exception of a unilateral increase in the CA3 stratum radiatum of kindled male rats). Interestingly, kindling decreased synapsin I immunoreactivity bilaterally in the basolateral and central amygdala of male rats but not female rats. Our

Table 2-1 Correlations between fearful behavior and synapsin I levels within various brain structures for male and female rats

	Line Crosses in First Minute		*Total Line Crosses*		*Total Time in Periphery*		*Resistance to Capture*		*Duration of Fleeing*	
Region	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*
Ipsilateral CA1	*-.19*	*-.02*	*.54***	*.51***	*.28*	*-.29*	*.39*	*.49***	*.20*	*.32*
Contralateral CA1	*-.26*	*-.05*	*.40*	*.31*	*.30*	*-.08*	*.44*	*.31*	*.28*	*-.19*
Ipsilateral CA3	*.01*	*-.02*	*.50***	*.49***	*.41*	*-.27*	*.41*	*.73***	*.35*	*.65***
Contralateral CA3	*-.05*	*-.01*	*.18*	*.35*	*.28*	*-.27*	*.16*	*.35*	*.05*	*.20*
Ipsilateral Hilus	*.02*	*-.04*	*.51***	*.43*	*.25*	*-.26*	*.16*	*.44*	*.11*	*.24*
Contralateral Hilus	*.38*	*-.14*	*.38*	*.27*	*.34*	*-.08*	*.29*	*.18*	*.26*	*.05*
Ipsilateral DG	*-.10*	*-.03*	*.68***	*.43*	*.29*	*-.22*	*.21*	*.43*	*.01*	*.04*
Contralateral DG	*-.07*	*-.01*	*.49***	*.40*	*.31*	*-.12*	*.29*	*.40*	*.01*	*-.02*
Ipsilateral BLA	*.31*	*-.13*	*-.72***	*-.01*	*-.62***	*-.34*	*-.63***	*.28*	*-.35*	*.21*
Contralateral BLA	*.06*	*-.19*	*-.83***	*-.04*	*-.59***	*-.27*	*-.61***	*.34*	*-.41*	*.08*
Ipsilateral CEA	*.12*	*-.13*	*-.51***	*-.01*	*-.68***	*-.30*	*-.54***	*.16*	*-.62***	*.20*
Contralateral CEA	*.05*	*.36*	*-.60***	*.20*	*-.53***	*-.47*	*-.45*	*.13*	*-.48***	*.08*

** $P < 0.05$ (Bolded values indicate statistically significant correlations)

correlational analyses revealed a greater number of significant correlations between fear behavior and synapsin I levels in the male rats compared to female rats. Taken together, these results suggest that the reorganization of synaptic terminals in hippocampal and amygdalar brain regions might serve as a substrate for the emergence of seizure-induced fear behavior, particularly in male rats.

4.1. Effect of long-term amygdala kindling on fear behavior in male and female rats

Previous experiments have repeatedly shown that kindling in male rats increases fear behavior in a number of different behavioral tests (Adamec et al., 2004; Adamec et al., 2005; Hannesson et al., 2005; Helfer et al., 1996; Murphy and Burnham, 2003). However, very few studies have focused on potential sex differences in kindled fear, despite the wealth of information showing sex differences in response to aversive situations (Archer, 1975). In this experiment, we found that kindling produced generally similar changes in fear behavior in male and female rats, with a few interesting sex differences in open-field behavior. For example, during the first minute of open-field testing, the kindled females explored at control levels, whereas the kindled males showed the typical increase in freezing that we have observed in a number of previous studies (Kalynchuk et al., 1997; Kalynchuk et al., 1998b; Kalynchuk et al., 2001). During minutes 2 through 5 of the open-field testing, the kindled females showed higher than control levels of exploration, but this effect was delayed in the kindled males until minute 3 of open-field testing. The fact that the kindled females showed less freezing in minute 1 and more active exploration in minutes 2 through 5 probably reflect inherent differences in how males and females respond to aversive stimuli, with males generally adopting passive responses and females generally adopting active avoidance strategies (Archer,

1975). It is important to note that this sex difference in open-field exploration only becomes apparent with this kind of minute-by-minute analysis. When we analyzed the total number of line crosses during the 5 min open-field test, there were no sex differences: kindled males and females were not significantly different from each other and both crossed significantly more lines than sham-stimulated males and females. This confirms previous reports of increased open-field exploration after amygdala kindling in males (Kalynchuk et al., 2006; Murphy and Burnham, 2003), and extends this finding to females.

Although locomotor hyperactivity can occur without concomitant changes in fear or anxiety-related behavior (e.g., Simon et al., 1994), we believe that the increased exploratory activity observed in this study is a manifestation of the general effect of amygdala kindling on fear reactivity to an unfamiliar environment. Support for this interpretation comes from our observation that kindled males spent significantly more time in the peripheral regions of the open-field compared to sham-stimulated males. Increased preference towards the walls of an open field (i.e., thigmotaxis; Barnett, 1968) is thought to reflect increased anxiety and fear in rodents (Valle, 1970). Consequently, it was surprising to find that kindled females engaged in less thigmotaxic behavior than the sham-stimulated females. However, the tendency for increased ambulation in kindled females in the open field (see Fig. 2-1A) raises the possibility that such a response might reflect a form of stress-induced locomotor hyperactivity. Although we did not directly assess movement velocity in the current study, preliminary evidence from our laboratory shows that kindled rats tested on a large elevated open-field apparatus show bursts of running behaviors (i.e., darting responses) and travel at greater velocities during their

exploration of center areas relative to peripheral areas of the open-field (Andersen et al., 2007).

Perhaps the strongest evidence that the altered open-field exploratory behavior of kindled rats reflects specific changes in emotional or fear-related behavior rather than non-specific changes behavioral hyperactivity is that kindling also dramatically increased resistance to capture and fleeing time in both male and female rats, consistent with our previous findings (Wintink et al., 2003). In contrast to more conventional measures of fear behavior in the rodent (e.g., elevated plus maze), resistance to capture is sensitive to extremely high levels of fear (Albert and Richmond, 1976). The resistance to capture behavior seen after long-term kindling is an extreme defensive response to retrieval from an unfamiliar experimenter. In this situation, the kindled rats engage in defensive jump attacks with excessive fleeing and loud vocalizations, and it can take 30 seconds or more for the experimenter to capture and remove the rat from the open field.

Long-term kindling produces particularly robust changes in open-field exploration and resistance to capture behavior; however, there have been reports of mixed effects of kindling on some measures of anxiety during open-field testing (e.g., (Adamec et al., 2004; Adamec et al., 2005; Helfer et al., 1996). For example, Helfer and colleagues (1996) showed that 15 class 5 kindled seizures in Wistar rats decreased open-arm exploration in the elevated-plus maze, but did not change exploratory behavior in the open-field. These apparent mixed effects are probably due to methodological differences between experiments, such as longer kindling protocols (e.g., 99 stimulations; results in >60 class 5 convulsions), shorter testing periods, and strain of rat (Long-Evans hooded rats vs. Wistar rats). It was recently shown that the anxiogenic effect of left BLA kindling

depends on an interaction between anterior-posterior axis location of the electrode tip and levels of anxiety prior to kindling (Adamec et al., 2005). Although this could ostensibly explain the mixed effects of short-term kindling paradigms on behavior, the long-term kindling paradigm employed in this study would presumably evoke more uniform and consistent changes in neuronal substrates (see below) and behavior, consequently masking the inherent effect of electrode location and premorbid anxiety on behavior.

4.2. Effect of long-term amygdala kindling on brain synapsin I expression

Long-term kindling had clear effects on synapsin I immunoreactivity in hippocampal regions in this experiment. With one exception (i.e., the CA3 stratum radiatum in male rats), both kindled male and female rats showed bilateral increases in synapsin I immunoreactivity relative to sham-stimulated rats in every quantified region of the hippocampus. We believe that the large number of generalized seizures (i.e., about 60 generalized seizures) experienced by the rats is responsible for the extent of these effects. In a previous study, Suemaru and colleagues (2000) reported a more restricted increase of synapsin I after short-term amygdala kindling (about 30 generalized seizures), with bilateral increases noted only in the CA3 stratum lucidum and hilus and contralateral increases noted in the CA1 stratum radiatum and stratum lacunosum-moleculare. Furthermore, intra-amygdalar administration of kainic acid, which is a more severe experimental model of temporal lobe epilepsy than short-term kindling, produced bilateral changes in synapsin I labeling in CA1 and CA3 regions that were comparable in pattern and magnitude to the kindling-induced changes in synapsin I seen in this experiment (Sato and Abe, 2001). In general, these findings suggest that more prolonged and repetitive activation of the hippocampal network by seizure activity can facilitate the

growth of new synaptic connections within hippocampal pathways in addition to the dentate gyrus-CA3 mossy fiber system—an area that was historically thought to be the primary hippocampal location of altered synaptic plasticity in rodent models of epilepsy (Scharfman et al., 2002b).

Kindling also had effects on synapsin I immunoreactivity in non-hippocampal brain regions. Many brain structures outside the hippocampus contribute to the effects of kindled seizures. Most notable is the piriform cortex, which is important for both the initiation of limbic seizures and development of amygdala kindling (Burazin and Gundlach, 1996; McIntyre and Plant, 1989; McIntyre and Plant, 1993). There is also evidence that seizure-induced changes in the striatum can promote the development of motor seizures in kindled animals (Uno and Ozawa, 1991). In addition, recent studies have shown that the motor map representation of the rat forelimb in the sensorimotor cortices is enlarged after kindling (van Rooyen et al., 2006). However, kindling had no effect on synapsin I immunoreactivity in any of these brain regions. Instead, kindling produced a significant bilateral decrease in synapsin I immunoreactivity in the BLA and CEA of male rats only. To our knowledge, there have been no previous studies comparing synapsin I expression in male and female rodents, and this makes it difficult to explain the differential effect of kindling on synapsin I levels in the amygdala. Our findings do raise the possibility that kindling may be more detrimental to amygdalar circuits in males than it is in females. This could be due to sex differences in ovarian hormone levels, which are known to possess neuroprotective effects in some animal models of epilepsy (Hoffman et al., 2003; Veliskova, 2006). Alternatively, it may be that the consequences of kindling on amygdala function in females do not include the forms

of synaptic plasticity visualized by synapsin I expression. At this point, we cannot rule out either of these explanations, but future studies employing multiple molecular markers of synaptic plasticity (e.g., GAP43, synaptophysin, etc.) will be needed in order to fully address the issue of why these differences occur and what their functional consequences might be.

4.3. Potential relationship between kindled fear and synapsin I expression

In this experiment, kindling clearly increased fearful behavior and it clearly produced widespread effects on synapsin I immunoreactivity throughout the hippocampus and amygdala. However, fearful behavior was only correlated with synapsin I levels in a subset of brain regions affected by kindling. In female rats, total line crosses and resistance to capture were positively correlated with synapsin I levels in the ipsilateral CA1 and CA3 regions and the duration of fleeing was positively correlated with synapsin I levels in the ipsilateral CA3. In male rats, total line crosses were positively correlated with synapsin I levels in the ipsilateral CA1, CA3, and hilus and bilateral dentate gyrus, and negatively correlated with synapsin I levels in the bilateral BLA and CEA. Interestingly, time spent in the periphery of the open field and the duration of fleeing and resistance to capture from the open field were also negatively correlated with synapsin I levels in the BLA and CEA, suggesting a possible relationship between altered amygdala plasticity and fearful behavior. What do these correlations mean? It is wise to be cautious when interpreting these effects, as correlations are only the first step in determining causality and the relationship between fear behavior and synapsin I expression could be coincidental. At the same time, it is possible that a functional relationship does exist between kindled fear and synapsin I. If this is the case,

then three interesting observations emerge from the pattern of these correlations. First, the main sex difference in the behavioral consequences of long term kindling—that kindled male rats show less exploration in the first min of exposure to an unfamiliar open field whereas kindled females rats do not—is probably not related to changes in synapsin I expression. Open-field exploration during the first min was not correlated with synapsin I levels in any brain region in either male or female rats. Second, the main sex difference in synapsin I alterations produced by long-term kindling—that kindled male rats show decreased synapsin I bilaterally in the BLA and CEA but kindled females do not—could be important for understanding the pathogenesis of kindled fear in the male rat. The negative correlations between amygdalar synapsin I levels and total line crosses, time in the periphery, duration of fleeing, and resistance to capture were particularly high in the kindled males. In fact, they were stronger than the correlations between these behaviors and synapsin I levels in hippocampal regions. Therefore, although temporal lobe epilepsy has been associated with reorganization of neuronal circuits specifically in hippocampal regions, these findings suggest that it would be premature to restrict our search for the neural correlates of interictal behavioral co-morbidities to hippocampal regions.

What are the functional implications of altered synapsin I levels? Synapsin I is a synaptic vesicle protein that is important for neurotransmitter release, synaptogenesis, and neurite extension (Greengard et al., 1987; Ryan et al., 1996). Fluctuations in synapsin I immunoreactivity are thought to indicate the growth or loss of functional synaptic connections respectively (Chi et al., 2003; Greengard et al., 1993). Accordingly, the results of this experiment suggest that some aspects of kindled fear could be related to increased synaptic plasticity in the hippocampus and decreased synaptic plasticity in the

amygdala, particularly in male rats. In previous experiments, we have found neurobiological changes in the hippocampus that are consistent with this idea. For example, we found that fearful kindled rats had increased benzodiazepine and 5-HT1A receptor binding in hippocampal regions indicating that kindling may increase a general inhibitory tone within hippocampal circuits (Kalynchuk et al., 2006; Kalynchuk and Meaney, 2003). It is possible that increased synapsin I could promote the sprouting of excitatory synaptic terminals upon inhibitory interneurons in order to facilitate a state of hyperinhibition that would limit the spread of seizures (Sloviter et al., 2006). Interictal fear might then arise as a consequence of this compensatory mechanism (Kalynchuk, 2000). This possibility is reinforced by the recent observation linking a mutation in the human synapsin I gene with an X-linked form of familial epilepsy. Interestingly, several members of the family with epilepsy also showed abnormal emotional behaviors (Garcia et al., 2004).

The implications of altered synapsin I levels in the amygdala after kindling are less clear. We are less confident in interpreting this finding because none of our previous experiments designed to identify alterations in receptors, genes and immediate early gene expression that may be involved in the pathogenesis of kindled fear revealed any changes in the amygdala (most other changes were located in the hippocampus). It is possible that decreased synapsin I labeling in the BLA and CEA could instigate the loss of local GABAergic terminals (Callahan et al., 1991; Lehmann et al., 1998) or the loss of dendritic spines (Nishizuka et al., 1991; Okada et al., 1993) seen in the amygdala after kindling. In either case, these dysfunctions could have significant consequences for the output of information from the central amygdala to other neuronal targets (Pare et al.,

2003). Specifically, kindling-induced loss of inhibitory circuitry within amygdalar pathways might facilitate the potentiation of amygdaloid efferents to the ventromedial hypothalamus or periaqueductal gray. This type of potentiation has previously been implicated in the pathogenesis of kindled fear (Adamec and Young, 2000). One other point to consider is that simultaneous changes in hippocampal and amygdalar synaptic plasticity would have greater functional consequences than changes in synaptic plasticity in one structure alone. In this case, the combination of increased hippocampal synaptic growth and decreased amygdalar synaptic growth could create an imbalance between excitatory and inhibitory processes in circuits that regulate fear behaviors. This type of imbalance has been reported to occur with epileptogenesis in some animal models of epilepsy (Rocha et al., 1996) and may be a fruitful avenue for further study in the context of the interictal behavioral co-morbidities associated with these forms of epilepsy.

4.5. Conclusions

We report here that kindled fear is associated with synapsin I immunoreactivity in both hippocampal and amygdalar regions, particularly in male rats. Because synapsin I is a marker of synaptic plasticity, these findings suggest that the synaptic reorganization that accompanies kindling might facilitate the development of a pathological architecture that serves to promote the development seizure-induced behavioral co-morbidities.

CHAPTER 3

The Effect of Amygdala Kindling On Hippocampal Neurogenesis Coincides With Decreased Reelin and DISC1 Expression in the Adult Dentate Gyrus

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1. Introduction

The involvement of seizure-induced neurogenesis in the pathophysiology of epilepsy has become a topic of considerable interest (Parent and Lowenstein, 2002). This is due in large part to the observation that seizure activity increases the birth of new neurons in the subgranular zone (SGZ) of the adult dentate gyrus and accelerates the morphological development and aberrant integration of these cells into hippocampal circuits (Overstreet-Wadiche et al., 2006b; Shapiro et al., 2007a). Interestingly, a small fraction of these newly generated neurons migrate ectopically into the hilus (Jessberger et al., 2007b; McCloskey et al., 2006; Mohapel et al., 2004; Parent et al., 1997). Electrophysiological studies have confirmed that these ectopic hilar granule cells exhibit epileptic burst discharges that are synchronized with CA3 pyramidal neurons (Scharfman et al., 2000). Because abnormal positioning of new neurons can promote hyperexcitability within adult hippocampal networks, extensive efforts have been made to identify candidate molecular mechanisms that may govern the inappropriate integration of adult generated neurons after seizures.

One candidate molecular mechanism for improper cell migration and integration is reelin. Reelin is a large extracellular matrix protein that provides a stop signal for migrating neurons. Although early work identified an important role for reelin in brain development (Curran and D'Arcangelo, 1998; D'Arcangelo et al., 1997), more recent studies have shown that reelin remains widely expressed in the adult vertebrate brain (Drakew et al., 1998; Perez-Garcia et al., 2001), where it facilitates normal adult hippocampal neurogenesis by promoting the correct migration and integration of newly born granule cells (Forster et al., 2006; Won et al., 2006)). Interestingly, reelin is synthesized and released by a diverse population of interneurons in the adult

hippocampus (Abraham and Meyer, 2003; Alcantara et al., 1998; Pesold et al., 1998) and many of these interneurons are also affected by epileptic seizures (Morimoto et al., 2004; Sayin et al., 2003). As a result, interest has now been directed at determining whether deficits in reelin signaling accompany seizure activity. Indeed, several groups have reported that reelin levels are lower after pilocarpine- (Gong et al., 2007) or kainate-induced seizures (Antonucci et al., 2008; Heinrich et al., 2006), and it is believed that reduced levels of reelin may underlie the progenitor cell migration deficits (Parent and Murphy, 2008) and granule cell layer dispersion (Frotscher et al., 2003) observed in these models of epilepsy.

Another candidate molecular mechanism for improper cell migration and integration is the gene Disrupted-In-Schizophrenia 1 (DISC1). DISC1 is a susceptibility gene for schizophrenia and mood disorders (Blackwood and Muir, 2004; Burdick et al., 2005; Porteous et al., 2006) that is widely expressed in the developing and adult vertebrate brain (Austin et al., 2004; Schurov et al., 2004). Duan and colleagues (Duan et al., 2007) have recently shown that downregulation of DISC1 leads to aberrant cell morphology, accelerated axonal and dendritic development, enhanced excitability, and impaired migration of adult generated neurons. The fact that both seizure activity and disrupted DISC1 expression produce similar impairments in cell migration suggests that the loss of DISC1 may also contribute to aberrant neurogenesis in the epileptic brain. However, there has been no study to date that has systematically explored the impact of seizure activity on DISC1 signaling.

The purpose of this experiment was to examine the effect of amygdala kindling on hippocampal neurogenesis, reelin, and DISC1 expression in the adult dentate gyrus and to

determine whether seizure-induced changes in reelin or DISC1 expression coincide with ectopic cell migration. Kindling is a commonly used animal model of temporal lobe epilepsy in which repeated electrical stimulation of specific limbic brain regions leads to the progressive development and intensification of electroencephalographic seizures and behavioral convulsions (Goddard et al., 1969). Short-term kindling (approximately 30 electrical stimulations) has been extensively used to study hyperexcitability and seizure susceptibility. Although short-term amygdala kindling increases hippocampal neurogenesis (Parent et al., 1998; Scott et al., 1998), it does not appear to provoke aberrant migration of newly generated neurons. However, epilepsy is a progressive process, in which the molecular, cellular, and behavioral consequences of repeated seizure activity require time to unfold. In past work we have shown that long-term kindling (i.e., 99 electrical stimulations) has greater effects than short-term kindling on markers of neuronal plasticity such as synapsin I and insulin-like growth factor I, and on behavioral measures related to anxiety and cognition (Fournier et al., 2008; Kalynchuk et al., 1997; Kalynchuk et al., 2001; Kalynchuk et al., 2002). This led us to hypothesize that long-term kindling might induce greater changes in reelin and DISC1 expression than short-term kindling and that these changes might be required in order to elicit aberrant neurogenesis and cell migration in this model of epilepsy. To examine this hypothesis, we assessed the effect of short and long-term amygdala kindling on several immunohistochemical markers. These markers included reelin and DISC1, as well as doublecortin, which selectively labels immature dentate granule cells and provides an effective way to quantify recent levels of neurogenesis as well as the dendritic growth of new neurons (Brown et al., 2003; Couillard-Despres et al., 2005). In addition, we also

examined the impact of kindling on the migration of new neurons by immunostaining for the transcription factor Prox1 (prospero-related homeobox-1), which selectively labels dentate granule cells (Pleasure et al., 2000; Steiner et al., 2008) and provides a way to quantify the ectopic migration of granule cells after seizures (McCloskey et al., 2006). Finally, we used the neuronal marker NeuN to examine potential cell loss in the hilus and to determine whether kindling causes volumetric changes in the dentate gyrus and/or dispersion of the dentate granule cell layer, which are common histopathological features of temporal lobe epilepsy (Blumcke et al., 2009; Houser, 1990; Mathern et al., 1997). Our results support the growing literature implicating reelin in the aberrant hippocampal neurogenesis observed after recurrent seizures and also reveals a potential role for DISC1 in the pathogenesis of temporal lobe epilepsy.

2. Materials and Methods

A total of 30 male Long-Evans rats (Charles Rivers, Montreal, QB, Canada) weighing approximately 200 to 300 g at the time of arrival served as subjects. They were individually housed in rectangular polypropylene cages with standard laboratory bedding and kept on a 12:12 h light:dark cycle with lights on at 0700h local time. Ambient temperature was maintained between 20ºC (±1ºC). Food and water was available *ad libitum* throughout the duration of the experiment. All animals were treated in accordance with the Canadian Council for Animal Care and the University of Saskatchewan Animal Care Committee. All efforts were made to minimize the number of animals used in this experiment.

2.1. Surgery

After a week of daily handling and habituation to colony conditions, the rats were individually anesthetized with isoflurane (5% initial, 2-2.5% maintenance) and placed into a stereotaxic apparatus. Prior to surgery, all rats were treated with an analgesic to minimize pain from the surgical procedure (Anafen, ketoprofen, 10 mg/kg, s.c., Merial, QB, Canada). An incision was made down the scalp and the underlying fascia was excised. A single bipolar electrode (MS-303-2, Plastics One, Roanoke, VA, US) was inserted into the left basolateral amygdala: -2.8 mm AP, +5.0 mm ML, and -8.5 mm V from the surface of the cranium (Fig. 1A, (Paxinos and Watson, 1998)). The electrode was secured to the skull with four stainless steel screws and dental acrylic. A topical antibacterial and antifungal agent (Hibitane, 1% chlorohexidine acetate, Ayerst Laboratories, Montreal, QB, Canada) was applied around the incision site to reduce the risk of infection.

2.2. Kindling Procedure

After a postsurgical recovery period of at least 2 weeks, the rats were habituated to the stimulation lead and handled for an additional 7 days, after which all subjects were randomly assigned into one of three conditions: long-term kindling (99 stimulations; n=10), short-term kindling (30 stimulations; n=10), or control (99 sham stimulations; n=10). To equalize the amount of handling received by the rats in each group, the rats in

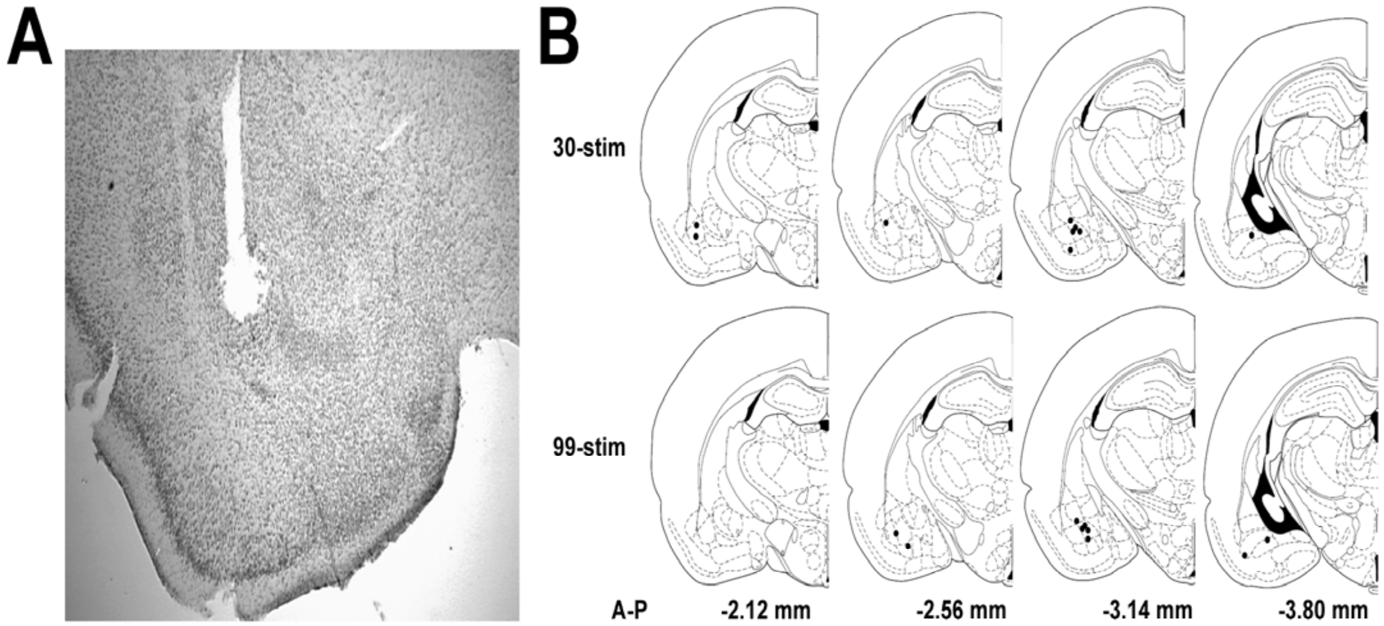

Figure 3-1 Location of electrode placements. Panel A) Representative photomicrograph of a stereotaxically implanted electrode penetrating the left basolateral amygdala nucleus. Panel B) Schematic depiction of the approximate location of stereotaxically implanted electrodes in the amygdala (-2.12, -2.56, -3.14, -3.80 mm from bregma) for short-term kindled rats (n=9) and long-term kindled rats (n=9). The representative sections were taken from the rat brain atlas of Paxinos and Watson (1998).

the short-term kindling group received 69 sham stimulations followed by 30 electrical stimulations (i.e., all rats in each group were handled 99 times). Prior to stimulation, each rat was placed in a plastic container containing a thin layer of commercial bedding, the wire lead was attached and the stimulation was delivered. Each stimulation comprised a 1 s, 60 Hz train of square-wave pulses with a biphasic amplitude of 800 μA (peak-to-peak) and a duration of 1 ms. The stimulations were delivered three times per day, 5 days per week, with a minimum of 3 h between stimulations. This stimulation protocol is well above the threshold current necessary to evoke epileptiform discharges from the amygdala (Racine, 1972a). The convulsion class elicited by each stimulation was classified according to a modified version of Racine's widely used scale (Racine, 1972b): Class 0 - freezing; 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 loss of equilibrium; Class 6 - multiple stage 5's. Control animals were treated similarly to the kindled rats with the exception that no electrical stimulation was delivered after the stimulation lead was attached.

2.3. Perfusion and Immunohistochemistry

Twenty-four hours after the final stimulation, all animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (240 mg/ml) and transcardially perfused with room temperature saline followed by ice-cold 4% (w/v) formaldehyde fixative (pH=7.4) that was freshly prepared from depolymerized paraformaldehyde. The brains were removed from the cranial vault and postfixed in the same fixative for up to 72 hrs at 4°C before being sectioned at 50μm on a vibrating

microtome in the coronal plane (Vibratome 3000, Vibratome Company, St. Louis, MO, USA). All sections were collected and stored at -20ºC in a cryoprotectant solution consisting of 30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, 30% (v/v) ethylene glycol in 0.1 M phosphate buffered saline (PBS; pH=7.4), until further processing.

Immunostaining was performed on free-floating sections with all rinses and incubations carried out under gentle agitation. To minimize endogenous peroxidase activity, the sections were rinsed in PBS and then incubated in 0.3% (v/v) hydrogen peroxide for 30 min. The sections were then rinsed several times in 0.1 M PBS and placed in a blocking solution containing 5% (v/v) normal animal sera, 1% (w/v) bovine serum albumin, and 0.3% (v/v) Triton X-100 in PBS for 60 min at room temperature. After blocking, the sections were incubated (at 4ºC) with one of the following primary antibodies (diluted in blocking solution): mouse monoclonal anti-NeuN (1:1000, incubated for 24 hrs, MAB377, Chemicon, Billerica, MA, US), mouse monoclonal anti-reelin G10 (1:2000, incubated for 48 hrs, MAB5364, Chemicon, Billerica, MA, US), goat polyclonal anti-doublecortin (1:250, incubated for 48 hrs, sc-8066, Santa Cruz Biotechnology, Santa Cruz, CA, US), goat polyclonal anti-DISC1 (1:1000, incubated for 24 hrs, sc-47990, Santa Cruz Biotechnology, Santa Cruz, CA, US), or rabbit polyclonal anti-Prox1 (1:5000, incubated for 72 hrs, PRB-238C, Covance, QB, Canada). Following the PBS rinses, the sections were incubated with appropriate biotinylated secondary antibodies diluted in 0.3% (v/v) Triton X-100 in PBS for 2 hrs at room temperature (1:200, Vector Laboratories, Burlington, ON, Canada). The sections were then placed in avidin-biotin-peroxidase complex (1:500, Vectastain ABC Elite, Vector Laboratories, Burlington, ON, Canada) and immunolabelling was visualized using DAB (0.033% (w/v)

3,3'-diaminobenzidine, 0.00786% (v/v) hydrogen peroxide diluted in PBS) to yield a brown product, or visualized using nickel-intensified DAB (0.065% (w/v) 3,3'-diaminobenzidine, 4.167% (w/v) nickel ammonium sulfate, 0.085% (v/v) hydrogen peroxide diluted in 0.175 M sodium acetate, pH=6.8) to yield a blue-black product. After sufficient coloration, the reaction was halted by washing in PBS several times. The sections were mounted onto glass slides and left to air dry overnight. Slides were dehydrated through a series of alcohols, cleared in xylene, and coverslipped with Entellan mounting medium. The specificity of each antibody was determined by running the immunostaining protocol in the absence of the primary antibodies. No staining was observed in these sections.

To determine if the electrode placement in each animal was correct, some of the immunohistochemically processed tissue was also counterstained with 0.1% cresyl violet acetate. For each animal, the electrode placement was verified using the Paxinos and Watson (Paxinos and Watson, 1998) stereotaxic atlas. Any animal with an incorrectly positioned electrode was removed from the study.

2.4. Analyses of Immunohistochemical Labeling

All analyses of the immunohistochemical reactions described above were conducted by individuals who were blind to all treatment conditions. These analyses were designed to assess several aspects of the effect of kindling on the adult dentate gyrus.

First, to examine the effect of kindling on hippocampal neurogenesis, the total number of doublecortin (DCX) labeled cells in the dentate gyrus was estimated using the unbiased optical fractionator method (West et al., 1991). Every sixth section was examined at 400X and 1000X (oil immersion) magnification on a Nikon E800

microscope equipped with a motorized stage and a computerized stereology system (StereoInvestigator v. 8.00, Microbrightfield, Baltimore, MD, USA). DCX-labeled cells in the granule cell layer and the SGZ were counted throughout the entire dentate gyrus (including dorsal and ventral hippocampus) starting at -2.56 and ending at -6.04 mm relative to bregma. The total number of DCX cells was estimated using the following formula: $N_{total} = \Sigma Q^{-} * 1/ssf * A(x,y\ step)/a(frame) * t/h$; where ΣQ^{-} is the number of counted cells; ssf is the section sampling fraction (1/6); A(x,y step) is the area associated with each x,y movement (5625 μm^2); a(frame) is the area of the counting frame (2500 μm^2); t is the weighted average section thickness; and h is the height of the dissector (12 μm). A guard zone height of 5 μm was used during cell counting to avoid sectioning artifacts. The coefficient of error was calculated and all values <0.10 were accepted (Gundersen and Jensen, 1987). All measurements were made ipsilateral and contralateral to the stimulation site. Morphological features, such as dendritic growth and branching, were also evaluated in DCX-labeled cells by visual examination of these cells at high-power magnification.

Second, to examine the effect of kindling on cell migration, ectopic Prox1-labeled granule cells were manually counted at 400X magnification in the ipsilateral and contralateral hilus across 8 to 10 sections through the entire dentate gyrus for each subject using a light microscope (Nikon Eclipse E600).

Third, to examine the effect of kindling on reelin expression, the total number of reelin-labeled cells was estimated using the same stereological analysis used to count the number of DCX-labeled cells. Reelin-labeled cells were counted in the dentate SGZ and the hilus (which excluded reelin cells located in the SGZ). The SGZ was defined as

extending approximately two cell layer widths deep into the hilus. The total number of reelin cells was estimated using the same formula presented above with the exception that the A(x,y step) was set to 22500 μm^2 and the a(frame) set to 6400μm^2 for the calculations. (All other parameters were held constant). A guard zone height of 5 μm was used during cell counting procedures in order to avoid sectioning artifacts. All measurements were made ipsilateral and contralateral to the stimulation site.

Fourth, to examine the effect of kindling on DISC1 expression, semi-quantitative densitometry was performed on the following regions of interest: 1) hilus, 2) granule cell layer, 3) SGZ, and 4) inner molecular layer across both ipsilateral and contralateral cortices. Sections were captured using a digital camera that was attached to a light microscope (Nikon Eclipse E600, 10× objective) with the camera exposure and gain settings held constant between images. The mean relative optical density for each quantified brain region was calculated from two to three adjacent coronal sections (300 μm distance between sections) and standardized between white (0) and black (255). Background staining was controlled by calculating the average optical density levels from the corpus callosum and subtracting these values from the area of interest. Standardized values were calculated and re-expressed as the percentage change from controls. Inter-rater verification was performed by a second researcher who was blind to all treatment conditions. Results indicated that the examined brain regions yielded inter-rater correlation coefficients between 0.84 and 0.96 respectively.

Fifth, three additional histopathological consequences of kindling were assessed. To examine the effect of kindling on dentate gyrus volume, the total volume of the dentate granule cell layer was calculated according to the Cavalieri's principle. A

systematic sampling grid with a superimposing lattice of test points was generated and placed over an image captured at 4X magnification. The number of points that crossed the granule cell layer was then counted. Following this, an unbiased estimation of dentate granule cell layer volume was calculated according to the following formula: $V_{est} = \Sigma P * a(p) * d$, where ΣP is the sum of test points counted across all sections, a(p) is the planar area associated with each test point (area per test point = 0.02 mm^2), and d refers to the distance between sections. The mean coefficient of error was less than 5%. To examine the effect of kindling on granule cell layer dispersion, the width of the dentate granule cell layer was measured at every 50 μm increment for the upper blade, lower blade, and crest across three coronal sections per animal at rostrocaudal levels -2.80, -3.80, -5.30 mm from bregma. Finally, to examine the effect of kindling on neuronal loss within the hilus, the number of neurons in the hilus (NeuN-labeled cells) was counted for 14 rats (sham n=5; 30-stim n=4, 99-stim n=5) using a modified optical fractionator procedure. The hilus was defined by the inner edge of the granule cell layer and lines connecting the tips of the two granule cell blades to the beginning of the CA3c/CA4 pyramidal cell layer of Ammon's horn. Briefly, all hilar neurons (mossy cells and interneurons) were exhaustively counted within the borders of the hilus and all measurements were made ipsilateral and contralateral to the stimulation site. Cells in the uppermost focal plane were excluded in order to avoid oversampling errors. The number of counted cells was then multiplied by the total number of sections that were counted from (i.e., 12 to 14 coronal sections) in order to provide a relative estimate of the number of NeuN-labeled hilar cells (Williams and Rakic, 1988).

2.5. Statistical Analyses

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS v 16.0, Chicago, IL, USA). Group differences were examined using a one-way analysis of variance (ANOVA) followed by Fisher's least significant difference *post hoc* tests when appropriate. Results are presented as mean ± standard error of the mean (S.E.M.). The criterion for statistical significance was set at $P < 0.05$.

3. Results

3.1. Electrode implantation and progression of kindling

Our histological analysis revealed that 28 rats had electrodes correctly implanted in the left basolateral amygdala. An example of a correct electrode placement into the amygdala is shown in Fig. 3-1A. A total of 2 rats were removed from the study because they had either an incorrect electrode placement or the loss of the electrode cap during the kindling procedures. Therefore, the final number of rats in each group was: 10 (sham controls), 9 (short-term kindling; 30-stim), and 9 (long-term kindling; 99-stim). The electrode placement for each rat in the short-term and long-term kindled groups is shown in Fig. 3-1B.

The kindling stimulations produced the typical progression of seizures in all rats. The initial stimulations elicited a short period of behavioral arrest followed by orofacial automatisms. With subsequent stimulations, these episodes progressed into class 5 generalized convulsions characterized by bilateral forelimb clonus, rearing, and loss of equilibrium. The total number of stimulations required to elicit the first class 5 convulsion was 12.3 (2.70) for the short-term kindled rats and 17.9 (2.04) for the long-term kindled rats. In addition, the total number of class 5 or higher convulsions

experienced by the rats in each group was 10.1 (1.36, range 4 to 16) for the short-term kindled rats and 68.9 (4.13, range 50 to 86) for the long-term kindled rats. Consistent with previous studies (Pinel and Rovner, 1978), we did not observe spontaneous motor seizures at any time during the experiment.

3.2. Kindling increases the number of DCX-labeled cells and alters cell morphology

The effect of kindling on DCX-labeled cells is shown in Fig. 3-2 (Panels A-I). A large number of DCX-labeled cells were located in the subgranular zone or in the inner one-third of the granule cell layer. Our statistical analyses revealed that the short-term kindled rats had significantly more DCX-labeled cells than control rats on both the side ipsilateral to the stimulation (i.e., 58% increase from controls) and the side contralateral to the stimulation (i.e., 34% increase from controls) [All Ps < 0.010, Fig. 3-1, Panel K]. In contrast, the long-term kindled rats did not differ significantly from the short-term kindled [$P = 0.087$] or controls rats [$P = 0.226$] in the number of DCX-labeled cells on either the ipsilateral or contralateral side.

As shown in Fig. 3-2 (Panel J), kindling also dramatically altered the morphology of DCX-labeled cells in the dentate SGZ. Our qualitative analyses revealed that the majority of DCX-labeled cells in the long-term kindled rats had elongated apical dendrites and greater dendritic branching compared to control rats. In addition, apical dendritic outgrowth was more complex in the long-term kindled rats and we could clearly

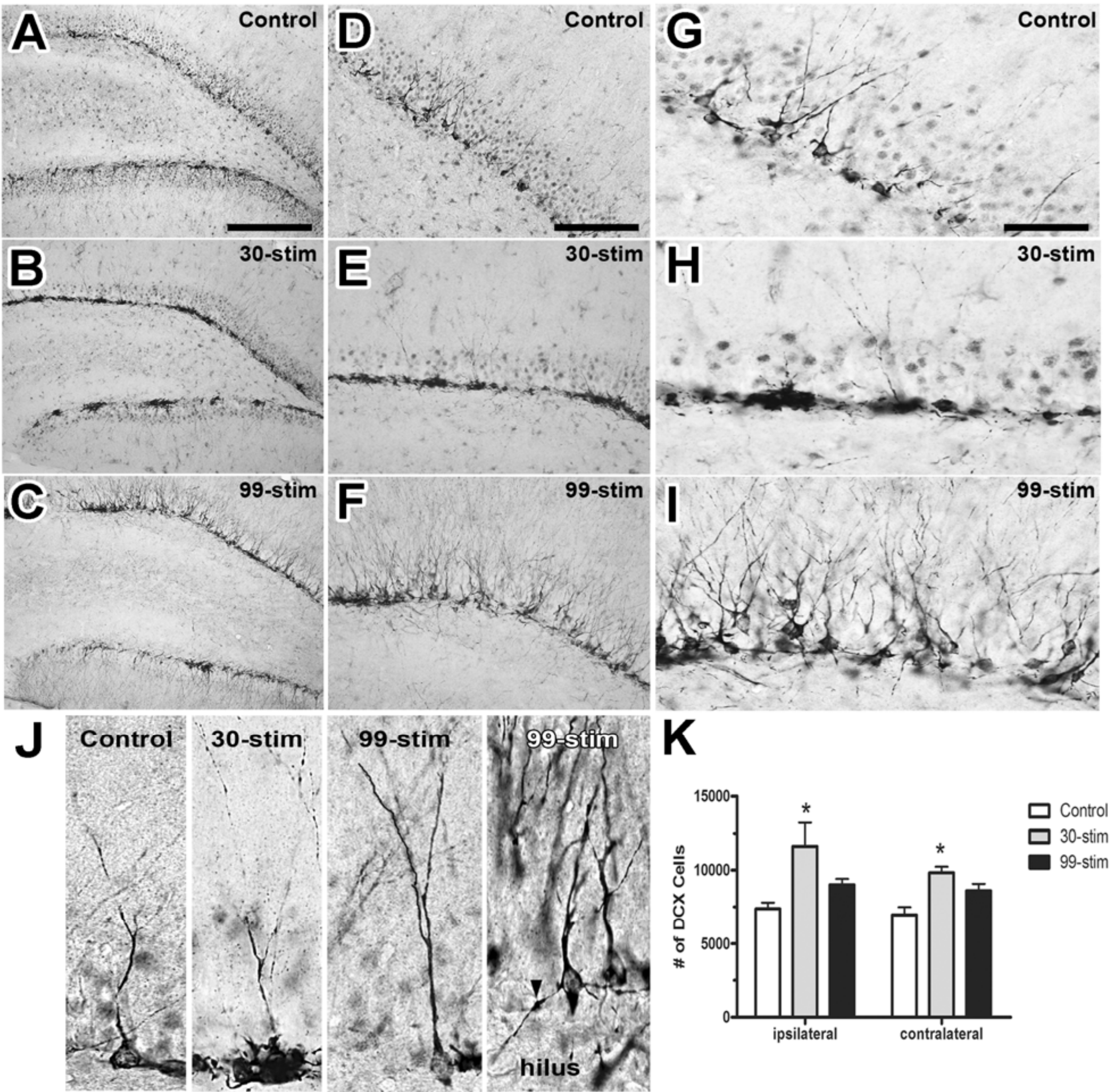

Figure 3-2 Effect of kindling on doublecortin immunolabeling in the adult dentate gyrus. Representative photomicrographs showing doublecortin immunolabeling in the adult dentate gyrus of rats in each condition: control (Panels A, D, G), short-term kindling (Panels B, E, H), and long-term kindling (Panels C, F, I). Examples of doublecortin-labeled cells from control rats, short-term kindled rats, and long-term kindled rats (Panel J). Note the doublecortin-labeled cell from a long-term kindled rat with a prominent basal dendrite extending into the hilus (arrow head). Quantitative stereological estimates of the number of immature doublecortin-labeled cells in the dentate subgranular zone (Panel K). Short-term kindling significantly increased the number of doublecortin-labeled cells in the dentate subgranular zone compared to control rats (* $P < 0.01$). There was no difference between long-term kindled and control rats. Error bars denote S.E.M. Scale Bar: 300 µm (A-C), 150 µm (D-F), and 55 µm (G-I).

see labeled dendrites extending into the middle and outer molecular layer, whereas the control animals generally had dendrites confined to the inner molecular layer. This effect was most prominent in the ipsilateral dentate SGZ. However, similar changes could be observed in the contralateral dentate SGZ to a lesser extent. We also observed the presence of basal dendrites on some DCX-labeled cells from the long-term kindled and control rats. Although basal dendrites from the control rats extended horizontally and then entered the granule cell layer, basal dendrites from long-term kindled rats often projected into the hilus. The morphology of DCX-labeled cells in the short-term kindled rats was significantly less complex than those of the long-term kindled and control rats. These cells were located mainly at the base of the granule cell layer suggesting that these cells were most likely newly proliferated neuroblasts (see Fig. 3-2, Panels B, E, and H).

3.3. Long-term kindling increases the number of ectopic hilar granule cells

The effect of kindling on ectopic Prox1-labeled cells is shown in Fig. 3-3. The long-term kindled rats had significantly more Prox1-labeled cells in the ipsilateral hilus than the control rats [104.9 ± 15.51 vs. 62.54 ± 14.86, $P = 0.047$] or short-term kindled rats [104.9 ± 15.51 vs. 57.42 ± 12.91, $P = 0.045$]. In contrast, there were no significant differences among any of the groups in the number of ectopic Prox1-labeled cells in the contralateral hilus [$P > 0.732$].

3.4. Long-term kindling decreases reelin expression in the SGZ

The effect of kindling on reelin is shown in Fig. 3-4. Large aggregates of reelin-labeled cells with clear and distinct immunolabeling were seen in the hilus and SGZ. The

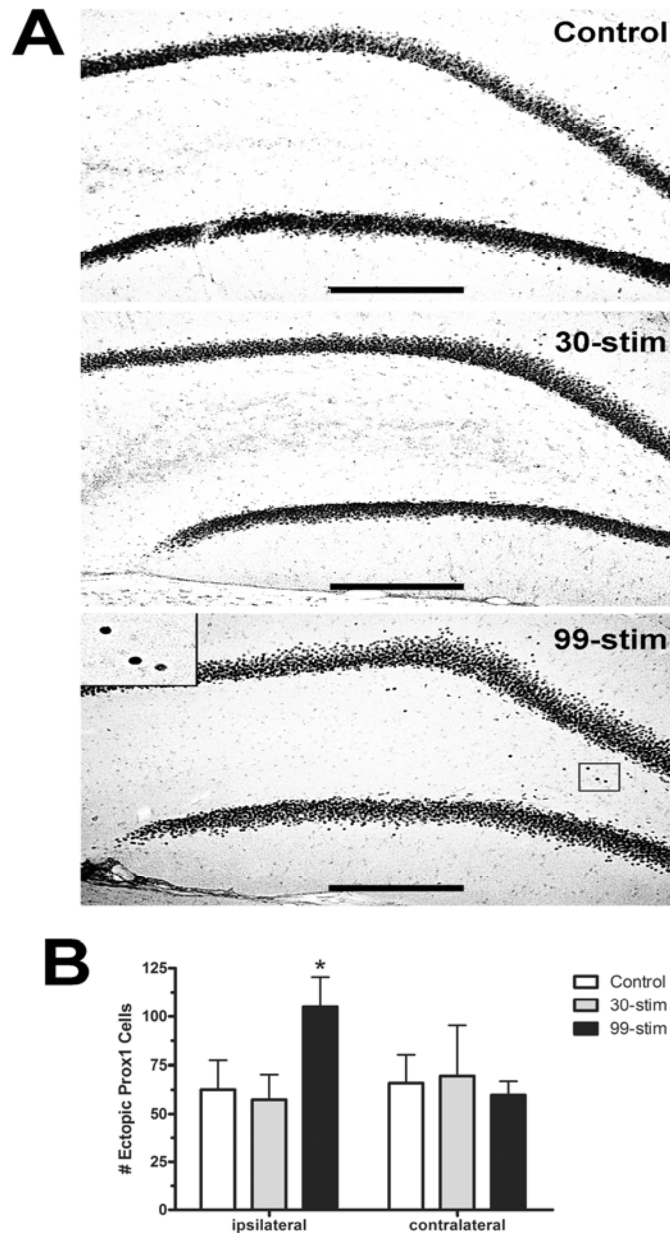

Figure 3-3 Effect of kindling on the distribution of Prox1-labeled ectopic granule cells in the dentate hilus. Panel A) Representative photomicrographs of the adult dentate gyrus showing Prox1-labeled ectopic granule cells in the ipsilateral hilus of control rats, short-term kindled rats, and long-term kindled rats. The small box indicates a few of the Prox1-labeled ectopic granule cells. Panel B) Quantification of Prox1 labeled ectopic granule cells in the hilus after kindling. A greater number of Prox1-labled granule cells accumulated in the ipsilateral hilus in rats that received long-term kindling compared to controls and short-term kindled rats (* $P < 0.05$). Error bars denote S.E.M. Scale Bar: 200 μm.

vast majority of these cells displayed morphological characteristics that are consistent with the profile of inhibitory interneurons previously described by Pesold and colleagues (1998). Reelin-labeled cells in the SGZ often clustered in groups of 3 to 5 cells and extended dendrites into the granule cell layer (Fig. 3-4, Panel B). However, there was no direct labeling of reelin in dentate granule cells indicating that staining was specific to interneuron populations.

Quantitative stereological analyses revealed that the long-term kindled rats had significantly fewer reelin-labeled cells in the ipsilateral SGZ compared to the short-term kindled and control rats [All Ps < 0.001, Fig. 3-4, Panel J]. There was no effect of kindling on the number of reelin-labeled cells in the contralateral SGZ [$P > 0.681$], ipsilateral hilus [$P > 0.343$] or contralateral hilus [$P > 0.465$] [Fig. 3-4, Panels J and K].

3.5. Long-term kindling decreases DISC1 expression in the SGZ

The effect of kindling on DISC1 expression is shown in Fig. 3-5 [Panels A-C]. DISC1 immunolabeling was particularly abundant within the dentate gyrus, whereas staining in the hilus was minimal. At high-power magnification, DISC1 immunolabeling was predominately found within the somatic compartment of granule cells. The long-term kindled rats had significantly less DISC1 expression in the ipsilateral dentate granule cell layer and SGZ than the control rats [All Ps < 0.031], and significantly less DISC1 expression in the contralateral dentate granule cell layer and SGZ than both the control and short-term kindled rats [All Ps < 0.012, Fig. 3-5, Panel D]. No other group comparisons in DISC 1 expression were statistically significant [All Ps > 0.309].

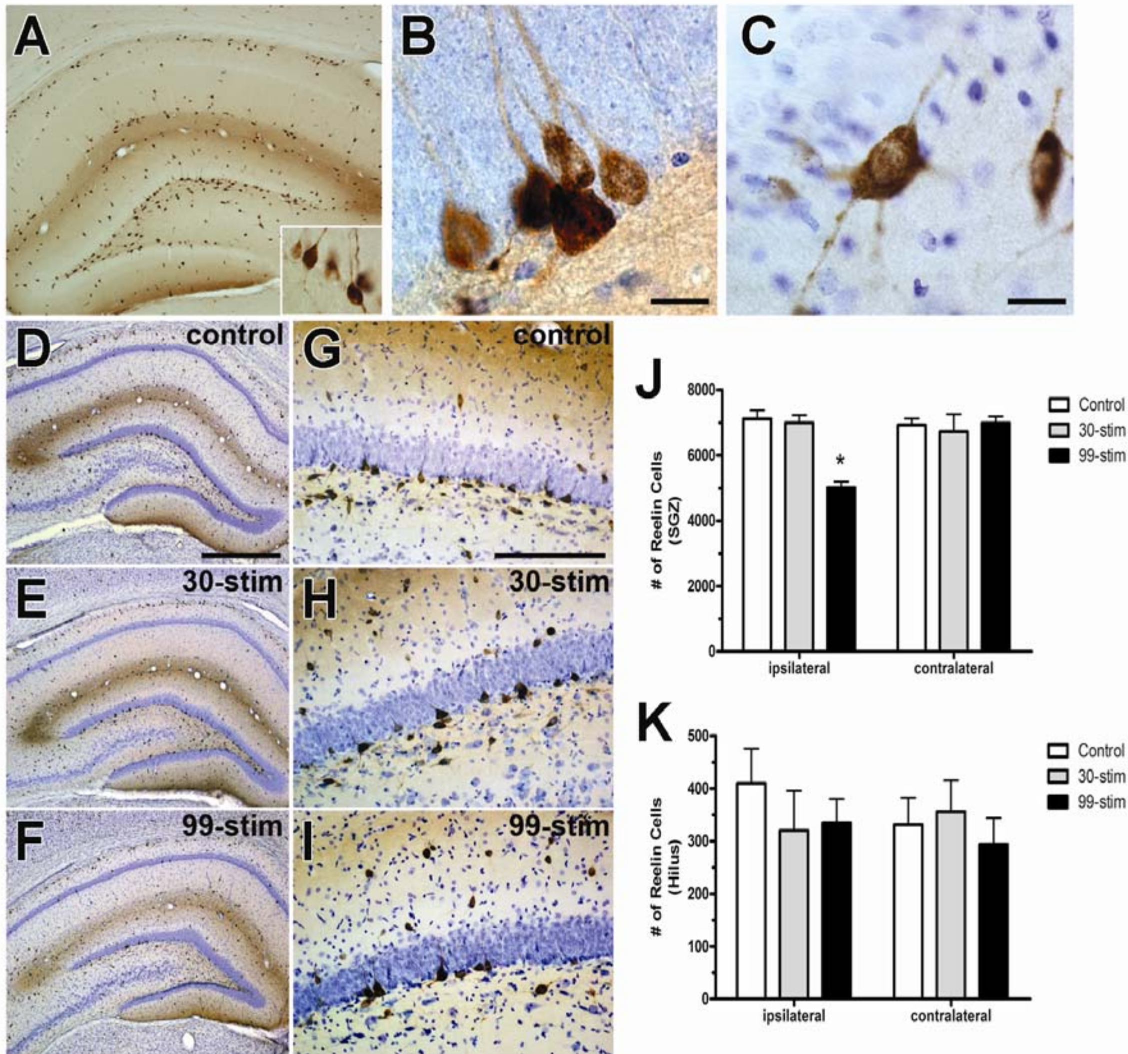

Figure 3-4 Effect of kindling on the distribution of reelin expressing neurons in the adult dentate gyrus. Panel A) A representative micrograph showing the distribution of reelin expressing neurons in the adult hippocampus. Panel B) A high power magnification image of a cluster of reelin expressing neurons located in the subgranular zone. A densely labeled dendritic process can be visualized extending through the dentate granule cell layer and terminating in the molecular layer. Panel C) An example of a reelin expressing neuron in the deep hilus. These cells morphologically resemble hilar mossy cells (Ribak et al., 1985). Panels D-I): Representative photomicrographs showing the distribution of reelin expressing neurons in the ipsilateral side of rats that received sham stimulation (Panels D, G), short-term kindling (Panels E, H), or long-term kindling (Panels F, I). Panel J) Quantitative stereological estimates of reelin expressing neurons in the dentate subgranular zone after kindling. Long-term kindled rats showed a significant decrease in the number of reelin expressing neurons compared to sham controls (* $P < 0.001$). Panel K) Quantitative stereological estimates of reelin expressing neurons in the hilus after kindling. There was no significant effect of kindling on ipsilateral or contralateral reelin expression in the hilus. Scale Bar: 20 μm (B, C), 800 μm (D-F), 200 μm (G-I).

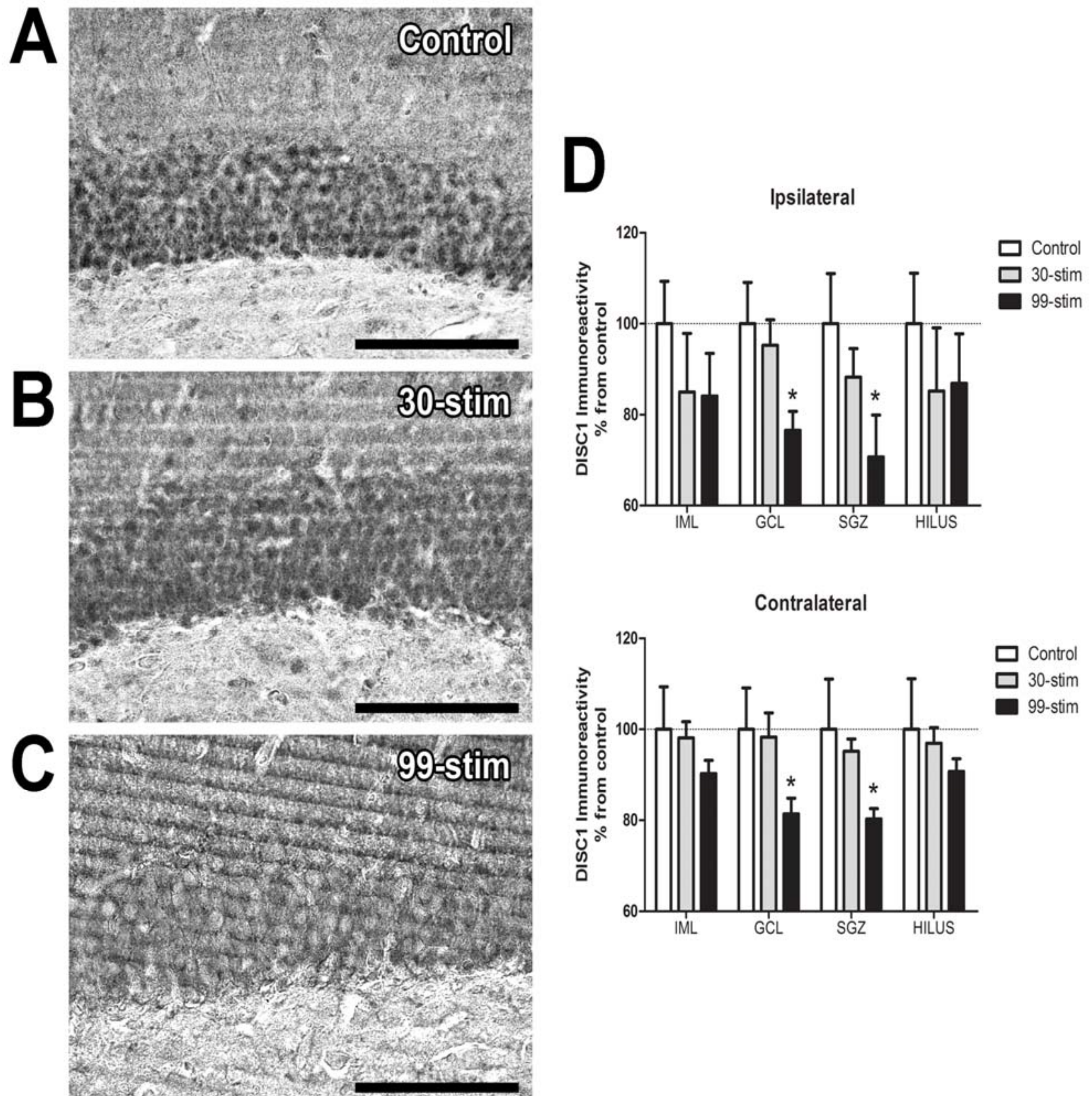

Figure 3-5 Effect of kindling on DISC1 immunolabeling within the dentate gyrus. Representative photomicrographs of the ipsilateral dentate gyrus showing DISC1 immunolabeling in control rats (Panel A), short-term kindled rats (Panel B), or long-term kindled rats (Panel C). Scale bar: 100 µm. Panel D) Quantitative densitometry for the ipsilateral and contralateral hilus, subgranular zone (SGZ), granule cell layer (gcl), and inner molecular layer (iml) after kindling. Long-term kindled rats had a significant decrease in DISC1 immunolabeling in the ipsilateral and contralateral dentate granule cell layer and subgranular zone compared to controls (* $P < 0.05$). Error bars denote S.E.M.

3.6. No effect of kindling on granule cell dispersion or hilar neuron numbers

The effect of kindling on dentate gyrus volume, granule cell layer width, and NeuN labeled cells in the hilus is shown in Fig. 3-6. Kindling had no significant effect on the volume of either the ipsilateral or contralateral dentate gyrus [All *P*s > 0.282, Fig. 3-6, Panel A]. In addition, quantitative evaluation confirmed that kindling had no significant effect on the width of the upper blade, crest, or lower blade [All *P*s > 0.382, Fig. 3-6, Panel B] of the dentate gyrus. Similarly, kindling had no effect on the number of NeuN-labeled cells in either the ipsilateral or contralateral hilus [All *P*s > 0.308, Fig. 3-6, Panel C].

4. Discussion

The results of this study make several important points about the effect of kindling on adult hippocampal neurogenesis. First, short term kindling had greater effects than long-term kindling on hippocampal neurogenesis as measured by DCX immunolabeling. However, long-term kindling increased dendritic outgrowth and complexity in DCX-labeled cells whereas short-term kindling did not. Long-term kinding also increased the number of ectopic granule cells in the ipsilateral hilus, which coincided with fewer reelin-labeled cells and less DISC1 expression in the SGZ of the dentate gyrus. These results extend past work by showing that amygdala kindling influences the positioning and morphological development of new neurons in a manner that parallels chemoconvulsant models of temporal lobe epilepsy. More importantly, they are the first to suggest a role for DISC1 in the pathophysiology of temporal lobe epilepsy and further suggest that

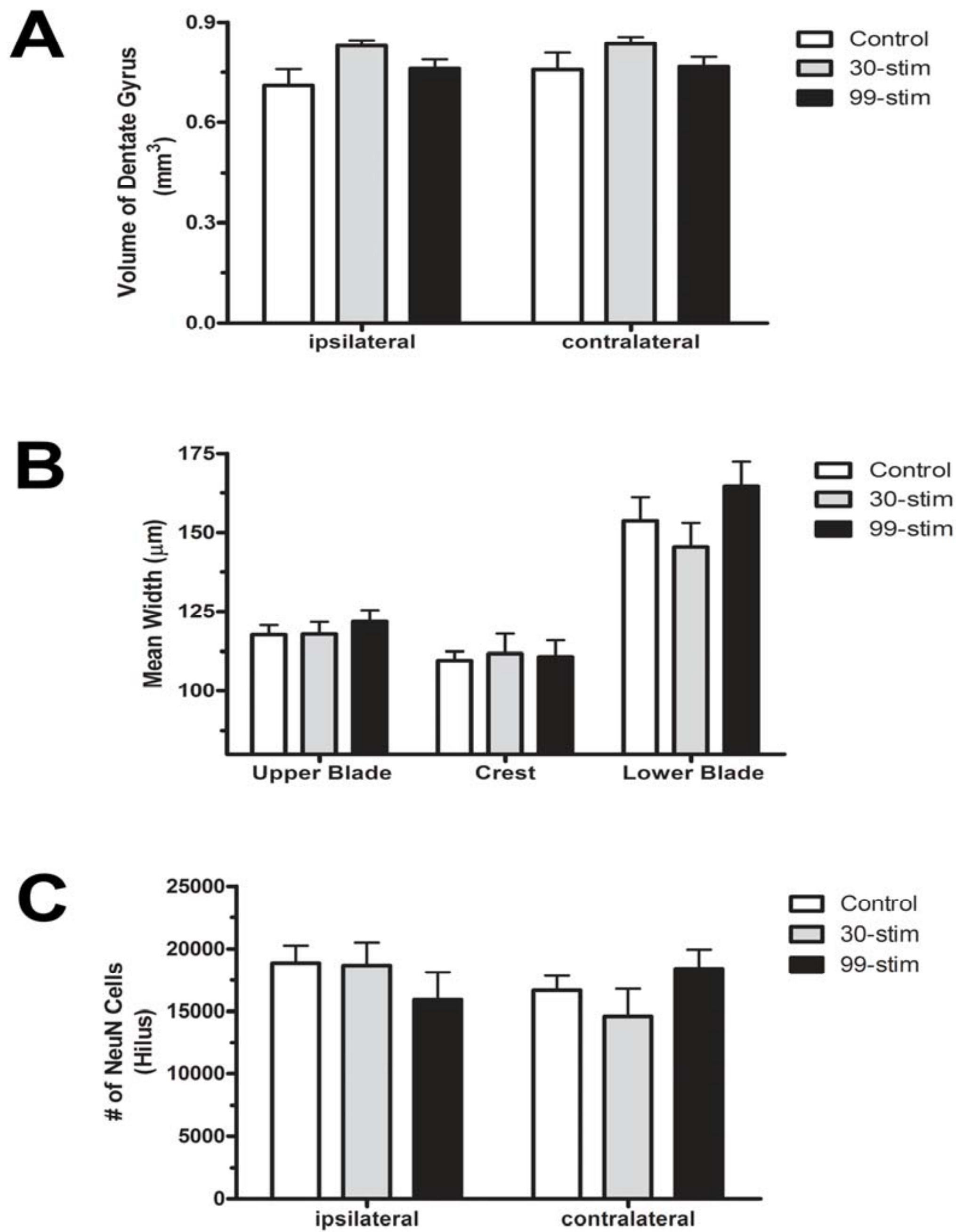

Figure 3-6 Effect of kindling on the dentate gyrus volume, width, and the number of neurons in the dentate hilus. Panel A) Total volume estimation of the dentate gyrus in after kindling. Panel B) The mean width of the granule cell layer for the upper blade, crest, and lower blade of the dentate gyrus after kindling. Panel C) The number of neurons (NeuN-labeled) in the hilus after kindling. There were no significant differences among the groups in these measures.

changes in reelin and DISC1 expression may contribute to aberrant neurogenesis after seizures.

4.1. Kindling-induced changes in hippocampal neurogenesis

In agreement with past studies (Parent et al., 1998; Scott et al., 1998), we found that short-term kindling impacted hippocampal neurogenesis by substantially increasing the number of DCX-labeled immature neurons in the SGZ. However, this effect was not maintained with long-term kindling as the long-term kindled rats had levels of DCX-labeled cells that were intermediate between the short-term kindled and control rats. It is not clear why the effect of kindling on neurogenesis would depend on the extent of kindling stimulation. It is possible that a reduction in the number of DCX-labeled cells associated with long-term kindling simply reflects decreased survival of early proliferating neurons (Mohapel et al., 2004) or decreased adoption of a neuronal fate. On the other hand, it is more likely that diminished neurogenesis after long-term kindling results from decreased levels of circulating mitogenic growth factor hormones (e.g., insulin growth factor, nerve growth factor, or fibroblast growth factor). Past work has shown that acute seizures can stimulate progenitor division through upregulation of various growth factors (Hattiangady et al., 2004; Katoh-Semba et al., 1999; Tirassa and Costa, 2007). In contrast, repeated seizures lead to a compensatory downregulation in growth factor expression thus diminishing progenitor responsiveness to further seizure activity. In fact, both fibroblast growth factor-2 and insulin-like growth factor-1 (IGF-1) levels are decreased in the hippocampus of chronically epileptic rats (Hattiangady et al., 2004) and IGF-1 binding sites in the dentate gyrus are significantly lower after long-term kindling (Kalynchuk et al., 2002). These observations suggest that the responsiveness of

progenitor cells to extrinsic stimulation declines with repeated kindling through a mechanism possibly involving altered or reduced mitogenic growth factor expression.

In addition to increasing the rate of neurogenesis, seizures also accelerate the maturation and assimilation of new neurons into hippocampal circuits (for review *see* (Parent, 2008; Zhao and Overstreet-Wadiche, 2008)). Our data are consistent with this idea. We found that kindling altered the morphology of DCX-labeled granule cells. Interestingly, this effect also depended on the extent of kindling. Long term-kindling increased the complexity of apical dendrites within DCX-labeled cells and stimulated the growth of hilar basal dendrites, which may be a key component in the development of recurrent excitatory circuitry following epileptic seizures (cf. (Shapiro and Ribak, 2005)). In contrast, short-term kindling had minimal effects on dendritic outgrowth and complexity of DCX-labeled cells. In fact, most of the DCX-labeled neurons in these rats possessed morphological features reminiscent of newly proliferated cells (*see* (Plumpe et al., 2006)). Because doublecortin is robustly expressed for up to 3 weeks in post-mitotic neurons (Brown et al., 2003), it is likely that DCX-labeled cells in the long-term kindled rats simply had more time to mature than DCX-labeled cells in the short-term kindled rats. Therefore, our finding that long-term kindling increases the dendritic outgrowth of newly born neurons appears to support previous studies using chemoconvulsant models of epilepsy (e.g., pilocarpine: (Overstreet-Wadiche et al., 2006b); kainate: (Jessberger et al., 2007b)) and further suggests that the repeated induction of seizures can greatly impact the structural plasticity of developing neurons.

4.2. Ectopic hilar granule cells after long-term kindling

Immunostaining with the granule cell-specific transcription factor Prox1 also revealed a greater number of hilar ectopic granule cells on the side of stimulation in rats that underwent long-term kindling compared to short-term kindling. Although we did not perform pulse chasing experiments with BrdU, the fact that these ectopic cells were not present after short-term kindling strongly argues that this population of Prox1 cells most likely reflects the aberrant migration of new granule cells that were born around the 30th electrical stimulation—a time point when robust hippocampal neurogenesis and proliferation occurs with kindling (see above, (Parent et al., 1998)). One reason that might explain why ectopic neurons were observed in this experiment is that our long-term kindled rats experienced a large number of motor seizures (~68 class 5 seizures). In contrast, previous studies (Parent et al., 1998; Scott et al., 1998) that have examined the effects of kindling on hippocampal neurogenesis and cell migration have typically employed short-term kindling protocols (i.e., between 4 and 10 class 5 seizures). Although these parameters may be sufficient to increase cell proliferation, they appear to be insufficient for triggering aberrant cell migration. This suggests that aberrant neurogenesis depends on the number of elicited seizures and the resultant circuitry rearrangement that follows the repeated induction of seizures, a finding that may help explain why past studies using the kindling model have not previously reported ectopic cell migration.

4.3. Potential molecular mechanisms regulating aberrant seizure-induced neurogenesis

How does the repeated induction of epileptic seizures prevent newly generated neurons from attaining their appropriate position? One possibility is that the induction of neuronal degeneration after repeated seizures may have altered the microenvironment of proliferating cells in such a way to promote improper migration (Mohapel et al., 2004). However, this appears to be an unlikely explanation because we did not observe any significant change in overall dentate granule cell volume, granule cell layer expansion, or in the number of hilar neurons in the ipsilateral or contralateral hemisphere after either short-term or long-term kindling. Instead, we believe that the migratory deficits found after long-term kindling might reflect direct changes in the expression of guidance proteins that are important in regulating neuroblast trajectories. Neurodevelopmental work has shown that reelin acts on clusters of migrating neuroblasts by serving as a detachment signal that promotes the correct positioning of neurons in laminated structures such as the hippocampus (Hack et al., 2002). In support of this view, we found that the presence of ipsilateral ectopic hilar granule cells after long-term kindling coincided with decreased reelin expression in the ipsilateral dentate SGZ. Importantly, we did not see a change in the number of reelin expressing neurons in the dentate SGZ after short-term kindling, a finding that may explain the lack of ectopic hilar granule cells in these animals. Although previous studies have shown that unilateral intrahippocampal kainate injections or systemic treatment with pilocarpine decreases reelin expression (Gong et al., 2007; Heinrich et al., 2006), we now extend these important findings to show that reelin expression is also decreased after long-term amygdala kindling, a model

of temporal lobe epilepsy that does not typically result in significant degeneration or cellular injury.

Changes in DISC1 expression may also contribute to the impaired migration and development of new neurons after seizures. DISC1 is highly abundant in the developing brain but its pattern of expression in the adult brain is restricted to regions that continuously generate new neurons in adulthood such as the hippocampus and olfactory bulb (Austin et al., 2004). We found that long-term but not short-term kindling decreased DISC1 immunolabeling within the dentate granule cell layer and SGZ on both ipsilateral and contralateral sides. There was no evidence of kindling-induced changes in DISC1 expression in the inner molecular layer or hilus. Among the many partners of DISC1, NDEL1 (nuclear distribution gene E homolog 1) is especially interesting because it is also a downstream target of the signal cascade derived from reelin (D'Arcangelo, 2006). This convergence between reelin signaling and DISC1-binding proteins may help shed light on our finding that long-term kindling only produces ectopic granule cells on the side of stimulation, and our finding that short-term kindling does not produce ectopic cell migration. In fact, Duan and colleagues (Duan et al., 2007) recently proposed that DISC1 does not function as a direct mediator of cell migration but that it functions to relay positional signals to migrating neurons, an interpretation that is consistent with our hypothesis that the collective decrease in reelin and DISC1 after a critical number of elicited seizures is required to provoke aberrant neurogenesis. Interestingly, retroviral silencing of DISC1 also accelerates morphological development of adult generated granule cells (Duan et al., 2007). These findings are extremely interesting in light of our observation of increased outgrowth and complexity of the apical dendrites in immature

DCX-labeled granule cells after long-term kindling. It is important to note that these findings occurred in both the ipsilateral and contralateral dentate SGZ and appear to parallel changes in DISC1 immunolabeling in both hemispheres. Interestingly, pilocarpine-induced seizures can also result in rapid dendritic growth of new neurons (Shapiro et al., 2007a) in a manner that parallels the effect of kindling on newly generated neuron. Importantly, these morphological changes also coincide with the accelerated integration of these cells into hippocampal networks (Overstreet-Wadiche et al., 2006b) suggesting that decreased levels of DISC1 in the epileptic brain may be an important molecular mediator in the aberrant development of new neurons in the epileptic hippocampus and in regulating the precocious integration of these cells into hippocampal networks following seizure activity.

4.4. Functional implications of aberrant neurogenesis

An outstanding question concerns the functional consequences of abnormal neurogenesis in the epileptic hippocampus. One consequence may be an enhancement of epileptiform activity. It is thought that the improper integration of neurons may contribute to the emergence of spontaneous recurrent motor seizures (Jung et al., 2004; Scharfman et al., 2000). Although we did not observe spontaneous seizures at any time during our study, extended kindling up to around 200 stimulations can elicit spontaneous seizures (Brandt et al., 2004; Michalakis et al., 1998; Pinel and Rovner, 1978) suggesting that our long-term kindled rats could represent an important transitional group for studying the progression of epileptogenesis. Our finding of increased ectopically located granule cells in the hilus before the onset of spontaneous seizures makes it tempting to

also speculate that the emergence of an abnormally situated granule cell network may provide a framework for the generation of spontaneous epileptiform activity.

A second potential consequence of abnormal neurogenesis in the epileptic hippocampus is that it may promote some of the cognitive and behavioral deficits associated with chronic epilepsy (Scharfman and Gray, 2007). We have previously shown that that there is a close relationship between the number of kindling stimulations and the development of anxiety and cognitive deficits (Kalynchuk et al., 1997; Kalynchuk, 2000). Whereas short-term kindled rats typically do not show robust changes in behavior after kindling, long-term kindled rats consistently show increased fear and anxiety-like behavior when placed in an unfamiliar open field and learning impairments when tested in a delayed-matching-to-sample water maze task (Fournier et al., 2008; Kalynchuk et al., 2001; Kalynchuk and Wintink, 2005). This is paralleled by the fact that only the long-term kindled rats had ectopic granule cell migration and decreased reelin and DISC1 expression. Interestingly, deficits in reelin and DISC1 are ostensibly linked to the etiologies of various neuropsychiatric disorders, including schizophrenia, depression, and bipolar disorder (Costa et al., 2002; Porteous et al., 2006). Considering that temporal lobe epileptic patients also report a higher incidence of depression, anxiety, and psychosis (Bear, 1985; Gilliam et al., 2004; Tebartz Van et al., 2002), it remains possible that deficits in reelin and/or DISC1 signaling may contribute to the emergence of behavioral and cognitive disturbances in chronically epileptic patients. If this is true, then developing strategies to "correct" migrational deficits after seizures might be prophylactic against the development of these problematic behavioral disorders.

4.5. Conclusions

Our study provides the first evidence that long-term kindling can result in a decrease in both reelin and DISC1 expression, two proteins important in aiding neuronal migration and development, within the adult dentate SGZ. These changes coincided with the appearance of hilar ectopic granule cells and with the aberrant growth and development of dendrites from immature neurons. Given that hippocampal neurogenesis can persist in the adult human hippocampus (Couillard-Despres et al., 2005; Eriksson et al., 1998) and patients suffering from temporal lobe epilepsy show evidence of progenitor cell migratory defects (Houser, 1990; Liu et al., 2008; Parent et al., 2006), our findings may have important implications for understanding the pathophysiology of human temporal lobe epilepsy. Future studies employing pulse-chase BrdU methodology in the kindling model will be important to help clarify the precise time point when potential ectopic neurons are generated and begin to display abnormal migrational properties. Finally, the results presented here also raise the intriguing possibility that signal transduction pathways instigated by reelin or DISC1 may be potential neurobiological targets for new therapeutic interventions for the treatment of seizure-induced migratory deficits and neuropathological changes within the hippocampal formation of patients with temporal lobe epilepsy.

CHAPTER 4

Decreased Levels of Disrupted-in-Schizophrenia 1 (DISC1) Are Associated With Expansion of the Dentate Granule Cell Layer in Normal and Kindled Rats

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1. Introduction

Disrupted-in-schizophrenia 1 (DISC1) has recently emerged as an important candidate gene involved with the development of schizophrenia (Camargo et al., 2007). Several studies have suggested that DISC1 is a multifunctional protein that plays a prominent role in extracellular signaling, neurite outgrowth, and migration (Morris et al., 2003). Although expression levels of DISC1 are particularly high during development (Austin et al., 2004), DISC1 mRNA continues to be expressed in the adult brain primarily in the neocortex, hippocampus, cerebellum, and olfactory bulbs (Ma et al., 2002; Meyer and Morris, 2008). Recently, Duan and colleagues (2007) showed that downregulation of DISC1 results in aberrant morphological development and migration of adult generated dentate granule cells. Interestingly, DISC1 downregulation also accelerated the integration of these new cells into hippocampal circuits suggesting that it may play an important role in regulating the functional incorporation of new neurons in the adult brain (Duan et al., 2007).

Seizure activity stimulates the birth of new dentate granule cells and can also influence the migration of these cells (Jessberger et al., 2007b; Parent et al., 2006). These ectopically located granule cells show hypersynchronicity with CA3 pyramidal neurons and it is thought that they may contribute to the process of epileptogenesis (Scharfman et al., 2000). Moreover, seizures have also been found to accelerate the integration of new neurons into circuitry of the adult dentate gyrus (Overstreet-Wadiche et al., 2006b). The obvious similarity between DISC1 downregulation and seizure-induced changes within the dentate gyrus suggests a possible link between impaired DISC1 expression and many of the histopathological features associated with temporal lobe epilepsy. To test this hypothesis, we evaluated whether changes in DISC1 immunolabeling within the dentate

gyrus was associated with expansion of the dentate granule cell layer in adult rats that underwent extended amygdaloid kindling (i.e., received 99-electrical stimulations). We chose an extended kindling paradigm for this study because it provides a way to examine the effect of a large number of generalized seizures on hippocampal morphology in the absence of the dramatic pathology often observed in the pilocarpine or kainate models of temporal lobe epilepsy (Brandt et al., 2004; Kalynchuk, 2000; Michalakis et al., 1998).

2. Materials and Methods

All procedures were carried out in strict accordance with the Canadian Council of Animal Care guidelines and were approved by the University of Saskatchewan Animal Care and Use committee. Twenty male Long-Evans rats, weighing approximately 250-300 g at the time of arrival, served as the subjects in this study. The rats were kept under controlled environmental conditions (20–22°C; 50–60% humidity; 12:12 h light:dark cycle; light on at 07:00 h) with free access to standard laboratory chow and tap water. After a 1-week acclimation period, a single bipolar electrode (MS-303-2, Plastics One, Roanoke, VA, US) was chronically implanted into the left basolateral amygdala (BLA) at the following stereotaxic coordinates: -2.8 mm AP, +5.0 mm ML, and -8.5 mm V from the surface of the cranium (Paxinos and Watson, 1998). The electrode was secured to the skull with four stainless steel screws and dental acrylic. A topical antibacterial ointment (Flamazine, 1% silver sulfadiozine) was applied around the incision site in order to reduce the risk for infection. After surgery, animals were kept warm until they were ambulatory.

Amygdaloid kindling initiated following a recovery period of at least 3 weeks. The rats were randomly divided into two groups: One group received 99 (n=10) kindling

stimulations and the other group received 99 sham stimulations (n=10). Each stimulation consisted of a 1 sec, 60 Hz train of pulses with a biphasic amplitude of 800 μA (peak-to-peak) and a duration of 1 ms. These kindling parameters are well above the threshold for eliciting afterdischarges in the rat amygdala (Racine, 1972a). The stimulations were delivered 3 times per day, 5 days per week, with a minimum of 2 hrs between consecutive stimulations. Rats receiving sham stimulations were treated in exactly the same manner except that no current was delivered. The behavioral convulsion elicited by each stimulation was classified according to Racine's popular five-stage scale (Racine, 1972b).

Twenty-four hours after the delivery of the final stimulation, all animals were transcardially perfused with room temperature phosphate-buffered saline (0.1 M PBS, pH 7.4) followed by ice-cold 4% (w/v) paraformaldehyde. Immunostaining was performed simultaneously on tissue from control and kindled rats using methodology previously described (Fournier et al., 2008). Briefly, free-floating 50μm thick sections were incubated at 4ºC with either a goat polyclonal anti-DISC1 (1:1000, incubated for 24 hrs, sc-47990, Santa Cruz Biotechnology) or a rabbit polyclonal anti-Prox-1 (1:5000, incubated for 72 hrs, PRB-238C, Covance) antibody that was dissolved in a solution consisting of 5% (v/v) normal animal sera, 1% (w/v) bovine serum albumin, and 0.3% (v/v) Triton X-100 and PBS. Following washes with PBS, sections were incubated with appropriate biotinylated secondary antibodies diluted in 0.3% (v/v) Triton X-100 in PBS for 2 hrs (1:200, Vector Laboratories) and then placed in avidin-biotin-peroxidase complex for 1 hr (1:500, Vectastain ABC Elite, Vector Laboratories) at room temperature. Immunolabelling was visualized using nickel-intensified DAB to yield a

blue-black product. The specificity of the antibodies was confirmed by simultaneously running tissue in the absence of the primary antibodies. No staining was observed. And finally, one series of tissue was Nissl-stained with cresyl violet in order to confirm electrode placements.

To examine the effect of kindling on DISC1 expression, semi-quantitative densitometry was performed on the following regions of interest: 1) hilus, 2) subgranular zone, 3) granule cell layer, and 4) inner molecular layer. The subgranular zone (SGZ) was defined as a two cell width region beginning from the inner margin of the dentate granule cell layer. Because our preliminary analyses showed that kindling had greater effects on DISC1 expression on the ipsilateral side compared to the contralateral side, we focused our analyses in this study on regions located on the ipsilateral side only (i.e., the same side as the electrical stimulation). Sections were captured using a digital camera (at 12-bit resolution) that was attached to a light microscope (Nikon Eclipse E600) with camera exposure and gain settings held constant between animals. Using an image-analysis software package (ImageJ 1.35, National Institute of Mental Health), the mean relative optical density was calculated for each region from three adjacent coronal sections (300 μm distance between sections) at the level of the dorsal hippocampus. Background staining was controlled by calculating the average optical density levels from the corpus callosum and subtracting these values from the regions of interest. Hilar ectopic Prox-1 immunolabeled cells were directly counted across 8 to 10 sections. In addition, the extent of granule cell dispersion was also evaluated in Prox-1immunolabeled tissue by directly measuring the width of the dentate granule cell layer

at 50 μm increments for the upper blade, lower blade, and crest using an image-analysis program (ImageJ 1.35, National Institute of Mental Health).

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS v 16.0, Chicago, IL, USA). The criterion for statistical significance was set at $P < 0.05$. All data are represented as mean ± standard error of the mean.

3. Results

All rats had electrodes correctly inserted into the left BLA. The initial electrical stimulations elicited short episodes of behavioral arrest that were often accompanied by repetitive head nodding and facial movements restricted mainly to the maxillary and mandibular regions. With subsequent stimulations, these episodes progressed into stage 5 generalized seizures that consisted of bilateral forelimb clonus, rearing, and loss of equilibrium (latency to first stage V: 14.2 ± 2.93). The total number of stage 5 seizures experienced by the kindled rats was 73.9 ± 5.32. In accordance with past work (Brandt et al., 2004; Michalakis et al., 1998; Pinel and Rovner, 1978), kindled rats that received 99-electrical stimulations did not demonstrate spontaneous motor seizures at any time during routine handling or during random observations throughout the duration of this experiment.

Consistent with the observations of Meyer and Morris (Meyer and Morris, 2008), DISC1 immunolabeling was prominent within the somata of dentate granule cells (Fig. 4-1 a,b). Semi-quantitative densitometeric analysis revealed a significant reduction in DISC1 labeling in the dentate granule cell layer and subgranular zone of kindled rats (Fig. 4-1c, all ps < .032, Student t-test). In contrast, there was no change in DISC1

expression in the hilus or inner molecular layer after kindling (all *p*s > .770, Student *t*-test).

The length of the granule cell layer for the upper and lower blades as well as for the crest of the dentate gyrus was determined after staining brain sections for the granule cell-specific transcription factor Prox-1 (Steiner et al., 2008) (Fig. 4-2a). Length measurements did not identify a significant change in width of the granule cell layer in the kindled rats (Fig. 4-2b, all *p*s > .132, Student *t*-test). However, the number of Prox-1 labeled ectopic granule cells in the hilus was significantly greater in the kindled rats compared to sham-stimulated controls (Sham: 44.0 ± 9.98, Kindled: 78.2 ± 12.1, $p<.045$, Student *t*-test, Fig. 4-3). Interestingly, a positive correlation was found between increased width of the upper blade and the number of ectopic granule cells in the hilus (r = .508, $p < .031$, Fig. 4-4a).

Correlational analyses revealed a significant association between levels of DISC1 expression and width of the dentate granule cell layer. More specifically, negative correlations were found for increased width of the upper blade of the dentate gyrus and DISC1 expression in the dentate granule cell layer (r=-.582, $p < .007$) and SGZ (r = -.520, $p < .019$), respectively. In addition, negative correlations were found for increased width of the crest and DISC1 expression in the inner molecular layer (r=-.512, $p < .031$) and the granule cell layer (r=-.462, $p < .048$). Finally, negative correlations were also found for the number of ectopic granule cells and DISC1 expression in the dentate granule cell layer (r=-.476, $p < .046$) and in the SGZ (r=-.539, $p < .015$) (Fig. 4-4b). No other correlations were statistically significant.

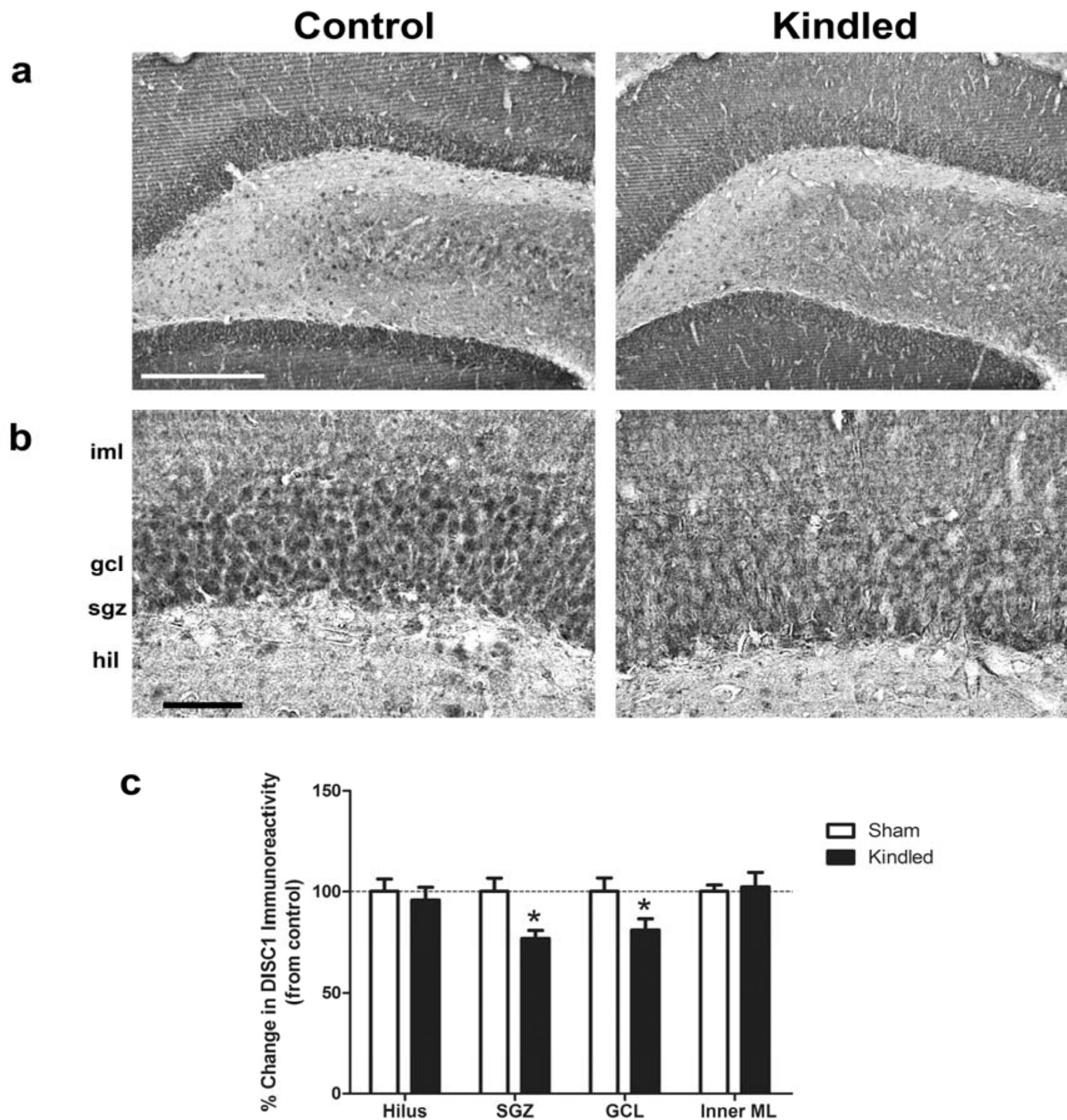

Figure 4-1 Effect of extended kindling on DISC1 expression in the adult dentate gyrus. a) Photomicrographs showing an example of DISC1 immunolabeling in the dentate gyrus of rats that received sham stimulations or extended kindling. Scale Bar: 300 µm. b) Higher power magnification showing an example of DISC1 immunolabeling in the dentate gyrus of rats that received sham stimulations or extended kindling. Scale Bar: 50 µm. c) Percentage change in DISC1 expression in the hilus, subgranular zone, granule cell layer, and inner molecular layer after extended kindling. Error bars represent mean ± standard error of the mean. * denotes a statistically significant difference from sham controls ($p<0.05$). Abbreviations: hilus (hil), subgranular zone (sgz), granule cell layer (gcl), and inner molecular layer (iml).

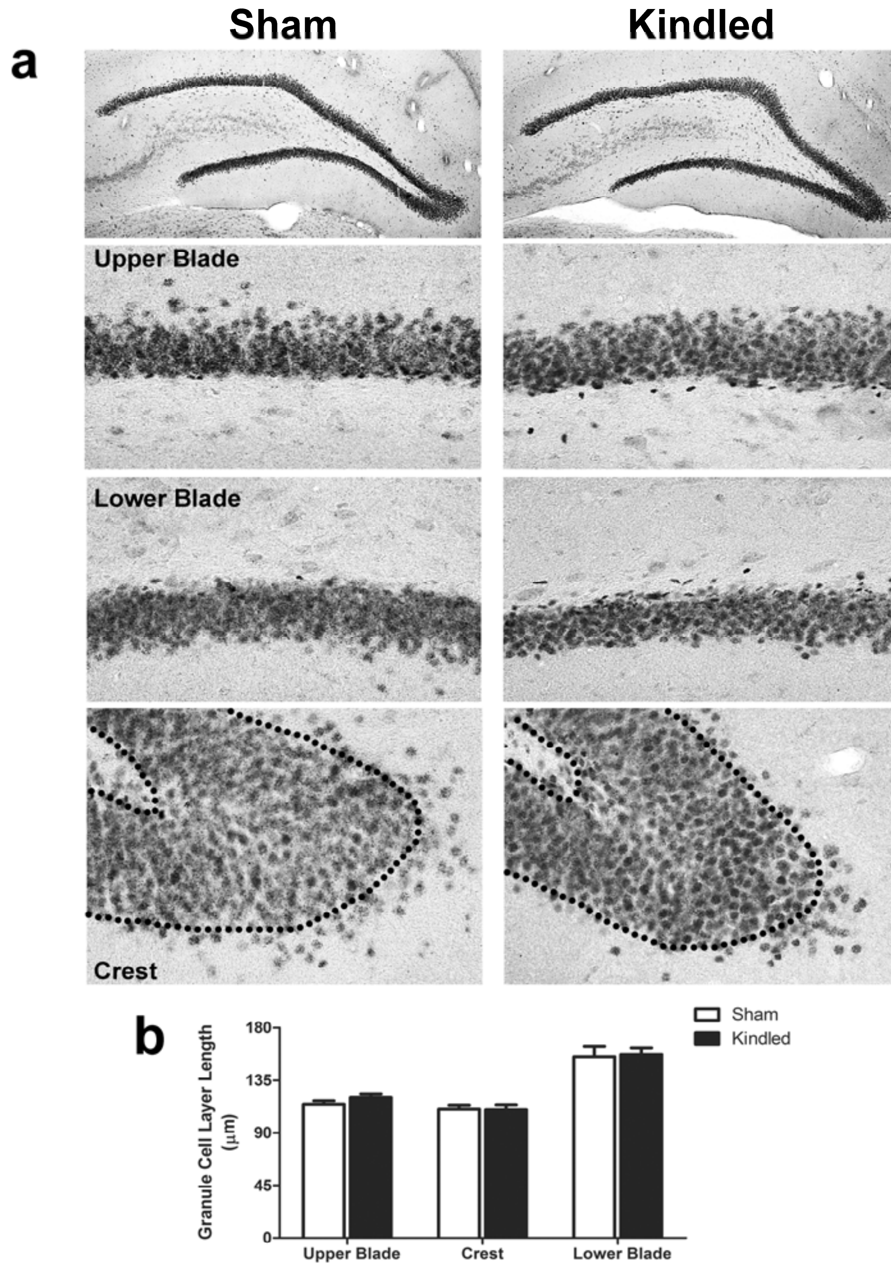

Figure 4-2 Effect of extended kindling on adult dentate gyrus morphology. a) Photomicrographs showing Prox-1 immunolabeling in the upper blade, lower blade, and the crest of the dentate gyrus of rats that received sham stimulations or extended kindling. The dotted line outlines the boundary of the crest of the dentate gyrus. b) Mean granule cell layer length for the upper blade, lower blade, and crest in rats that received sham stimulations or kindling. Error bars represent mean ± standard error of the mean.

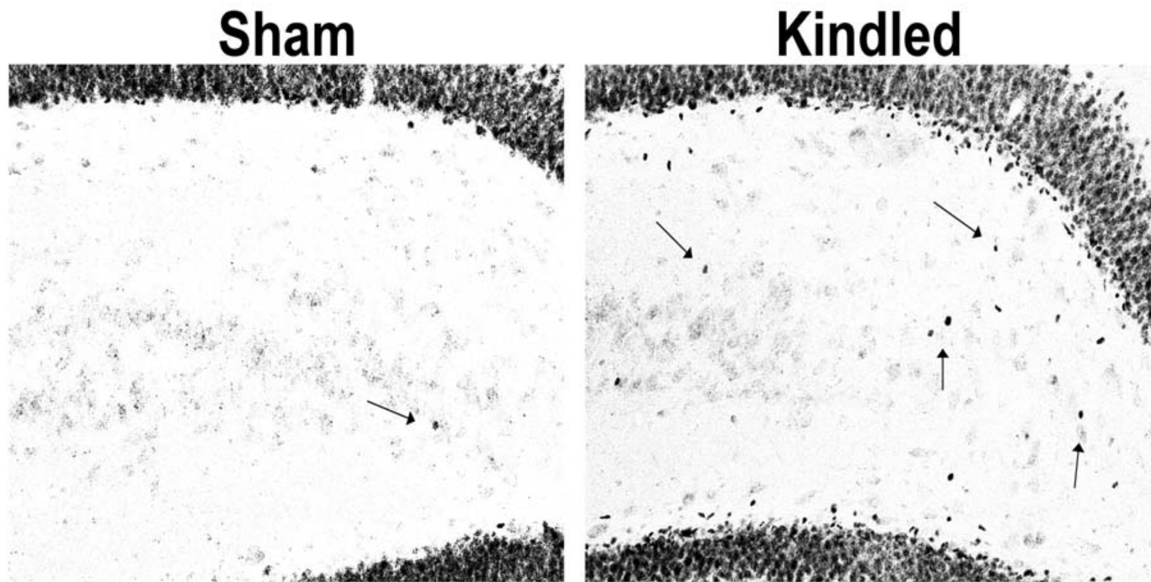

Figure 4-3 Effect of extended kindling on ectopic cell migration. Phototomicrographs showing ectopic Prox-1 immunolabeled cells in the hilus of rats that received sham stimulations or extended kindling. Arrows point to examples of ectopic Prox-1 granule cells in the hilus.

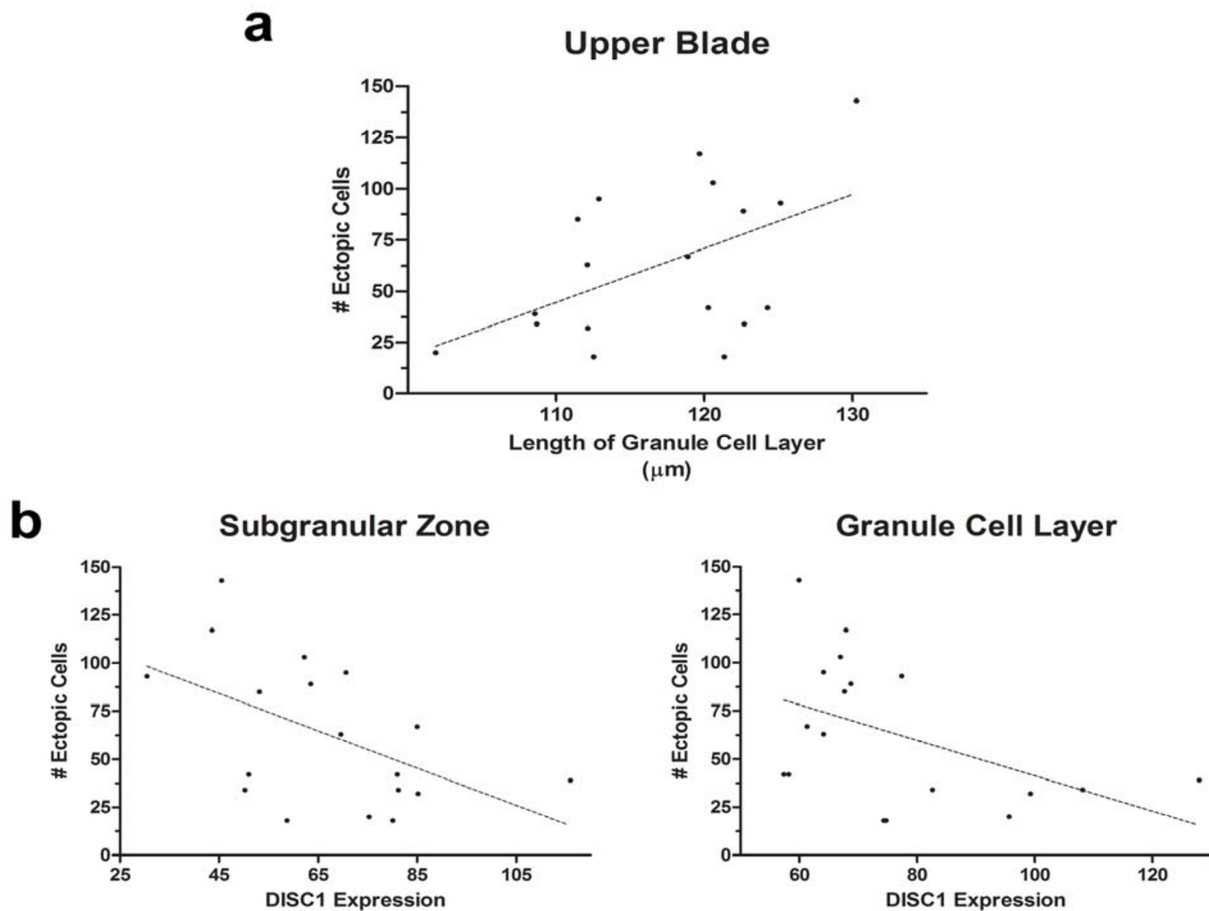

Figure 4-4 Correlations between DISC1 expression and cytoarchitectural organization of the adult dentate gyrus. a) Scattergram plot displaying the relationship between the number of ectopic granule cells and length of the granule cell layer in the upper blade of the dentate gyrus ($r^2 = 0.26$, $p<0.03$). b) Scattergram plots displaying the relationship between the number of ectopic granule cells and DISC1 expression in the SGZ ($r^2 = 0.29$, $p<0.02$) and granule cell layer ($r^2 = 0.22$, $p < 0.05$).

4. Discussion

The present study provides the first evidence that the repeated induction of seizure activity through electrical kindling of the amygdala results in decreased DISC1 immunolabeling within the dentate granule cell layer and subgranular zone. Although the width of the granule cell layer was virtually identical in the sham-stimulated and kindled rats, an association was found between declining levels of DISC1 expression throughout the granule layer and subgranular zone, and expansion of the granule cell layer in the upper blade and crest of the dentate gyrus. In addition, kindled rats also showed a greater number of ectopic granule cells in the hilus, which in turn was associated with decreased DISC1 expression in the SGZ and granule cell layer for the upper blade. Taken together, the results from this study suggest that alterations in DISC1 expression may be associated with changes in the shape of the dentate gyrus and may contribute to aberrant migration of neurons after seizure activity.

Architectural disturbances of the dentate gyrus, including granule cell layer expansion and cell loss, are frequently observed in patients suffering from medically refractory temporal lobe epilepsy (El Bahh et al., 1999; Houser, 1990; Thom et al., 2005). Although extensive dispersion of granule cells is commonly observed in experimental status epilepticus models associated with significant neuronal injury (Mello et al., 1992; Nitta et al., 2008), we did not detect granule cell layer expansion after extended kindling. Instead, subtle expansion of the dentate granule layer was found to be associated with lower levels of DISC1 across the dentate granule cell layer (including the subgranular zone) in both normal and kindled animals. We cannot exclude possibility that in addition to changes in the dentate gyrus, DISC1 levels may be impacted in other areas of hippocampal formation after extended kindling. However, in the adult rat hippocampal

formation, DISC1 expression tends to be highest in the dentate gyrus. While moderate staining is found in CA3 subfield, minimal staining is found in CA1 subfield. For instance, CA3c tends to show a decrease in DISC1 immunolabeling after extended kindling (Fournier & Kalynchuk, unpublished observations). Interestingly, DISC1 labeling has been confirmed in GABAergic interneurons (Meyer and Morris, 2008). Considering that the loss of selective subtypes of interneurons in the dentate gyrus may be a key cause in the initiation of seizures (Dudek and Sutula, 2007), it will be worthwhile for subsequent studies to investigate the potential relationship between DISC1 and interneuronal populations in the epileptic brain.

Extended kindling also increased the number of ectopically positioned granule cells in the hilus. Although we did not perform cell proliferation measurements in the present study, others have shown that amygdala kindling significantly increases hippocampal neurogenesis (Parent et al., 1998; Scott et al., 1998). As a result, it is likely that the ectopic hilar granule cells found in our study reflects the aberrant migration of new neurons generated during the kindling process. However, because vigorous stereological procedures were not employed in our study, the total number of ectopic neurons generated after extended kindling cannot be fully addressed and thus our quantification of Prox-1 labeled ectopic cells must be viewed with some caution. Nonetheless it is interesting that higher numbers of ectopic granule cells were associated with lower levels of DISC1 expression in both normal and kindled animals. This may be particular noteworthy because decreased expression of DISC1 in newly generated neurons has been shown to cause aberrant migration of newly generated granule cells (Duan et al., 2007). Importantly, seizure activity also promotes aberrant migration (Parent

et al., 2006). Therefore, it is conceivable that impaired DISC1 signaling in newly generated neurons may contribute to altered migration of these cells and subsequent changes in dentate granule cell layer width following seizures.

At present, we do not know what the functional significance that subtle expansion of granule cell layer might have on the normal operation of the dentate gyrus. However, granule cell expansion is typically associated with enhanced cellular excitability and increased propensity for seizures (Rougier et al., 2005). Although speculative, considering the marked individual differences present in the risk for developing epilepsy after traumatic brain injury (Frey, 2003), it is possible that even slight variations in dentate gyrus structure across a normal population may have an understated effect in increasing the likelihood of generating epileptiform activity in some people after injury has occurred.

It is also plausible, however, that these associations are simply spurious in nature. Although we cannot presently rule out this possibility, we do believe that this conclusion is unlikely considering the specificity associated with these effects (i.e., significant and robust correlations were only found for certain regions of the dentate gyrus). Future studies will be necessary in order to examine the full extent of granule cell dispersion in the normal population, and possibly through the use retroviral transfection systems, whether intrinsic differences in the level of DISC1 expression contribute to this process.

To date a number of studies have also linked impairments in DISC1-related pathways with the development of several neuropsychiatric complications, including schizophrenia and mood disorders (Blackwood and Muir, 2004; Millar et al., 2004). These findings are of particular interest because architectural disturbances of the dentate

gyrus have been found in some patients suffering from schizophrenia (Arnold, 1999). For instance, post-mortem examination have revealed a particularly high incidence of granule cells with basal dendrites in schizophrenic patients (~46%) compared to controls (~28%) (Senitz and Beckmann, 2003), a finding that has also been shown in patients with temporal lobe epilepsy (von Campe et al., 1997). Moreover, it well known that schizophrenic patients can benefit from antiepileptic medication (Rogawski and Loscher, 2004). Interestingly, patients suffering from temporal lobe epilepsy also have an elevated risk for developing psychosis and depression compared to patients with non-temporal lobe seizure disorders (Kanner, 2008b; Sachdev, 1998). Despite the considerable evidence linking these seemingly diverse conditions, the underlying mechanism that give rise to the frequent occurrence of psychoses and seizures is poorly understood. Our finding that DISC1 levels are decreased in the dentate gyrus after extended kindling may shed a new light on a potential molecular mechanism that could be involved in the co-morbidity between epilepsy and psychosis.

In conclusion, the findings of this study provide the first evidence that seizures can directly influence DISC1 expression in the dentate gyrus and that variation in DISC1 expression may contribute to subtle cytoarchitectural abnormalities of the dentate gyrus.

CHAPTER 5

Impaired Functional Integration of Seizure-Generated Granule Cells in Behaviourally-Relevant Networks of the Adult Dentate Gyrus

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1. Introduction

New neurons are continuously generated in the dentate gyrus throughout adult life (Altman, 1962; Eriksson et al., 1998; Kempermann et al., 1997; Kempermann and Gage, 1999; Kronenberg et al., 2003; Schinder and Gage, 2004). The majority of these newly generated dentate granule cells appear to be synaptically integrated into the existing hippocampal circuitry within 1 month after their birth (Jessberger and Kempermann, 2003; Snyder et al., 2001; van Praag et al., 2002). Due to the robust capacity for synaptic plasticity displayed by adult generated granule cells, it has been speculated that neurogenesis may be essential for various aspects of hippocampal function (e.g., Clelland et al., 2009). Indeed, several recent studies using c-fos and Arc expression as indicators for neuronal activation have confirmed that new dentate granule cells may be readily recruited into hippocampal circuits that are critical for spatial learning and memory formation (Farioli-Vecchioli et al., 2008; Kee et al., 2007b; Ramirez-Amaya et al., 2006; Tashiro et al., 2007; Trouche et al., 2009).

One of the most extreme forms of neuronal activation, seizure activity, has been shown to significantly increase the birth of new dentate granule cells (Fournier et al., 2009; Jessberger et al., 2005; Nakagawa et al., 2000; Parent et al., 1997; Parent et al., 1998; Parent et al., 2006). Prolonged seizures also accelerate the functional maturation of newly born granule cells permitting them to integrate into network circuits sooner than under normal conditions (Overstreet-Wadiche et al., 2006b). However, many of these cells also display altered morphological characteristics compared to normal granule cells, such as greater dendritic outgrowth (Arisi and Garcia-Cairasco, 2007; Overstreet-Wadiche et al., 2006b) and the sprouting of basal dendrites (Ribak et al., 2000; Shapiro and Ribak, 2005). In addition, a sizeable fraction of new granule cells migrate ectopically

into the hilus or molecular layer (Crespel et al., 2005; Parent et al., 1997; Parent et al., 2006). Electrophysiological studies have confirmed that these mispositioned cells display an unusual propensity for burst-firing synchronization with CA3 pyramidal neurons suggesting that they may contribute to the generation of spontaneous epileptiform activity and general hippocampal dysfunction (Scharfman et al., 2000; Scharfman and Gray, 2007).

Although seizures are the primary problem for many epileptic patients, one of the most frequent complainants in children and adults with epilepsy concerns learning and memory impairments (Chaix et al., 2006; Gleissner et al., 2005; Hendriks et al., 2004; Jokeit et al., 2001; Lah et al., 2006; Marques et al., 2007; Vannucci, 2007). Given the strong association between adult neurogenesis and hippocampal-dependent learning (Madsen et al., 2003; Saxe et al., 2006; Shors et al., 2001; Snyder et al., 2001), it is possible that aberrant neurogenesis observed in chronic epilepsy may adversely impact cognition (Scharfman and Gray, 2007). In prior studies, we have shown (Fournier et al., 2009; Kalynchuk et al., 1997; Kalynchuk et al., 1998; Kalynchuk, 2000; Wintink et al., 2003), as well as others (Adamec and Young, 2000; Adamec, 1990; Cammisuli et al., 1997; Hannesson et al., 2008; Helfer et al., 1996; Nieminen et al., 1992), that amygdaloid kindling affects a variety of behavioural measures in rats, such as increasing anxiety-like behaviour and impairing spatial learning. Kindling refers to the process by which initially subconvulsant electrical stimulation to a brain region, such as the amygdala, results in the progressive development of seizure activity and epileptic convulsions when stimulations are repeatedly administered (Goddard et al., 1969; Racine, 1972b). This method of seizure induction offers great advantages over conventional chemoconvulsant models of

epilepsy because important variables such as seizure frequency and interval between seizures can be experimentally controlled (Morimoto et al., 2004).

Previously, we have shown that kindling-induced molecular (i.e., changes in $GABA_A$ and $5\text{-}HT_{1A}$ receptor binding) and structural alterations (i.e., growth of new synaptic terminals) within the dentate gyrus and surrounding hippocampus are associated with the development of these behavioural changes (Fournier et al., 2008; Kalynchuk et al., 2001; Kalynchuk and Wintink, 2005). Importantly, there appears to be a strong relationship between the number of "kindled" stimulations and the development of behavioural changes, in which long-term amygdaloid kindling (i.e., 99-electrical stimulations) typically produces the most robust alterations in behaviour (Kalynchuk et al., 1997). These findings are particularly noteworthy as it has been found that during the initial stages of kindling (i.e., up to around 30-electrical stimulations), stimulations robustly increase hippocampal neurogenesis (Chapter 3) (Parent et al., 1998; Scott et al., 1998), but only mildly affect behavioural functioning (Kalynchuk et al., 1997)

Given the observation that newly born neurons require approximately 4 to 6 weeks to fully integrate into adult dentate networks, it is likely that neurons born after the 30th kindling stimulation would have a sufficient amount of time to differentiate, migrate, and begin circuit integration by the time the 99th kindling is delivered (i.e., ~4 to 5 weeks after the 30th kindled stimulation). However, because of the pathological features associated with seizure-generated neurons described earlier, it is also possible that the inappropriate integration of seizure-generated neurons into functioning circuits may interfere with normal hippocampal function and, thus, result in the development of maladaptive behaviour (Kalynchuk and Fournier, 2009; Scharfman and Gray, 2007).

In the following experiments, we used the long-term amygdala kindling model to test the propensity for seizure-generated dentate granule cells to become incorporated into functional circuits that support hippocampal-dependent learning. We labeled adult-generated granule cells with the DNA synthesis marker 5-bromo-2'-deoxyuridine (BrdU) at a time point when hippocampal cell proliferation was highest during the process of kindling (i.e., around the 30th stimulation) but continued delivering stimulations until the completion of the experiment. To determine if the seizure-generated neurons in the dentate gyrus could be activated during memory retrieval of learnt information acquired in a behavioural task, we quantified co-expression of the activity-dependent protein c-fos and BrdU following testing in a hippocampal-dependent trace fear conditioning paradigm. Auditory trace fear conditioning is a learning task that requires animals to associate an auditory conditioned stimulus (CS) and an unconditioned fear-producing stimulus (US; e.g., mild foot-shock) that had been separated by a temporal gap or 'trace' and past work has identified a critical role for the hippocampus in this task (Burman et al., 2006; Chowdhury et al., 2005; McEchron et al., 1998; Misane et al., 2005; Rogers et al., 2006). Our results show that despite the evidence that seizure-generated neurons are functionally integrated into adult dentate networks, the repeated induction of seizures appears to impair the recruitment of new neurons into circuits that support normal learning.

2. Materials and Methods

A total of 31 male Long-Evans rats (Charles Rivers, Montreal, QB, Canada) weighing approximately 200 to 300 g at the time of arrival served as subjects. They were individually housed in rectangular polypropylene cages with standard laboratory bedding

and kept on a 12:12 h light:dark cycle with lights on at 0700h local time. Ambient temperature was maintained between 20ºC (±1ºC). Food and water was available *ad libitum* throughout the duration of the experiment. All animals were treated in accordance with the Canadian Council for Animal Care and the University of Saskatchewan Animal Care Committee. All efforts were made to minimize the number of animals used in this experiment.

2.1. Surgery

After a week of daily handling and habituation to colony conditions, the rats were individually anesthetized with isoflurane (5% initial, 2-2.5% maintenance) and placed into a stereotaxic apparatus. Prior to surgery, all rats were treated with an analgesic to minimize pain from the surgical procedure (ketoprofen, 10 mg/kg, s.c., Merial, QB, Canada). A single bipolar electrode (MS-303-2, Plastics One, Roanoke, VA, US) was stereotaxically inserted into the left basolateral amygdala: -2.8 mm AP, +5.0 mm ML, and -8.5 mm V from the surface of the cranium (Paxinos and Watson, 1998). The electrode was secured to the skull with four stainless steel screws and dental acrylic. A topical antibacterial and antifungal agent (Hibitane, 1% chlorohexidine acetate, Ayerst Laboratories, Montreal, QB, Canada) was applied around the incision site to reduce the risk of infection.

2.2. Kindling Procedure

Following a two to three week recovery period, the rats were habituated to the stimulation lead and handled for an additional 7 days, after which all subjects were randomly assigned into one of three conditions: long-term kindling (99 stimulations;

n=12), short-term kindling (30 stimulations; n=9), or control (99 sham stimulations; n=9). To equalize the amount of handling received by the rats in each group, the rats in the short-term kindling group initially received 69 sham stimulations followed by 30 electrical stimulations. Prior to stimulation, each rat was placed in a plastic container containing a thin layer of commercial bedding, the wire lead was attached and the stimulation was delivered. Amygdala kindling consisted of a single 800 μA (peak-to-peak) electrical stimulation (60 Hz square-wave pulse; 1 msec pulse duration) with 1 sec train duration. The stimulations were delivered three times per day, 5 days per week, with a minimum of 3 h between stimulations. This stimulation protocol is well above the threshold necessary to elicit epileptiform discharges from the amygdala (Racine, 1972a). The convulsion class elicited by each stimulation was classified according to a modified version of Racine's widely used scale (Racine, 1972b): Class 0 - freezing; 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 loss of equilibrium; Class 6 - multiple stage 5's. Control animals were treated similarly to the kindled rats with the exception that no current was delivered after the stimulation lead was attached.

2.3. BrdU Injections

BrdU (BA-5002; Sigma-Aldrich) was dissolved in warm physiological saline (50°C) and then sterile filtered. Long-term kindled rats received three separate injections of BrdU (75 mg/kg; 20 mg/ml) approximately 4 hours after their 30th, 33rd, and 36th kindling stimulation. For the controls and short-term kindled rats, BrdU injections took

place during the corresponding period of sham stimulation (Fig. 5-1A). Thus, all animals received injections of BrdU at the same time point during the process of kindling.

2.4. Conditioning Chambers

The chambers (30.5 × 24.1 × 21.0 cm) contained a grid floor (19 parallel 0.48-cm diameter stainless steel rods, 1.6-cm apart) above a stainless steel waste pan (ENV-008; Med Associates, USA) and a loudspeaker (ANL-926; Med Associates, USA) mounted in the chamber wall. All rods were wired to a shock generator and scrambler (ENV-414S; Med Associates, USA). Each chamber was located inside a sound-attenuating cubicle with an internal fluorescent light source (ENV-022; Med Associates, USA). A ventilation fan provided a constant background noise of 60 dB. Delivery of stimuli was controlled with a PC and Video Freeze software through a SmartCTL Interface System (DIG-700F; Med Associates). Each conditioning chamber was equipped with a miniature high speed firewire monochrome video camera that was mounted on the inside of the cubicle door in order permit observation of behaviour during training and testing sessions. The video signals were captured at a sampling rate of 30 frames per second using Video Freeze software (Med Associates, USA) and directly stored onto a PC-type microcomputer for later off-line analysis by a blind observer.

For the tone test, the conditioning room was altered by hanging darkly colored curtains from the ceiling and using dim floor lamps as the main source of ambient light. The context of the chamber was also modified by including colored Plexiglas insert frames that altered the shape of the conditioning chamber from a rectangular to a semicircular frame. The top of conditioning chamber was also fitted with a black and white stripped polypropylene insert. The floor of the chamber was modified by providing

a solid white polypropylene base. A stainless steel pan scented with a small amount (~1.5 to 2 ml) of a mild vanilla solution was inserted under each floor in order to provide a distinct olfactory cue. A white noise generator (65 dB) was used to provide a distinct background noise. An assortment of various colored shapes was also placed strategically throughout the walls and floors cubicle in order to modify its context. Finally, transportation cues were also altered. For the conditioning and contextual test, the rats were transported to conditioning room in their home cages on a trolley using a predefined route. For the CS (conditioned stimulus, i.e., tone) test, the animals were placed in a novel clear Plexiglas container and transported by hand with a cover over top of the cage using a modified route from the previous training day.

2.5. Procedure

The day after the final kindled stimulation, rats began training on a trace fear conditioning paradigm. On day 1, the habituation day, two rats at a time were brought into a brightly lit experimental room and placed individually into one of two similar conditioning chambers and permitted to freely roam the conditioning chamber for a period of 10-min in order to acclimate the animals to the testing room and conditioning apparatus. The floor of the conditioning chambers was cleaned with a dilute 0.6% (v/v) acetic solution between subjects in order to minimize olfactory cues.

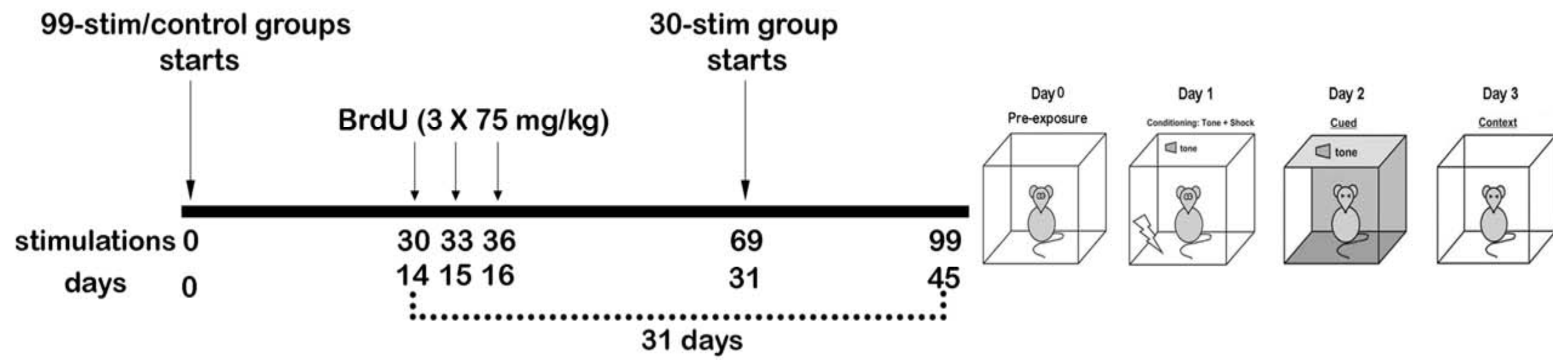
On day 2, the conditioning day, the rats were transported to the conditioning room and placed into their previous chamber. After being placed in the chamber for a 180 sec acclimation period, they received seven tones and seven footshocks delivered using a trace fear conditioning procedure (Quinn et al., 2005). The trace conditioning comprised a 16 s tone (CS; 85 dB, 2 kHz, 5 ms/rise fall time) followed by a 30 sec trace period and

then a 2 sec footshock (US; 0.9 mA) (Fig. 5-1B). The inter-trial interval was held constant at 180 sec (tone onset to tone onset). The floor of the conditioning chambers was cleaned between subjects with a dilute 0.6% acetic acid solution.

For the tone test (Day 3), the rats were returned to the modified conditioning room (see above) twenty-four hours after training and placed into the opposite conditioning chamber from the one that they were trained in on previous day. Following a baseline period of 180 s, they received 4 discrete presentations of the tone (16 s duration, 85 dB, 2 kHz, 5 ms/rise fall time) that were separated by an inter-trial interval of 198 s (tone onset to tone onset). No footshocks were delivered during this test. The chambers were cleaned between subjects using a 1% NaOH solution. The following day (Day 4), the rats were returned to the original conditioning chamber for a 480 sec context test during which no tones or footshocks were delivered. The chambers were cleaned using a dilute 0.6% acetic acid solution.

Defensive freezing, which was defined as the absence of observable movement, except for those necessary for respiration (Bolles, 1970), was measured for all sessions. During training (Day 2), defensive freezing was recorded every 2 sec while the tone was on and also for every 2 s during the trace interval (while the tone was off). Following the delivery of the footshock, postshock freezing was examined every 8 s until the onset of the next tone stimulus. During the novel context test, pre-tone freezing behaviour was examined every 4 s for the 3 min baseline period. In addition, freezing to the tone stimulus was recorded every 2 s, whereas after the termination of the tone (i.e., tone offset), freezing was measured every 4 s for three successive 60-s increments in order to examine conditioned freezing to a novel context. Finally, during the context test, freezing

A



B

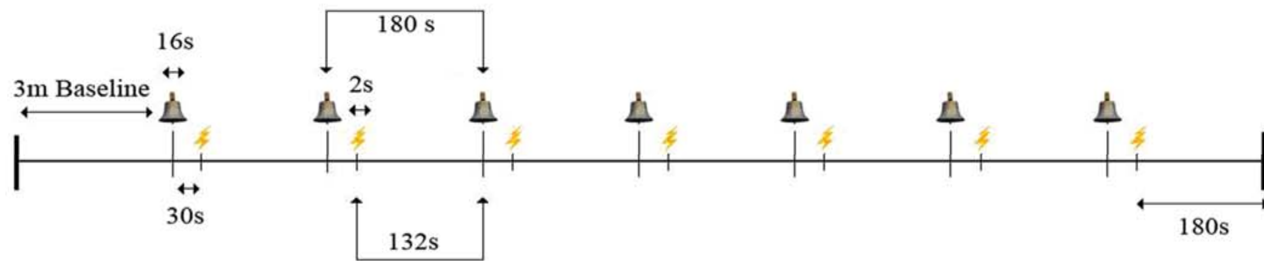


Figure 5-1 Experimental Design. A) A schematic showing the general outline for the proposed experiment. All animals are injected with BrdU (75 mg/kg) 3 to 4 hrs after their 30-sham or 30-electrical kindling stimulations. Approximately twenty-four hours later, animals received a second injection (i.e., 3 to 4 hrs after their 33 sham or electrical kindling stimulation) followed twenty-four hours again by a third injection of BrdU (i.e., 3 to 4 hrs after their 36 sham or electrical stimulation). Animals continue to receive sham or kindling stimulations until the completion of the experiment. Note: that the short-term kindled rats begin receiving stimulation after 69th sham stimulation. Twenty-four hours after the final stimulation animals were pre-exposed to the conditioning environment. The following day testing began in which animals were required to as associate a mild foot shock with an auditory stimulus that was separated by a 30-s trace interval. The next day animals were tested for their freezing to the conditioning tone by placing the animals in a novel environment and examining freezing behaviour after the presentation of several tone trials. Finally, twenty-four hours after the tone test, animals were returned to the original training environment and freezing to the context was examined. Approximately, two to two and half hours later animals were sacrificed in order to examine neuronal activation associated with memory performance in the context test. The time from BrdU to completion of kindling was approximately 31 days. B) A schematic showing the details of the training protocol for the trace fear conditioning paradigm administered on Day 1. Refer to text for details.

was examined every 8 s during the entire 480 sec period. All freezing observations were transformed into the percentage of time spent freezing by adding the specific number of individual freezing episodes and dividing this value by the total number of freezing observations for each period and multiplying by 100 (Fournier and Persinger, 2004).

2.6. Perfusions and Immunohistochemistry

Approximately 2-2.5 hours (i.e., 4 to 5 weeks after BrdU injections) after the completion of the context test, all animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (240 mg/ml) and transcardially perfused with room temperature saline followed by ice-cold 4% (w/v) formaldehyde fixative (pH=7.4) that was freshly prepared from depolymerized paraformaldehyde. The brains were extracted and postfixed in the same fixative for up to 72 hrs at 4°C before being sectioned at 50µm on a vibrating microtome in the coronal plane (Vibratome 3000, Vibratome Company, St. Louis, MO, USA). All sections were collected and stored at -20ºC in a cryoprotectant solution consisting of 30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, 30% (v/v) ethylene glycol in 0.1 M phosphate buffered saline (PBS; pH=7.4), until further processing.

For single labeling BrdU immunohistochemistry, sections were treated with 1 N HCl at 45 °C for 30 min in order to denature DNA and expose the BrdU antigen. Sections were then incubated for 1 hr at room temperature in a blocking solution comprised of 5% normal horse serum, 1% bovine serum albumin (BSA), and 0.3% Triton X-100 dissolved in 0.1 M phosphate buffered saline (PBS). Following this, sections were then incubated with a primary anti-mouse BrdU monoclonal antibody (1:500, 48 hrs, 4°C, Roche Diagnostics) diluted in blocking solution, followed by secondary biotinylated antibody

(horse anti-mouse, 1:500, 2 hrs, room temperature, Vector Laboratories) and then avidin-biotin peroxidase complex (1:200, 1 hr, room temperature Vectastain ABC Elite, Vector Laboratories). Immunolabelling was visualized using DAB (0.033% 3,3'-diaminobenzidine, 0.00786% (v/v) hydrogen peroxide diluted in PBS) to yield a brown product. Sections were mounted onto charged glass slides and lightly countered stained with 0.1% cresyl violet acetate.

For single labeling c-fos immunohistochemistry, sections were processed according to previously published methods (Fournier et al., 2009). Briefly, sections were incubated with a primary anti-rabbit c-fos polyclonal antibody (1:15,000, Calbiochem) for 72 hours at 4°C in a solution containing 5% normal goat serum (NGS), 1% BSA, and 0.3% Triton X-100 dissolved in PBS. Following this, sections were incubated in a biotinylated goat anti-rabbit secondary antibody (1:500, room temperature, 2 hrs, Vector Laboratories) and then avidin-biotin peroxidase complex (1:200, 1 hr, room temperature Vectastain ABC Elite, Vector Laboratories). Immunolabeling was visualized using nickel-DAB (2.5% nickel sulfate, 0.02% DAB, 0.000083% H_2O_2) in order to produce a blue-black product. Sections were mounted onto charged glass slides and dehydrated through ascending ethanol rinses and finally cleared in xylene.

For double immunofluorescence labeling, sections were first treated with 1 N HCl at 45 °C for 30 min and then simultaneously incubated for 48 hrs a 4°C with c-fos (1:5000, anti-rabbit c-fos polyclonal antibody, Calbiochem) and BrdU (1:500, rat anti-BrdU monoclonal antibody, Accurate Chemicals), or BrdU (1:500 rat anti-BrdU) and NeuN (1:1000, mouse anti-NeuN monoclonal antibody, Chemicon) antibodies. All primary antibodies were dissolved in a PBS solution containing 5% NGS, 1% BSA, and

0.3% Triton X-100. Following this, sections were incubated with Alexa-488 goat anti-rat and Alexa-566 goat anti-rabbit or Alexa-566 goat anti-mouse (all 1:500, 2 hrs room temperature, Molecular Probes) secondary fluro-conjugated antibodies. Sections were then mounted onto charged slides with Citifluor anti-fade mounting medium.

To determine if the electrode placement in each animal was correct, some of the immunohistochemically processed tissue was also counterstained with 0.1% cresyl violet acetate. For each animal, the electrode placement was verified using the Paxinos and Watson (Paxinos and Watson, 1998) stereotaxic atlas. Any animal with an incorrectly positioned electrode was removed from the study.

2.7. Quantification

The total number of BrdU-positive cells in the dentate subgranular zone (defined here as a two granule cell width zone bordering the granule cell layer and the hilus) and granule cell layer was exhaustively counted bilaterally at 40X for every 6th section (300 μm apart) using a light microscope (Nikon E800). In addition, BrdU-positive (BrdU+) cells located in the dentate hilus and the molecular layer were also counted separately. In order to reduce oversampling, a modified optical fractionator method was employed in that cells that were sharply in focus within the uppermost focal plane were not counted. The resulting numbers were multiplied by 6 to obtain the estimated total number of BrdU+ per granule cell layer, hilus, or molecular layer.

For every 6th section, the number of Fos-positive (Fos+) cells was manually counted at 20X for ipsilateral and contralateral dentate gyrus. The regions were traced at 4X or 10X magnification using an automated computer program (NeuroLucida 8.0, Microbrightfield, Baltimore, MD, USA). The density of labeled cells was then

determined by dividing the total number of Fos+ cells by the total cross-sectional area (Knapska and Maren, 2009). Cell counts were expressed as the number of Fos+ cells per mm2.

The number of double-labeled BrdU+/Fos+ was determined in the dentate granule cell layer using an epifluorescence microscope (Nikon E800) with a 40X objective. Only cells that fluoresced brightly within the appropriate channel were included. Because of the low frequency of Fos labeling after behavioural testing (typically <2.5% of all cells show Fos expression after a given behavioural task), the probability of overlap of multiple Fos+ in the z-axis plane is extremely unlikely (Kee et al., 2007a). Therefore, confocal microscopy was deemed unnecessary for our preliminary analysis. Cells counts were expressed as the percentage of BrdU+ cells that co-labeled with the activation marker c-Fos (i.e., BrdU+/Fos+ cells). Finally, the percentage of BrdU-labeled cells in the dentate granule cell layer that co-expressed the neuronal phenotype marker NeuN was determined by examining 50 BrdU-labeled cells distributed across 5 sections (Mullen et al., 1992). In addition, the phenotype of ectopic cells (i.e., BrdU+ cells located in the hilus or molecular layer) was also assessed by examining the percentage of BrdU/NeuN co-labeling within at least 50 BrdU-labeled cells.

2.8. Statistical Analysis

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS v 16.0, Chicago, IL, USA). All data were analyzed using a one-way analysis of variance (ANOVA) followed by Fisher's LSD *post hoc* tests in order to determine the direction of significant group differences. Results are presented as means ±

standard errors of the mean (S.E.M.). The criterion for statistical significance was set at $P < 0.05$.

3. Results

3.1. Electrode implantation and progression of kindling

There were 26 rats that had electrodes correctly implanted in the left basolateral amygdala. However, 5 rats were removed from the study because they had either an incorrect electrode placement, died during surgical recovery, or lost their electrode cap during the course of the experiment. Therefore, the final number of rats in each group was: 8 (non-kindled controls), 7 (short-term kindling; 30-stim), and 11 (long-term kindling; 99-stim).

The total number of stimulations to elicit the first class 5 convulsion was 12.85 (3.33) for the short-term kindled rats and 13.18 (1.25) for the long-term kindled rats. In addition, the total number of class 5 or higher convulsions experienced by the rats in each group was 5.66 (1.25) for the short-term kindled rats and 64.90 (5.46) for the long-term kindled rats. Consistent with previous studies (Pinel and Rovner, 1978), we did not observe spontaneous motor seizures at any time during the experiment.

3.2. Effect of kindling on hippocampal cell survival in the dentate gyrus

We previously showed that hippocampal neurogenesis was differentially affected at various stages of kindling (Fournier et al., 2009). We found that the number of doublecortin positive cells, an immature neuronal marker, was significantly increased after rats received 30-electrical kindling stimulations compared to non-kindled controls. However, there was no difference in number of doublecortin positive cells between rats

that received 99-electrical kindling stimulations and controls suggesting that the stimulatory effect of seizures on hippocampal neurogenesis dissipates somewhat over time. In the present study, we decided to examine the ability of newly born cells to integrate into dentate networks when seizure stimulations are continued after cell birth. We did this by administrating the proliferation marker BrdU at the 30th kindling stimulation and continuing stimulations until 99 electrical stimulations were delivered. A group of short-term kindled rats, that received 30-electrical stimulations, were also included. Initially these animals received 69 sham stimulations followed by kindling (i.e., 30 electrical stimulations). However, they were treated with BrdU after the delivery of 30 sham stimulations in order to permit cellular analysis with long-term kindled rats.

Immunostaining for BrdU revealed a significantly greater number of BrdU+ cells in both the ipsilateral and contralateral dentate granule cell layer and SGZ in rats that underwent long-term kindling and received injections of BrdU after their 30-stimulation compared to non-kindled controls (Fig. 5-2) [All Ps <.001]. As expected, short-term kindled rats that received injections of BrdU after 30-sham stimulations did not show a difference in the number of BrdU+ cells compared to controls [P=.404]. Interestingly, the number of ectopic BrdU+ cells in the hilus and molecular layer was higher in the long-term kindled rats compared to all other groups for both the ipsilateral [All Ps < .004] and contralateral side [Ps <.001]. Double immunofluorescent labeling indicated that 95.2% (±4.03%) of BrdU+ cells located in the dentate granule cell layer and over 55.2% (±4.03%) of ectopic cells located in the hilus co-labeled with the neuron specific marker NeuN, confirming that a large proportion of new cells born after kindling differentiated into neurons (data not shown).

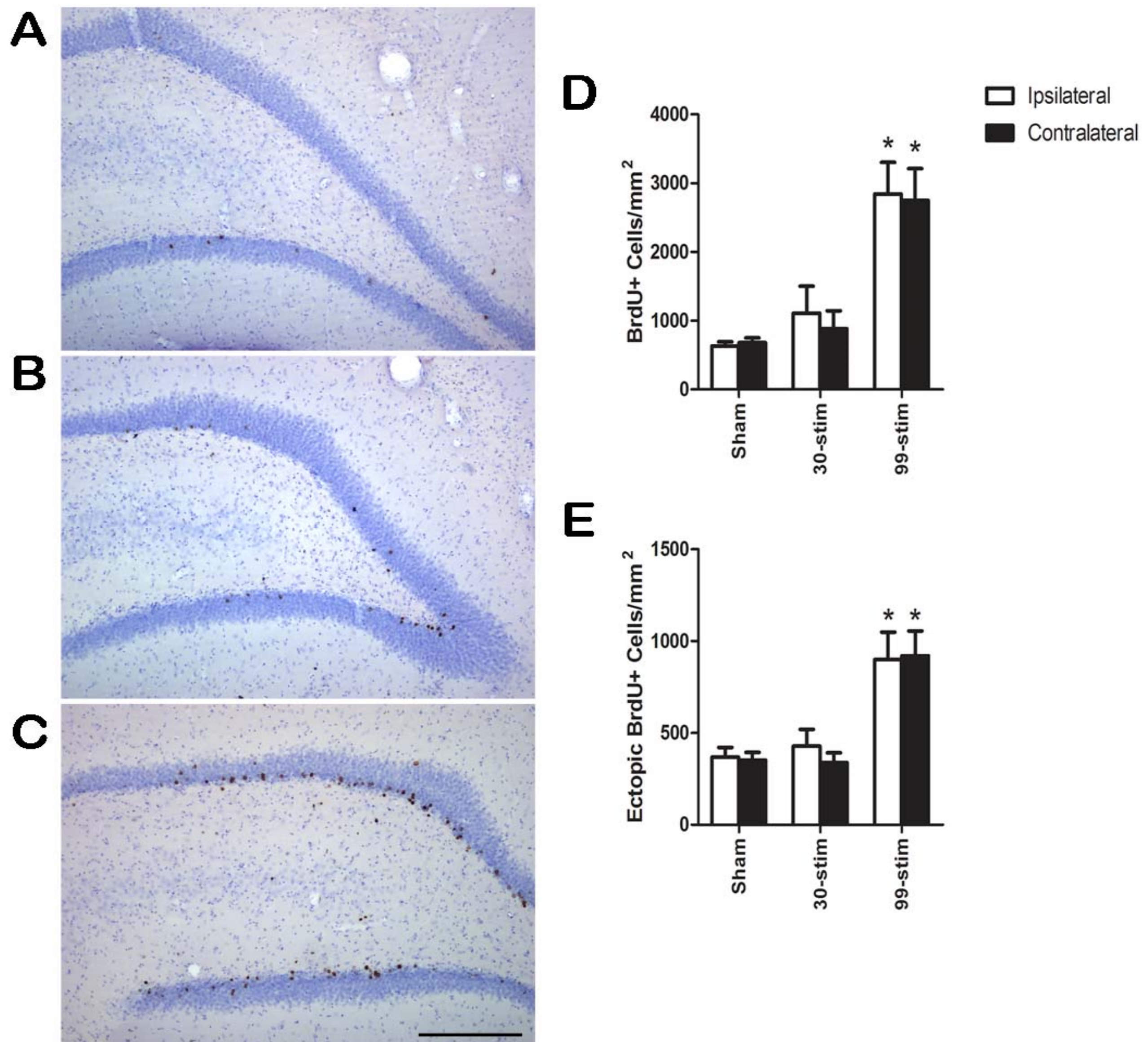


Figure 5-2 Effect of kindling on hippocampal cell survival. (A-C) Representative photomicrographs showing the effect of kindling on hippocampal cell survival for sham (A), short-term kindled (B), and long-term kindled rats (C). All groups were labeled with BrdU after 30 sham or 30 electrical kindling stimulations and sacrificed approximately 4 weeks later. Scale bar: 250 µm. (D) Quantification of the total number of BrdU+ cells in the ipsilateral and contralateral dentate granule cell layer and subgranular zone. There was a significant increase in the BrdU+ for both the ipsilateral and contralateral dentate subgranular zone and granule cell layer in long-term kindled rats ($P<.05$) compared to all other groups. (E) Quantification of the total number of ectopic granule cells for long-term kindled rats, short-term kindled, and non-kindled controls. Ectopic cells were defined as BrdU+ cells that were located within the hilus or molecular layer regions. There was a significant increase in the number of ectopic BrdU+ cells for long-term kindled rats (P <.05) compared to all other groups.

3.3. Effect of Kindling on Auditory Trace Fear Conditioning

Twelve hours after the completion of kindling, animals began training in a conditioning chamber. The first day of training comprised a 10-min habituation to the conditioning chambers. Analysis of the midline crossovers indicated no significant differences in defensive freezing between kindled rats and non-kindled controls [$F < 1.00$, $P = .831$] suggesting that all animals exhibited a similar level of ambulatory activity in the conditioning chambers during the first day of chamber exposure.

3.3.1. Training

During the 3-min acclimation period, there were no significant differences in the number of midline crossovers between kindled rats and non-kindled controls [$F(2,23)=1.92$, $P = .172$; Fig. 5-3]. In addition, postshock freezing (averaged across the 7 CS-US trials) was not significantly different between the groups [$P=.771$]. To determine if there were initial differences in postshock freezing between the groups, responses were partitioned into two equal increments of 66 s and freezing was examined every 8 s to yield 8 separate observations per increment. There were no differences in freezing during either increment indicating that all rats displayed an appropriate level of freezing following administration of the US [All $Ps>.75$]. Finally, no significant group differences were found for freezing during the presentation of the tone [$P=.848$] or during the trace interval [$P=.797$]. However, a significant group difference for the total number of fecal boli during the training session was found [$F(2,23)=3.84$, $P=.036$]. *Post hoc* analyses revealed that long-term kindled rats had a fewer number of fecal boli during training compared to short-term kindled rats [$P=.029$] and non-kindled controls [$P=.030$].

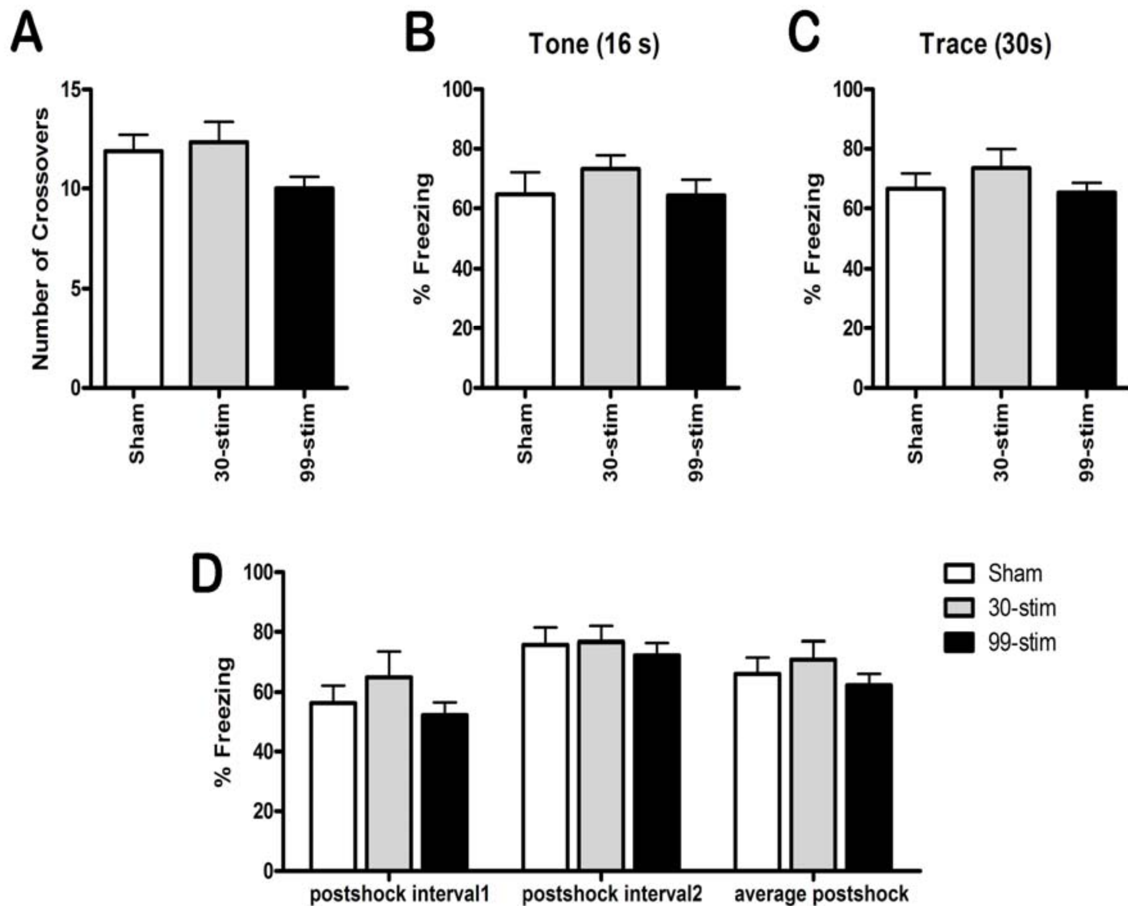


Figure 5-3 Effect of kindling on training in a trace fear conditioning paradigm. A) The total number of midline chamber crossovers during the 3-min habituation period. There was no significant difference for the number of midline chamber crossovers for any of the groups. B) The mean percentage of freezing displayed during each 16 auditory tone stimulus during training (averaged across all 6 CS tone presentations). There was no difference in the freezing response of any of the groups. C) The mean percentage of freezing during the 30 s trace period before onset of the aversive footshock stimulus ((averaged across all 6 CS trace presentations). There was no significant difference for any of the group in the percentage of freezing displayed during the trace period. D) The average percentage of postshock freezing displayed by the groups for the first and second 66 s interval , as well as the average postshock freezing across the entire 132 s interval. There was no statistically significant difference between the groups.

3.3.2. Tone Test

One day following training, rats were tested in a novel context in order to examine their freezing response to the discrete CS (tone) stimulus displayed during training. Freezing during the tone test is represented in Fig. 5-4. During the first 180 s of the test session, baseline freezing was examined prior to the presentation of the first test tone. Although baseline freezing levels were relatively low during this period (mean < 21%), both long-term kindled and short-term kindled rats froze significantly less than the non-kindled controls [F(2,23)=4.21, P=.027]. This difference in baseline freezing indicated that some generalization between the conditioning and tone test contexts may have taken place, especially for control rats.

During the presentation of the first tone (CS1), there was a significant increase in freezing responses for all groups relative to pre-tone baseline levels [Fig. 5-4]. However, there was no significant difference between kindled or non-kindled controls in the level of freezing during the 16 s of CS1 [F(2,23)=1.98, P=.158] or during the 16 s presentation of the second tone CS2 [F(2,23)=1.51, P=.243]. Following tone offset, long-term kindled rats froze significantly less than non-kindled controls for each of the 1 minute intervals until the CS2 test trial [All Ps<.020]. In addition, there were no differences between non-kindled controls and short-term kindled rats, or between long-term kindled and short-term kindled rats during this period [All Ps>.124]. However, following CS2 tone offset, long-term kindled rats froze significantly less than both non-kindled controls and short-term kindled rats for each of the 1 minute intervals until the CS3 test trial [All Ps<.014].

During the 16 s presentation of the third tone (CS3), both short-term kindled rats and long-term kindled rats froze less than non-kindled controls, however, only long-

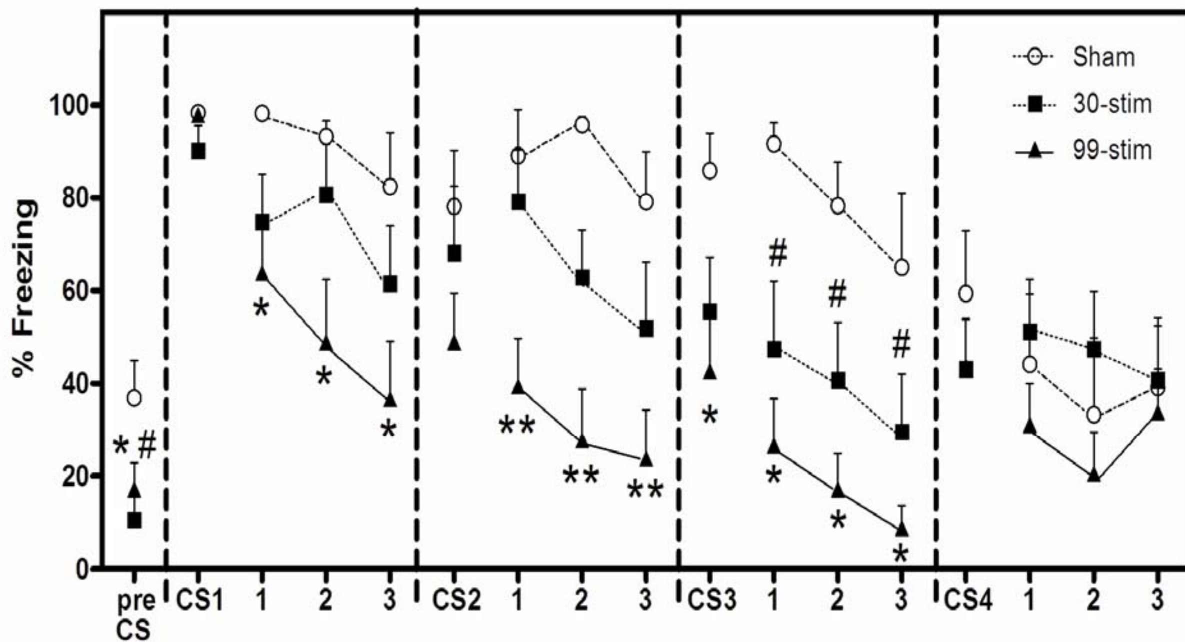


Figure 5-4 Effect of kindling during testing in a novel context in a trace fear conditioning paradigm. The mean percentage freezing of kindled and sham control rats during the testing in a novel context. * indicates that long-term kindled rats are statistically significant from non-kindled controls, # indicates that short-term kindled rats are statistically different from non-kindled controls, and ** indicates that long-term kindled are statistically different from both short-term and sham control rats.

term kindled rats were significantly different from non-kindled controls [P=.01]. Differences in freezing levels between the short-term kindled rats and non-kindled controls during the presentation of CS3 approached statistical significance [P=.079]. Following tone offset, both short-term kindled and long-term kindled rats froze significantly less than non-kindled controls for each 1 minute interval [All Ps<.037]. In addition, there were no statistical differences between short-term and long-term kindled rats during this period [All Ps>.091]. Finally, during the 16 s presentation of the last tone (CS4), there were no differences in freezing behaviour between kindled and control rats. Interestingly, following the tone offset, both kindled and controls rats showed similar levels of freezing for each of the 1 minute interval until the completion of testing.

A significant group difference for the total number of fecal boli during the tone test was also found [$F(2,23)=6.82$, P=.004]. *Post hoc* analyses indicated that long-term kindled rats had fewer fecal boli during training compared to short-term kindled rats [P=.012] and non-kindled controls [P=.002].

3.3.3 Context Test

One day after the tone test, the rats were returned to the original training context and defensive freezing to the context was examined during an 8-min extinction test [Fig. 5-5A,B]. A one-way ANOVA revealed a significant group difference for total freezing during the extinction test. *Post hoc* analyses revealed that the major source of the difference was diminished freezing in the long-term kindled rats compared to controls [P<.001]. Although the difference in freezing level between short-term kindled and long-term kindled rats approached statistical significance [P=.069], there was no significant difference between short-term kindled and control rats [P=.082]. When each minute

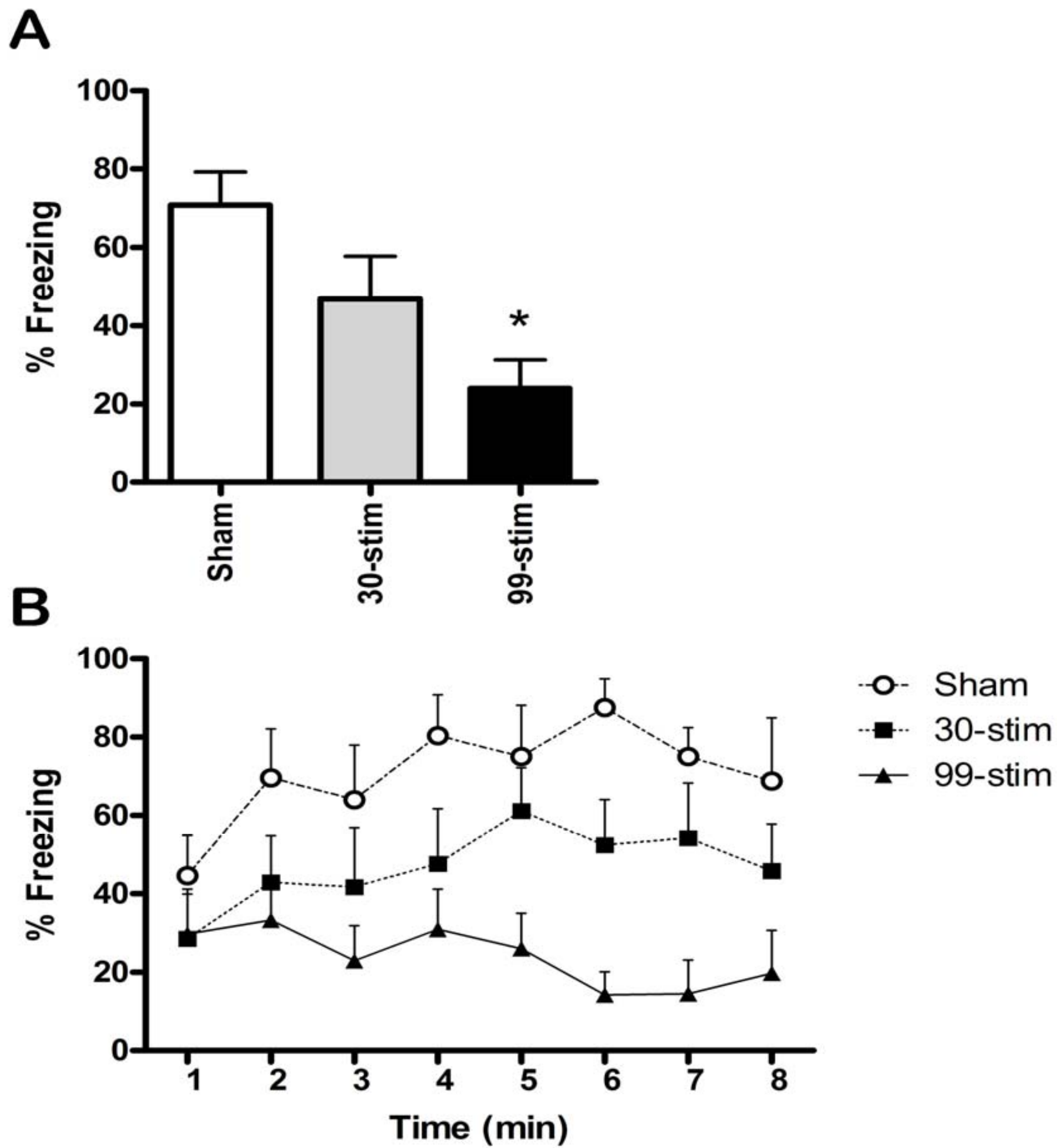


Figure 5-5 Effect of kindling on freezing during the context test. A) The mean percentage freezing during the 8 min extinction test in the original context. Long-term kindled rats froze significantly less than sham control animals. (* $P< .05$). B) The mean percentage of freezing for each 1-min increment during the 8-minute extinction test. Long-term kindled rats froze significantly less than non-kindled controls after the second minute in the context. This effect persisted throughout the duration of testing.

during the context was analyzed separately, long-term kindled rats froze significantly less than control rats by the second minute of testing and remained more ambulatory until the end of the extinction test [All Ps<.026]. Finally, long-term kindled rats also had fewer fecal boli than non-kindled controls during the extinction test [P=.015].

3.4. Effect of kindling on the activation of newly born cells in the dentate after testing

To determine if kindling impacted the activation of dentate granule cells during testing, we examined the expression of the cellular activity marker c-Fos between 2 and 2.5 hrs after the completion of contextual fear testing. Because previous work had shown a greater number of Fos+ and Zif628+ cells in the dentate gyrus following testing in the original context compared to testing tone-related freezing in a novel environment during trace fear conditioning (Weitemier and Ryabinin, 2004), we speculated that evaluating Fos-immunoreactivity after testing in the original context would maximize the likelihood for us to identify Fos+ cells, as well as newly born cells (BrdU+), that were activated (i.e., Fos+) during behavioural testing.

Immunostaining for the cellular activation marker c-fos revealed that long-term kindled rats had a significantly fewer Fos+ cells in the ipsilateral dentate granule cell layer [All Ps<.030] and contralateral dentate granule cell layer [All Ps<.007] than non-kindled controls and short-term kindled rats [Fig.5-6]. There was no statistically significant difference for the number Fos+ cells between short-term kindled and controls [All Ps>.312]. We then examined whether newly generated neurons born during the process of kindling could actively respond during testing conditions. Because of the low number of BrdU+/Fos+ cells, hemispheric differences could not be reliably analyzed;

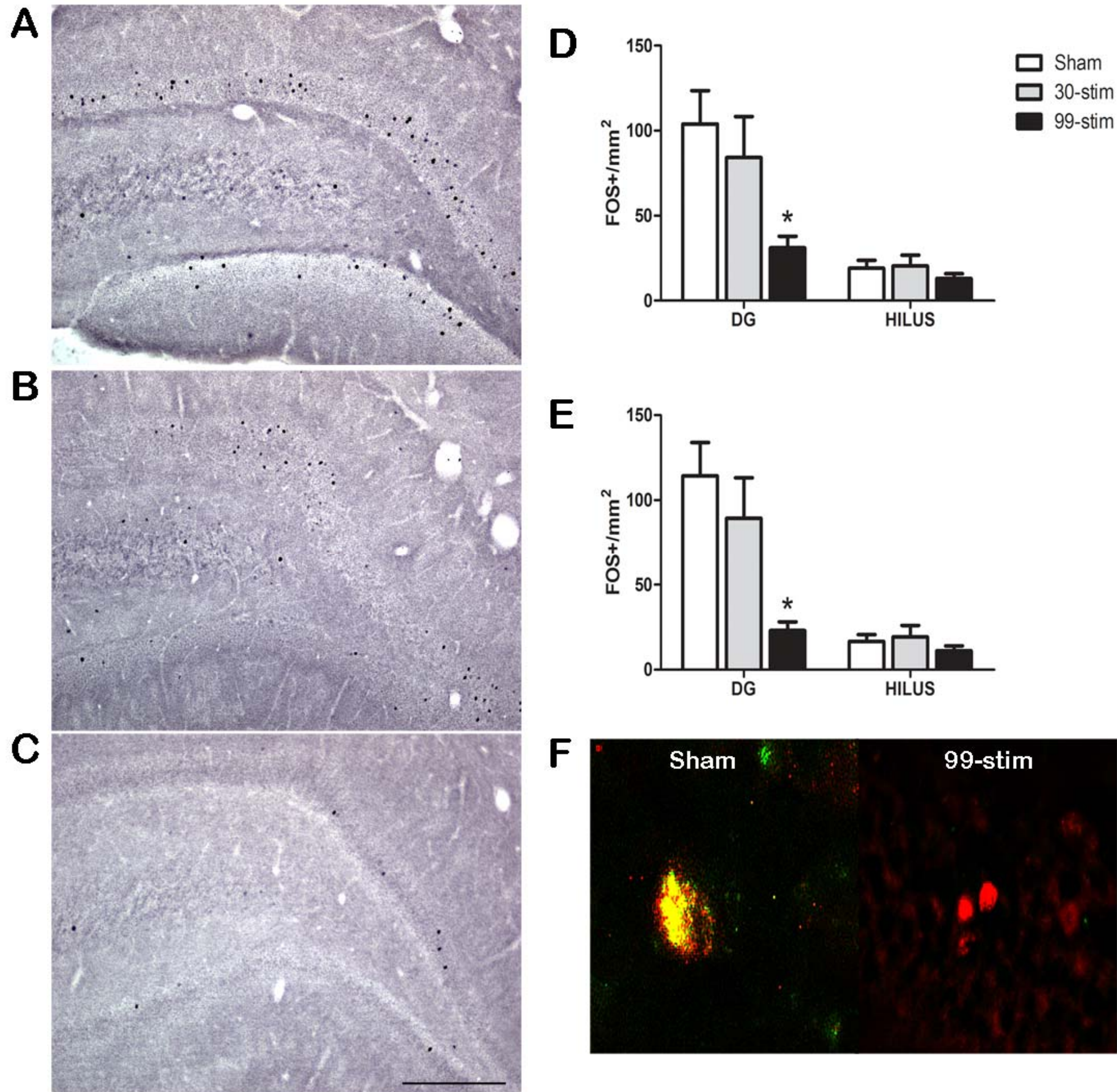


Figure 5-6 Effect of kindling on c-fos expression in the dentate gyrus. (A-C) Photomicrographs demonstrating the pattern of c-fos express in the dentate gyrus of non-kindled controls (A), short-term kindling rats (B), and long-term kindled rats (C). Scale bar: 250 µm. D)The total number of Fos+ cells in the ipsilateral dentate granule cell layer and hilus after kindling. There fewer Fos+ cells in the ipsilateral dentate of long-term kindled rats compared to non-kindled controls. E) The total number of Fos+ cells in the ipsilateral dentate granule cell layer and hilus after kindling. There were fewer Fos+ cells in the contralateral dentate of long-term kindled rats compared to non-kindled controls. F) Photomicrograph showing examining the presence of Brdu+/Fos+ colocalization two hours after behavioural testing. Approximately 2% of the total number BrdU+ cells from non-kindled controls showed co-expression with the neuronal marker c-fos after testing. By contrast, there were no BrdU+ cells from long-term kindled rats that co-expressed c-fos.

therefore, cell counts for both hemispheres were combined. Analysis indicated that in both control and short-term kindled animals, approximately 1.1 to 2.3% (mean 2.04%) of all labeled BrdU+ cells in the dentate gyrus showed Fos+ expression after testing. However, despite the greater number of BrdU+ cells born after long-term kindling, no BrdU+/Fos+ cells could be found in the dentate gyrus of long-term kindled rats, indicating that most of the newly generated cells did not respond during the behavioural test. Additional, no ectopic BrdU+ cells were found be to Fos+ after testing.

4. Discussion

There are four important findings that arise from the present study. First, we showed that during the early stages of kindling, there is an increase in new neurons in the adult dentate gyrus. Second, a significant proportion of these new neurons migrated ectopically into the molecular layer and hilus after long-term kindling. Third, long-term kindled rats also showed significant learning impairments in trace fear conditioning as reflected by a decrease in general levels of freezing during testing, whereas short-term kindled rats showed comparable learning to that of non-kindled control animals. And finally, a small percentage of BrdU+ cells (~2%) in the adult dentate gyrus of non-kindled rats were activated during recall of a fear conditioned memory in the original training context, whereas newly born cells from long-term kindled rats failed to show activation during testing. The present findings provide evidence that kindled seizures promote the survival of newly born neurons, but they also impacts the functional incorporation of these cells into neuronal networks involved in hippocampal-dependent learning.

4.1. Effect of kindling on hippocampal neurogenesis

We found that rats treated with BrdU after delivery of the 30th electrical stimulation showed a significant increase in the number of newly born neurons in the dentate gyrus when examined after the completion of kindling (i.e., after 99-electrical stimulations had been delivered). These results are consistent with previous studies showing that during the early stages of kindling, repeated epileptiform stimulation of the amygdala induces hippocampal neurogenesis (Fournier et al., 2009; Parent et al., 1998; Scott et al., 1998). Importantly, rats treated with BrdU after 30 sham stimulations, as in the short-term kindled and non-kindled groups, did not show a change in the number of new neurons suggesting that the increased survival of new neurons observed in the long-term kindled group was a direct consequence of seizure activity.

There was a significant fraction of BrdU+ cells that appeared to migrate ectopically into the hilar and molecular regions when long-term kindled rats were sacrificed ~4 weeks after injection with BrdU (i.e., administered after their 30th stimulation). Our previous work suggested that these ectopic cells were most likely newly generated dentate granule cells because they are immunoreactive for the granule-cell specific marker Prox-1 and they do not appear during the early stages of kindling (Fournier et al., 2009). We also speculated that these ectopic granule cells were most likely new neurons that were born around the 30th stimulation, a hypothesis that is further supported by the present study. Electrophysiological studies have indicated that aberrantly located granule cells show unusual electrical characteristics, such as increased propensity for hyper-synchronous burst-firing with CA3 pyramidal neurons (Scharfman et al., 2000). Interestingly, these ectopic cells also show activation during spontaneous seizures, as determined by increased c-fos expression, indicating that they receive

functional synaptic contacts (Scharfman et al., 2002). When taken together, these results suggest that seizure-generated neurons are functionally integrated into limbic circuits where they can contribute to complex pathological network processes, such as the generation of epileptiform activity.

4.2. Effect of kindling on auditory trace fear conditioning

Previous work has shown that changes in emotional behaviour are a common consequence of long-term amygdala kindling (Kalynchuk and Fournier, 2009). These animals typically show decreased exploratory behaviour in a novel environment (Kalynchuk et al., 1997; Kalynchuk et al., 1999; Nieminen et al., 1992), decreased open-arm exploration on an elevated plus maze (Adamec and Morgan, 1994; Helfer et al., 1996), and increased immobility during a social interaction test (Helfer et al., 1996). However, very little is known about the consequences of long-term kindling on conditioned fear learning. The sparse data that are available suggest that partial kindling or repeated non-convulsive amygdaloid stimulation enhances conditioned startle responses (Kellett and Kokkinidis, 2004; Rosen et al., 1996).

In the present study, we showed that long-term kindled rats trained in an auditory trace fear conditioning task display less conditioned freezing when tested in the original training context (Fig. 5-5). However, during the tone test, which was conducted in a different context from the training, the long-term kindled rats displayed high levels of freezing during the presentation of the first CS tone (Fig. 5-4). In fact, the level of freezing during the first tone was comparable to that of non-kindled controls providing strong evidence that conditioned associational learning of the tone took place in long-term kindled rats. After termination of the first tone, non-kindled rats showed persistent

freezing but the long-term kindled rats did not. This pattern of results continued for subsequent presentations of the tone, with increasing smaller differences in freezing among the groups. Following the final tone test (i.e., CS4), all groups showed a marked reduction in freezing response suggesting a comparable final level of extinction occurred. By contrast, short-term kindled rats were not significantly different from either non-kindled controls or long-term kindled rats during the context test. They showed similar performance as non-kindled rats during the tone test, but displayed a reduction in freezing after the presentation of the third tone test indicating that extinction occurred sooner in these animals in comparison to control animals.

Because kindling disrupts hippocampal circuitry and function (Fournier et al., 2008; Kalynchuk et al., 2001; Morimoto et al., 2004), it is possible that kindling-induced changes in the hippocampus may play a role in producing the learning deficits in the long-term kindled rats described above. Interestingly, long-term kindled rats showed evidence for some associative learning in which the conditioned stimulus (i.e., tone) evoked freezing during the initial period of testing in the novel context. However, the associative mnemonic representation between the tone and the footshock may be partly mediated by intact functions involving thalamo-amygdala and/or cortico-amygdala circuits that are independent of the hippocampus (Boatman and Kim, 2006; Li et al., 1996; Romanski and Ledoux, 1992). Therefore, these circuits may form a weak associative link between the tone and the footshock during conditioning (Campeau and Davis, 1995). Since the repeated presentation of the tone is not followed by an aversive stimulus during the novel context test, it is conceivable that this associative tone-shock memory quickly dissipates with each presentation of the tone, thus accounting for the

diminished freezing of long-term kindled rats to each tone presentation during the tone test. Finally, another important observation was that kindled rats showed significantly less freezing when the tone terminated. This finding is difficult to interpret, but it has been recently shown that post-tone freezing may be supported by the hippocampus (Quinn et al., 2008). Thus, a disruption in normal hippocampal functioning after long-term kindling could potentially account for most of the mnemonic deficits displayed by long-term kindled rats during both the context and tone tests. Alternatively, this finding might be interpreted as evidence that long-term kindled rats may have higher levels of extinction compared to all other groups. In other words, a higher rate of extinction in the long-term kindled rats would reflect diminished freezing during the post-tone period. Presently, we cannot rule out this possibility. However, there is evidence that long-term kindled rats show impaired habituation to a novel open-field environment, a process which is also related to intact hippocampal functioning (Andersen et al., 2007).

With respect to trace fear conditioning, the acquisition of hippocampal-dependent CS–US associations relies on the interaction between the hippocampus and the amygdala, a structure known to be critically involved in both the acquisition and expression of fear conditioning (Ledoux, 2000). Kindling is thought to sensitize amygdaloid circuits responsible for the behavioral manifestation of fear (Rosen and Schulkin, 1998). Exposure to novel environments, which will activate the amygdala and related circuitry, strongly elicits anxiety-like responses from kindled rats (Kalynchuk et al., 1997). Because of this potential confounding issue, we decided to pre-expose all animals to the conditioning context prior to training. The level of locomotor activity during the pre-exposure period, as measured by midline chamber crossovers, was comparable for all

groups (data not shown). Importantly, previous work has shown that pre-exposure to the conditioning context is known to facilitate subsequent contextual fear conditioning (Kiernan and Westbrook, 1993; O'Reilly and Rudy, 2001), an effect that depends on the functional integrity of the hippocampus and reduces the contribution of the amygdala to fear learning (Huff and Rudy, 2004).

An alternate interpretation for these data is based on the evidence that hippocampal lesions increase hyperactivity (Anagnostaras et al., 1999; Blanchard et al., 1977; Good and Honey, 1997). Therefore, it is possible that the freezing deficit displayed by long-term kindled rats resulted from a performance deficit, rather than a mnemonic deficit (McNish et al., 1997). This interpretation is unlikely for several reasons. First, long-term kindled rats did not show any deficits in postshock freezing during the conditioning session. Second, long-term kindled rats also showed increased freezing during the presentation of the first tone during the novel context test. In fact, when compared to their level of initial freezing (~17%) during the 3 min habituation (i.e., pre-tone) period in the novel environment, long-term kindled rats typically showed a 30 to 90% increase in freezing during the presentation of the tone stimuli. However, it is possible that changes in anxiety-related behaviour after kindling might have contributed to the deficits displayed by long-term kindled rats during trace fear conditioning. For instance, long-term kindled rats initially show decreased exploration when placed into a novel open field, but this is then followed by excessive locomotion that resembles anxiogenic-like responses (Fournier et al., 2008).

Because innate species-specific defense reactions, such as fleeing and freezing, lie on a response continuum (Bolles, 1970), it is possible that the decreased immobility

displayed by long-term kindled rats simply reflects an adoption towards more active coping strategies (i.e., fleeing) to a threat-provoking situation compared to the typical passive coping strategy (i.e., freezing) displayed by normal (non-kindled) animals. The issue of differences in fear-related responses to threatening stimuli has been long recognized as a potential problem in the interpretation of fear conditioning data (Maren, 2008). Although we cannot directly rule out this possibility, we have found that the magnitude of freezing behaviour is highly dependent on the topography of the testing situation. For example, long-term kindled rats show greater ambulation and exploratory velocity when traveling in a larger elevated open-field compared to a more enclosed smaller environment (Andersen et al., 2007). In addition, our evidence from this study that kindled rats display appropriate levels of freezing during conditioning strongly argues that these animals can engage in the appropriate defensive freezing response following an aversive stimulus, such as footshock. When taken together, our findings suggest that the freezing deficits displayed by long-term kindled rats are most likely related to memory-related impairments involving the hippocampus rather than due to non-specific changes in locomotor activity or alterations in anxiety-like behaviour.

4.3. Effect of kindling on the functional integration of new neurons

Following exit from the cell cycle, new neurons begin extending axons into CA3 and dendritic processes into the molecular layer (Hastings and Gould, 1999; van Praag et al., 2002). At this stage, they exclusively receive GABA-mediated excitatory synaptic inputs (Overstreet-Wadiche et al., 2006a). But as these new cells develop over the next few weeks, they begin receiving excitatory glutamatergic connections from the perforant path. It is during this period (i.e., two to four weeks after birth) that immature granule

cells display highly unique cellular properties compared to fully mature granule cells, such as enhanced excitability and increased Ca^{2+} conductance, as well as a lower threshold to induce long-term potentiation (Ambrogini et al., 2004; Schmidt-Hieber et al., 2004). Interestingly, Ge and colleagues (Ge et al., 2007) demonstrated that new granule cells between 4 and 6 weeks old display a striking increase in the magnitude of LTP compared to both younger and older granule cells, indicating that there may be a critical period when neuronal properties are particularly sensitive to synaptic input and particularly susceptible to modification by experience. Consistent with these findings, adult-born neurons with a cell age between 1 and 2 months, but not those younger than 3 weeks, are preferentially activated by hippocampal-dependent behaviours (Farioli-Vecchioli et al., 2008; Kee et al., 2007b; Ramirez-Amaya et al., 2006; Tashiro et al., 2007).

The critical period outlined above closely overlaps with the development of cognitive deficits in long-term kindled animals (Kalynchuk and Fournier, 2009). This prompted us to ask if the large number of seizure-generated neurons born at a point when proliferation was highest could be functionally recruited during a behavioural experience despite maturing in a pathogenic environment. Because a robust form of behavioural experience involves the learning of a fear provoking stimulus with a particular associative stimulus (Ledoux, 2000), such as a distinct context and/or an auditory tone, we set out to examine the functional activation of new neurons in an auditory trace fear conditioning paradigm. Previous work had shown that exposure to trace fear conditioning increases immediate early gene expression in the dentate gyrus (Weitemier and Ryabinin, 2004) and also increases the rate of neurogenesis in this structure (Gould et al., 1999).

Importantly, ablation of new neurons is associated with diminished performance on this task (Shors et al., 2001; Shors et al., 2002) suggesting that functionally intact neurogenesis within the dentate gyrus plays a role in the ability to successfully acquire and store information important for associative learning. In support of these findings, we found that a small percentage of newly generated neurons (~2%) in non-kindled rats were activated when conditioned animals were returned to the original training environment. This percentage of activated new neurons was also within the range previously reported by other studies (Kee et al., 2007b; Ramirez-Amaya et al., 2006; Tashiro et al., 2007) providing strong evidence that a very select proportion of newly generated cells is activated or recruited during a memory-recall experience. Yet despite the large number of neurons generated after long-term kindling, we were unable to find any activation of new neurons in the long-term kindled rats following exposure to the original training context. In addition, we also detected a reduction in overall Fos+ immunolabeling in the dentate of long-term kindled rats sacrificed two hours after context testing. These findings parallel the decreased freezing behaviour displayed by long-term kindled rats during the context test providing at least partial support for the idea that the impaired activation of the dentate gyrus and new neurons are associated with diminished performance in this task. By contrast, for the short-term kindled rats, we did find some newly generated neurons that were activated during exposure to the training context. It is important to note that short-term kindling animals were not significantly different from non-kindled rats.

The present study raises an important question: Why are seizure-generated neurons that develop in a pathological environment unable to be activated during hippocampal-dependent behavioural testing? It is well known that seizure stimulation not

only increases the number of adult generated neurons, but also alters the way these cells function in hippocampal circuits (Zhao and Lang, 2009; Jessberger et al., 2007a). In an elegant set of experiments, Overstreet-Wadiche and colleagues (Overstreet-Wadiche et al., 2006b) unexpectedly showed that seizures dramatically accelerated dendritic maturation of new neurons that corresponded with their early circuit integration. Under normal conditions, the development of newborn granule cells takes much longer in the adult brain than in neonates (Overstreet-Wadiche et al., 2006a) suggesting that the prolonged development might be partially due to the reduced depolarizing GABA-mediated network activity in the adult brain (Ben-Ari et al., 2007). However, seizures also increase local ambient GABA levels (Esclapez and Houser, 1999; Gutierrez and Heinemann, 2006; Schwarzer and Sperk, 1995), thus it is possible that elevated GABAergic transmission following seizures may have simply accelerated the maturation and integration of the new neurons in the epileptic dentate.

Although speculative, if seizures accelerate the maturation of newly generated neurons, then it may also be possible that the development of GABAergic mediated inhibitory events occurs earlier for these cells than those that develop in a non-pathological environment. There is some experimental data to support this hypothesis. For example, a recent study by Jakubs and colleagues (Jakubs et al., 2006) showed that the excitatory drive on newly generated dentate granule cells was reduced in the epileptic brain, whereas inhibitory input on to these same cells was increased. Interestingly, these changes were directly related to seizure activity because similar changes were not found in cells generated after a wheel-running episode. It appears that local changes in the microenvironment encountered by these new neurons at the time of development

significantly impacts their maturation and functional properties. If seizure-generated neurons receive inhibitory GABAergic synaptic potentials sooner than cells from corresponding normal (non-pathological) conditions, then it is conceivable that the critical window of enhanced plasticity displayed by these young cells would quickly dissipate as they are assimilated into functional circuits (Ge et al., 2006). This point is important because it is well known that LTP is difficult to induce in dentate granule cells, unless GABA-mediated tonic inhibition can be removed (Wigstrom and Gustafsson, 1983). Therefore, the rapid transition from excitatory to inhibitory GABAergic action following epileptic seizures may make new neurons less plastic and less likely to participate in learning—a finding that would reflect the observation from the present study that decreased Fos expression in seizure-generated cells occurs following behavioural testing. This hypothesis could also explain why short-term kindled rats did not differ significantly from non-kindled controls in terms of the number of BrdU+/Fos+ activated cells after testing since in these animals, the newly generated cells developed and matured in a mostly seizure-free environment and still maintained the elevated propensity for plasticity. Thus, under these conditions, a small pool of newly born neurons that still display enhanced plasticity could be recruited during learning.

A corollary to the above hypothesis is that testing at an earlier time point during kindling (for instance after 60 stimulations) might be associated with enhanced or at least normalized trace fear learning because a potentially larger pool of seizure-generated neurons could be recruited. However, this possibility rests on the basic assumption that seizure-generated neurons possess normal morphological and functional characteristics. It is clear that seizure-generated neurons display several unique pathophysiological features

that might actively impede normal circuit operation once they integrate (Jessberger et al., 2007a). For example, the presence of basal dendrites, which are abundant on new neurons from chronically epileptic animals, is associated with increased hyperexcitability (Shapiro and Ribak, 2005). In addition, a number of seizure-generated granule cells migrate ectopically into the dentate hilus and molecular layer, where they may be involved in the epileptic condition (Scharfman et al., 2000). Unfortunately, we did not find any evidence for c-fos expression in ectopic BrdU+ cells suggesting that these cells are not likely recruited during behavioural testing.

4.4. Conclusion

The present study demonstrates that long-term amygdaloid kindling is associated with reduced performance on a hippocampal trace fear conditioning task. Our study also revealed that kindled seizures trigger the formation of new dentate granule cells and that some of these new neurons ectopically migrate into the dentate hilus and molecular layer. Although a small percentage of newborn neurons from non-kindled rats were active during testing, we could not find any evidence for the recruitment of seizure-generated neurons when long-term kindled animals were placed in the test environment. These findings suggest that the surrounding neuronal activity and direct stimulation elicited by seizures during the process of maturation can impact the functional recruitment of new neurons into neuronal networks that mediate normal learning and memory.

Our results support the view of a unique contribution of new dentate granule cells in memory processes related to trace fear conditioning and that the repeated induction of epileptiform activity during the process of kindling can impact the activation of these new neurons during memory retrieval. Future studies will be necessary in order to

determine the precise cellular and molecular changes in the epileptic brain responsible in mediating the impaired recruitment of new neurons during learning. By understanding how seizure activity leads to a reduction in the recruitment of seizure-generated neurons during behaviour, we hope to understand the role that abnormal neurogenesis might play in the development of cognitive impairment after seizures. These findings may one day lead to more efficacious treatments that target the reduced activation of newly generated granule cells as a selective therapeutic means to reverse the cognitive deficits experienced by epileptic patients.

CHAPTER 6

General Discussion

1. An Overview of the Main Findings

The objective of this dissertation was to gain greater insight into the potential structural and functional changes in the hippocampus and dentate gyrus that occur after the repeated seizure activity. In order to study this, we examined the effects of amygdaloid kindling on several different outcomes including behaviour, structural plasticity, and adult hippocampal neurogenesis.

In **Chapter 2**, we evaluated the effects of long-term amygdaloid kindling on structural plasticity in the hippocampus and surrounding areas in male and female rats. In addition, we further examined the effects of kindling on exploratory and fear behaviour in a novel open-field. We found that long-term kindling significantly increased ambulation and fear-like behaviour during a 5-min exposure to the open-field and increased synapsin I immunolabeling within the various laminae of the hippocampal subfields CA1 and CA3, as well as in the dentate gyrus. Synapsin I immunolabeling in the central and basolateral amygdaloid complex was decreased after kindling in male rats only, whereas female kindled rats were generally unaffected. However, structures implemented in the spread of epileptiform activity during kindling, such as the striatum, piriform cortices, and sensorimotor cortex, did not show changes in synapsin immunolabeling after kindling, indicating that seizure-induced changes in synapsin I expression occur within specific brain regions. Finally, we also showed that changes in synapsin I immunolabeling within specific brain regions was also associated with different aspects of fearful behaviour in the open field. For instance, for male rats, increased synapsin I

immunolabeling primarily in the dentate gyrus, CA3, and CA1 on the side of stimulation was associated with increased locomotor activity in the open field, whereas decreased levels of synapsin I immunolabeling in the basolateral and central amygdala was associated with higher levels of resistance to being captured. In contrast, for female rats, structures associated with alterations in exploratory activity and resistance to being captured were only related to changes in synapsin I immunolabeling involving the CA1 and CA3 subfields.

The results from **Chapter 2** provided an important starting point for all subsequent experiments. One particularly interesting finding was that kindling produced significant changes in synapsin I expression within the dentate gyrus (DG) and CA3 regions in the male rat. In addition, the locomotor hyperactivity displayed by kindled males during exposure to a novel open-field environment was also related to changes in synapsin I expression in both of these regions.

Previous studies in our laboratory have revealed that the DG may be particularly sensitive to the effects of long-term kindling. For example, changes in various receptor subtypes are significantly altered in this region after kindling, and often at a greater level than what is observed in the other hippocampal subfields (Kalynchuk et al., 2001). When considering: 1) that the loss of specific populations of dentate hilar neurons after seizure activity can increase dentate excitability and seizure susceptibility (Lothman et al., 1992; Lothman, 1994), 2) that the dentate granule cell layer is morphologically altered in patients suffering from pharmacoresistant forms of TLE (El et al., 1999; Houser, 1990; Lurton et al., 1997; Lurton et al., 1998; Mello et al., 1992), 3) that dentate granule cell axons display an extensive degree of structural plasticity (i.e., sprouting) in response to

epileptogenic stimulation (Masukawa et al., 1999; Sutula et al., 1989; Sutula et al., 1992), and 4) that seizure activity can stimulate the production of new dentate granule cells in the adult DG (Mohapel et al., 2004; Parent et al., 1998; Scharfman and Gray, 2007; Scott et al., 1998; Siebzehnrubl and Blumcke, 2008), it was clear to us that examining the consequences of kindling on dentate gyrus structure and function might provide an area of exciting research.

In **Chapter 3**, we decided to examine the consequences of amygdaloid kindling on perhaps one of the most interesting findings involving the DG—the proclivity of this brain region to continually generate new neurons in response epileptiform stimulation. We found that short-term kindling significantly increased the number of immature (doublecortin-labeled) neurons in the dentate SGZ, whereas long-term kindling did not. However, immature neurons from long-term kindled rats showed significantly greater dendritic growth compared to non-kindled control rats with portions of the apical dendritic tree extending deep into the inner to middle molecular layer and showing considerable branching. In addition, many of these neurons frequently displayed a prominent basal dendrite that projected into the hilus. In contrast, doublecortin-labeled cells from short-term kindled rats were less complex than in long-term kindled or non-kindled controls, and were frequently found at the base of the granule cell layer suggesting that these cells were most likely newly proliferated neuroblasts. We also found that long-term kindling decreased reelin and DISC1 expression in the dentate granule cell layer and SGZ. Interestingly, kindling-induced changes in reelin and DISC1 expression coincided with the appearance of ectopically located granule cells in the hilus. These effects occurred independently of alterations in granule cell layer length, dentate

volume, or the number of hilar neurons. Taken together, these findings provide evidence that aberrant neurogenesis can occur after long-term amygdaloid kindling and that changes in reelin and DISC1 expression, two proteins that are critical in neuronal migration and structural development of new neurons (D'Arcangelo, 2006; Ishizuka et al., 2006; Meyer and Morris, 2009), may contribute to these effects.

In **Chapter 4**, we decided to further evaluate the effect of amygdaloid kindling on DISC1 expression. In this study, we examined whether alterations in DISC1 expression after kindling were related to aberrant neuronal migration of dentate granule cells and whether changes in DISC1 contributed to disturbances in the morphological organization of the DG. We supported our previous findings by showing that long-term kindling (referred to as extended amygdala kindling in this study) significantly decreased DISC1 labeling in the dentate granule cell layer and SGZ. Interestingly, DISC1 expression in the dentate granule cell layer and SGZ was negatively correlated with the number of ectopic or mispositioned dentate granule cells in the hilus. In to order determine if alterations in DISC1 expression were associated with changes in dentate granule cell layer morphology, we examined whether long-term kindling produced dentate granule cell layer dispersion (a frequent histopathological observation in hippocampal resected tissue from patients with refractory mesial TLE (Houser, 1990). Although we did could not find any evidence of kindling-induced expansion of the dentate granule cell layer, we did find that decreased levels of DISC1 in the dentate granule cell layer and SGZ were associated with enlargement of the upper blade and crest of the dentate gyrus in both kindled and non-kindled control rats. These finding suggest that seizure activity can affect DISC1 signaling in the adult dentate gyrus and that intrinsic differences in DISC1

expression may impact the cytoarchitectural organization of the dentate granule cell layer.

Finally, for **Chapter 5**, because of the considerable evidence that kindled seizures increase hippocampal neurogenesis (Parent et al., 1998; Scott et al., 1998) and that these new neurons display greater levels of plasticity compared to mature granule cells (Schmidt-Hieber et al., 2004), we decided to evaluate the functional consequences of aberrant seizure-induced neurogenesis on behaviour. We examined this issue by determining whether seizure-generated neurons, born at a period during kindling when proliferation is highest (i.e., around 30 electrical stimulations), could be activated at a later point in their maturation during acquisition of a hippocampal-dependent learning task. We found that non-kindled control rats that received three separate injections of BrdU and then were trained on a trace fear conditioning task approximately 4 weeks later (i.e., after they received 99 sham-stimulations), showed a small number of new neurons that co-expressed the neuronal activation marker c-fos when animals were tested in the original training context and sacrificed 2 hours later. In contrast, long-term kindled rats showed no recruitment or activation of new neurons when the rats were tested in the original training context. Importantly, although long-term kindled rats showed normal levels of postshock freezing during training, they displayed significant freezing deficits during recall testing suggesting that these animals exhibited a memory impairment. Finally, we found that the short-term kindled rats were not significantly different from non-kindled rats during testing. Importantly, these animals showed a similar number of new neurons that were activated during the context test. When taken together, the results from this study suggest that although kindled seizures can trigger the production of new

dentate granule cells, many of these cells are not responsive or functionally recruited into neuronal networks that mediate learning and memory.

The overall results of this dissertation provide strong evidence that amygdaloid kindling can affect hippocampal and dentate gyrus structure and function. There are two mechanisms that might contribute to this process. One mechanism may involve the functional growth of new synaptic contacts within key areas of the hippocampal formation, the other may involve the production of aberrant new neurons after seizures. We will now discuss some of the major implications of these findings.

2. Seizure-induced Axonal Plasticity: Going Beyond the Classical Dentate-CA3 Mossy Fiber Pathway

Studies investigating synaptic growth in the hippocampus have generally relied on the fact that the large terminals of mossy fibers of the dentate granule cells contain the highest amount of zinc in the brain (Nadler, 2003). Through the use of sulfide-silver staining, commonly referred to as Timm staining, mossy fiber projections from the dentate to the CA3 stratum lucidum can be visualized and quantified with relative ease (Danscher, 1981; Frederickson et al., 2000; Sloviter, 1982). It is known that Timm staining decreases in all areas of the DG gradually after the development of constant hippocampal afterdischarges in mice treated with an intrahippocampal injection of kainate suggesting the possibility of previously sprouted mossy fiber terminal loss (Bouilleret et al., 1999; Suzuki et al., 1995). However, additional markers of synaptic growth and plasticity, such as synapsin I, vesicular glutamate transporter 1, and zinc-transporter 3 are all increased in mossy fiber boutons indicating that the reduction is the result of decreased zinc content and not necessarily the loss of the sprouted mossy fibers

(Mitsuya et al., 2009). Therefore, Timm stains may not always provide reliable measures of synaptic sprouting and are less effective outside of the zinc-rich mossy fiber networks of the DG-CA3 projection. Other techniques must be considered, including the immunolabeling of synaptic proteins associated with growth and plasticity. For example, one particularly interesting synaptic-related protein involves the synapsin family that shows a wide-spread distribution within the brain. The synapsin genes, termed synapsin I, II, and III, code for the synapsin phosphoproteins that are located in nerve terminals, where they are linked to synaptic vesicles and vesicle trafficking. During depolarization, activation of calmodulin kinase II leads to phosphorylation of synapsin I, which weakens the link between the cytoskeleton and the synaptic vesicles thereby promoting the availability of synaptic vesicles for exocytosis (Baines, 1987; Greengard et al., 1987; Torri et al., 1992). Synapsin I plays a key role in the release of glutamate from mature synaptic terminals and in axonal elongation during synaptogenesis (Chin et al., 1995; Ferreira et al., 1998; Nichols et al., 1992). Thus, high levels of synapsin I gene expression and protein are typically observed in neurons that maintain a high rate of activity within the networks they participate in (Melloni, Jr. et al., 1993).

Although there has been limited study of sprouting and synaptic reorganization beyond the mossy fiber pathway in both experimental models of epilepsy and in humans with intractable TLE, there is evidence that the CA1 pyramidal neurons show persistent cellular hyperexcitability and a pattern of neuronal changes similar to those observed in the epileptic dentate gyrus (Babb et al., 1984; Babb et al., 1988; Meier et al., 1992; Meier and Dudek, 1996). Perez and colleagues (1996) observed the presence of local sprouting from CA1 pyramidal cell axons into stratum oriens and alveus after kainate

administration suggesting that these connections may provide recurrent excitation via newly formed synaptic, and possibly through autaptic contacts with pyramidal cell dendrites. Using fluorescent tracing methods, Lehmann et al., (2001) also found evidence for axonal sprouting between CA1 and CA3, as well as increased recurrent excitatory collaterals among spatially proximal CA1 neurons after pilocarpine seizures. In addition, the authors also reported evidence of increased sprouting between neurons in the subiculum complex and the hippocampus (Lehmann et al., 2001).

By contrast, sprouting beyond the mossy fiber-CA3 field has not been typically observed using kindling paradigms. One possible explanation for this is that although the kindling model may accurately depict the periodicity of chronic epileptic seizures in humans, this model may produce less severe alterations in brain organization than those observed after convulsive status epilepticus, such as those changes seen after kainate or pilocarpine administration. For example, Suemaru and colleagues (2000) showed that short-term amygdaloid kindling increased synapsin I immunolabeling within the mossy fiber terminals innervating the stratum oriens and stratum lucidum/radiatum of CA3, but they were unable to detect any change in synapsin I expression within CA1. Interestingly, these changes synapsin I immunolabeling after short-term kindling were extremely similar to the changes in hippocampal synapsin I mRNA that occurs following LTP-induction of the perforant pathway (Morimoto et al., 1998; Sato et al., 2000) supporting the idea that increased levels of synapsin I reflect persistent enhancement of synaptic activity within neural networks (Chi et al., 2003; Greengard et al., 1987), such as those involving dentate granule cells, which can participate in both LTP and kindling (Bekenstein and Lothman, 1991; Sutula et al., 1986). By contrast, kainate administration

was associated with a substantial increase in synapsin I immunolabeling throughout the various hippocampal subfield laminae. These findings suggest that different modes in the propagation of neural plasticity and synaptic activity may occur between kindling and the kainate model.

In the kainate model, the prolonged and excessive seizure activities led to additional synaptic inputs to CA3 and CA1 via the stratum oriens, whereas in kindling, the transient nature and duration of the seizure stimulation only led to enhanced synaptic inputs from the DG to CA3 via the mossy fiber projection. These results conflict with our findings (Chapter 3) that long-term kindling is associated with increased synapsin I expression in both hippocampal CA1 and CA3 subfield laminae. We believe that because our animals experienced a greater number of epileptic seizures compared to the animals from the Suemaru study, there was simply a greater potential in eliciting further transsynaptic propagation of plasticity and modification within distributed networks of the hippocampus. In support of this idea, expression of GAP-43 is increased in area CA1 following perforant path stimulation suggesting that sprouting can occur beyond the mossy fiber system in the kindling model (Dalby et al., 1995) and that the ability to elicit transsynaptic changes outside of the classical mossy fiber-CA3 network may critically depend upon the excessive activation of the hippocampus by seizure activity and site of stimulation.

Another important observation is that although the initial mode of the propagation of synaptic plasticity and growth may differ between early stages of kindling and status epilepticus, later stages of kindling, such as those associated with long-term or extended kindling, may produce additional changes within the brain that reduce the discrepancy

between these models. In other words, long-term or extended (i.e., greater than 150 electrical stimulations) amygdaloid kindling may produce changes in the neuronal and synaptic organization of the hippocampus that are comparable to those observed after the induction of severe seizures only after a critical number of seizures have been triggered. Finally, it is presently unclear how long-lasting the changes in synapsin I expression might persist after the last kindling stimulation. Although we did not directly examine this question in the present study, previous work has shown that synapsin I mRNA expression decreases rapidly towards baseline levels shortly after the induction of kainate seizures (Sato and Abe, 2001). Interestingly, the decrease in synapsin I mRNA expression occurs just before the emergence of spontaneous seizures and at a point when cell loss is not complete in this model of epilepsy suggesting that spontaneous seizures may appear once a critical amount of synaptic growth and loss has proceeded in the brain. In addition, previous studies have shown that extended kindling beyond 150 stimulations can result in the development of spontaneous seizures in rats (Brandt et al., 2004; Michalakis et al., 1998; Pinel and Rovner, 1978). Thus, in our model of epilepsy (long-term kindling; 99-electrical stimulations), the fact that we can detect a similar degree of synaptic growth and reorganization in the brain that parallels findings from chemoconvulsants raises the extremely interesting idea that this point in the process of kindling might reflect an important transition stage toward the development of an epileptogenic network that supports the generation of spontaneous seizure activity.

2.1. Synaptic Remodelling Outside of the Hippocampus

The extent of structural remodeling seen after kindling also does not appear to be restricted to the hippocampus. When we evaluated synapsin I expression in the

amygdaloid complex after long-term kindling, we were surprised to find a decrease in synapsin I immunolabeling in the basolateral and central amygdala in male rats only. By contrast, long-term kindled female rats were not significantly different from non-kindled female controls suggesting that differences in circulating levels of sex hormones might exert sex-specific protective effects against the seizure-induced synaptic and cellular remodeling and injury (Scharfman et al., 2008).

The loss of synapsin I immunolabeling within these two structures after kindling is difficult to explain. One possibility is that the loss of synapsin I immunoreactivity might reflect the loss synaptic innervation from local interneurons, which function to increase levels of inhibition and dampen excitation after seizures (Bausch, 2005; de Lanerolle et al., 1989). Interestingly, the central amygdala is the fastest amygdaloid nucleus to develop kindled convulsions (Mohapel et al., 1996). However, the initial AD threshold is fairly high for this nuclei (i.e., up to 350 μA) compared to the other amygdaloid nuclei. Large retrograde injections into the central amygdala provide relatively little cortical labeling; however, there is evidence for dense labeling of the perirhinal cortex suggesting that the central amygdala receives strong input from this structure (Deacon et al., 1983). This projection may have important consequences for the development of kindled seizures, as the perirhinal cortex has one of the fastest kindling rates in the rodent brain (McIntyre et al., 1993; Mohapel et al., 2001). Interestingly, there is evidence for prolonged loss of dendritic synapses in cells of the central and medial amygdala over 100 days after 5 consecutive class five convulsions through kindling (Okada et al., 1993). It has been speculated that the loss of these dendritic spines reflects the loss of local GABAergic terminals within these amygdaloid regions.

By contrast, the basolateral amygdaloid complex is the most frequent site for kindling experiments. Interestingly, Handforth and Ackermann (1995) reported that the basolateral amygdala was recruited during the initial stages of seizure development, regardless of where stimulation occurred in the limbic system. Although the basolateral amygdala has one of the lowest AD thresholds (~60 μA) of the amygdaloid nuclei, it develops kindled convulsions at a rate that is intermediate between the fast-kindling central nucleus and the slow-kindling medial nucleus, a finding that might be explained by the extensive interneuronal innervation (Cunningham et al., 2008) that neurons in this region receive (Truitt et al., 2009). An impressive set of studies by Rainnie and colleagues (1991; 1992) demonstrated that: 1) both low Mg^{2+} perfusion of an amygdala-piriform slice preparation and prior *in* vivo kindling readily evokes spontaneous epileptiform burst firing from neurons of the BLA that are both N-methyl-D-aspartate (NMDA) and non-NMDA dependent, and 2) the kindling-induced enhancement of glutamatergic synaptic transmission and epileptiform bursting *in vitro* in BLA neurons was related to changes in intrinsic inhibitory mechanisms (Asprodini et al., 1992; Rainnie et al., 1991; Rainnie et al., 1992). When taken together, these findings suggest that re-modeling of intrinsic local network inhibition within the central and basolateral amygdala may underlie the development of hyperexcitability and seizure generation during kindling.

An important study by Li and colleagues (Li et al., 2002) demonstrated increased synaptophysin labeling in the hippocampus following perforant path kindling. Whereas Timm granule density (mossy fiber staining) increases in the stratum oriens of CA3 of kindled rats compared to controls (Adams et al., 1997; Van der Zee et al., 1995), levels of

synaptophysin immunoreactivity were increased in the stratum lucidum/radiatum of CA1 and CA3, but remained unchanged in the entorhinal cortex. Interestingly, these authors also found increased synaptophysin labeling in the piriform cortex after perforant path kindling. The piriform cortices are well known to play a critical role in the initiation and spread of epileptiform activity in the kindling model (Loscher and Ebert, 1996). However, we were unable to find any evidence that synapsin I immunolabeling was changed in the piriform cortex after long-term amygdaloid kindling. This suggest that different synaptic vesicle membrane-related proteins (synaptophysin vs. synapsin I) can show differential changes in expression after seizures. Although the functional implications of these differences are not known, future studies should be aimed at using immunohistochemical (protein) and gene (mRNA) expression techniques to examine a wide-range of potential synaptic vesicle and plasticity markers that may show dynamic regulation within various brain regions and time points during the development of epilepsy.

When taken together, these findings suggest that the effect of kindling on brain reorganization may not be limited to synapses within the hippocampus but that these changes may be transmitted to other brain regions involved in the circuitry of kindled seizures. The increased sprouting of recurrent excitatory connections may facilitate bursting activity and spread of epileptiform activity between synaptically and non-synaptically (electrotonic or ephaptic) connected neurons thus contributing to the development of epilepsy and cognitive dysfunction frequently found in chronically epileptic patients.

3. Role of Reelin and DISC1 in Aberrant Seizure-induced Neurogenesis

3.1. Reelin and Ectopic Cell Migration

The DG is one of the few brain areas that continuously undergoes neurogenesis throughout life, and after seizures, neurogenesis increases dramatically. This has been shown for a variety of seizure types, including kindling, status epilepticus following chemoconvulsant administration (pilocarpine, kainate), and electroconvulsive shock (Chapter 3) (Bengzon et al., 1997; Gray and Sundstrom, 1998; Jessberger et al., 2007b; Parent et al., 1997; Parent et al., 1998; Scott et al., 1998; Scott et al., 2000). Growing evidence has suggested that although many of the newly born cells appear to survive and migrate to a position in the granule cell layer that is similar to newly born granule cells in non-epileptic animals (Markakis and Gage, 1999), a sizeable fraction of newly born cells also lose their way during migration, often ending up misplaced in the dentate molecular layer or hilus (Parent et al., 2006; Scharfman et al., 2000). In both cases, the intrinsic membrane properties of the neurons appear to be identical despite their different locations (Jakubs et al., 2006; Scharfman et al., 2000; Scharfman et al., 2003). However, ectopic granule-like cells exhibit epileptiform discharges spontaneously, a finding that is abnormal for granule cells (Scharfman and Schwartzkroin, 1990), which in the normal brain require unusual conditions to discharge spontaneously (such as increased extracellular potassium or the presence of $GABA_A$ receptor antagonists). Because of these unusual properties, hilar ectopic granule cells have been implicated as key contributors to the abnormal network of the epileptic hippocampus (Parent and Murphy, 2008).

Why some granule cells migrate aberrantly into inappropriate locations in the epileptic brain is not entirely clear. However, guidance cues that influence neuronal

migration during brain development are believed to be strong candidates (D'Arcangelo, 2006). One likely candidate is the extracellular glycoprotein reelin. Reelin is well known to regulate neuronal migration and lamination of the developing brain (Curran and D'Arcangelo, 1998). Although the exact mechanism governing layer formation are still not fully understood, one model suggests that reelin produced near the marginal surface by early-generated Cajal-Retzius cells diffuses down into the developing cortical plate or hippocampus in a gradient-like function, which in turn influences the migratory behaviour of neuroblasts (Curran and D'Arcangelo, 1998; Del Rio et al., 1997; Fatemi, 2001; Jossin et al., 2003; Kim et al., 2002; Schiffmann et al., 1997). Importantly, available data suggests that reelin acts as a stop signal for migrating neuroblasts (Chai et al., 2009). At low levels, reelin promotes the radial migration of early target cells along the radial glial scaffold towards the marginal zone, which has a high concentration of reelin. Once the migrating neurons have reached the marginal zone, they detach from radial glial fibers and start to differentiate into their prospective neuronal phenotypes. It has also been shown that phosphorylation of Dab1, the major cytoplasmic integrator of the reelin signal, at tyrosine residues 220 and 232 leads to detachment of the migrating neuron from the radial fiber, thus providing termination of the migration process (Sanada et al., 2004). For later neurons to reach this same stop signal, they must migrate past the early generated neurons and approach the reelin-containing marginal zone, thus forming the characteristic inside-out lamination of the cortex and hippocampus.

Consistent with previous reports (Gong et al., 2007; Heinrich et al., 2006), we found that long-term kindling decreased the number of reelin-expressing cells in the dentate SGZ (Chapter 3). The decrease in reelin expression observed in this study

coincided with the appearance of ectopically located (Prox-1 labeled) granule cells in the hilus. Moreover, it also appears that these ectopic cells are born during the early stages of amygdaloid kindling (i.e., around the 30th electrical stimulation), when cell proliferation is highest (Chapter 5). The loss of reelin after kindling may interfere with dentate granule cell migration in many ways. For example, the absence of reelin signaling may directly affect the radial glial scaffold that is known to be important for cell migration during development (Forster et al., 2006; Frotscher et al., 2003; Gierdalski and Juliano, 2002; Schaefer et al., 2008). Importantly, this radial glial scaffold persists in the adult rodent DG (Gubert et al., 2009). Although Dab1 mRNA is predominately found in neurons, GFAP-positive cells in the hippocampus may also express Dab1 mRNA suggesting radial glial cells may directly respond to reelin-mediated signaling (Forster et al., 2002). Previous work had shown that in addition to the mispositioning of neurons, the absence of reelin also causes impaired development of radial glial cells, including defasciculation of radial glial fibers and malformation of their apical processes (Caviness, Jr. and Rakic, 1978; Hartfuss et al., 2003; Pinto-Lord et al., 1982; Super et al., 2000). Using light and electron microscopy, Shapiro and colleagues (2005b) showed that new neurons are found proximal to GFAP-expressing radial glial cells in the SGZ that have an apical process that extends through the granule cell layer. However, these cells also possess non-radial processes that directly envelope the cell body of the new neurons. These authors speculated that the radial glial process that extends through the granule cell layer might be utilized by new neurons as a support structure during their migration through the dentate granule cell layer. Therefore, the loss of reelin signaling after seizures may result

in alterations in the radial glial scaffold which in turn causes impaired migrational behavior of new neurons.

Although radial glial fiber atrophy cannot be directly excluded as a cause for ectopic cell migration, there is evidence that reelin deficiency could impact migrating neurons directly. For instance, Gong and colleagues (2007) recently performed a series of *in vitro* experiments using Matrigel explants of postnatal day 7 mouse dentate gyrus. They showed that when dentate gyrus explants were incubated in control media, there was greater migration of immature neurons (β-tubulin immunoreactive) out of the explants into the matrix over a 24 hr period. By contrast, following incubation with a reelin-conditioned media, a significantly greater number of individually detached cells and fewer chains of neuroblasts migrating outside the explant were found. Interestingly, neutralization of the reelin with a CR-50 antibody resulted in a significantly greater chain migration and fewer detached cells indicating that reelin directly prevents chain migration of dentate progenitor cells.

It should also be noted that reelin-expressing cells are also abundantly found within the dentate SGZ. Preliminary experiments in our laboratory have revealed that radial glial-like GFAP expressing astrocytes in the SGZ are closely positioned to reelin-synthesizing basket cells (Fournier and Kalynchuk, unpublished observations). Because reelin serves as a detachment signal for migrating neuroblasts, it is possible that as new neurons begin migrating through the granule cell layer, they encounter reelin (that is released by neighboring reelin-synthesizing inhibitory basket cells) of an appropriate concentration that causes them to detach from the radial scaffold and terminate migration. The loss of reelin signaling after seizures will no longer provide this important stop signal

to the migrating neuroblast causing some of them to over-migrate into the molecular layer. In addition, there is evidence that an ectopic glial scaffold forms in the hilus subsequent to cell loss in this region after seizures (Shapiro et al., 2005a). It has been speculated that the new neurons may incorrectly use this ectopic radial glial scaffold during migration causing them to deviate towards the hilus rather correctly migrating into the granule cell layer. The model presented above argues that the close proximity of neuroblasts, radial glial cells, and reelin-synthesizing basket cells in the dentate SGZ forms an important tripartite system in the adult SGZ that serves to regulate and induce the correct migration of newly generated dentate granule cells.

Recent studies by Frotscher and colleagues (2002; 2006) have found that reelin mRNA expression in Cajal-Retzius cells of the DG is reduced in some patients with TLE. Furthermore, the amount of reelin loss was shown to be positively correlated with the extent of dentate granule cell layer dispersion suggesting that granule cell layer dispersion may develop due to the loss of reelin and the subsequent impaired migration of granule cells (Fahrner et al., 2007; Heinrich et al., 2006). These findings have been replicated in animal models of epilepsy. For example, unilateral injection of kainate into the hippocampus of mice is associated with a dramatic decrease in reelin expression and the development of dentate granule cell layer dispersion only on the side injected; the contralateral side shows no evidence of dispersion or loss of reelin-synthesizing cells (Heinrich et al., 2006). Interestingly, the local reelin deficiency that causes granule cell layer dispersion could be mimicked by chronically infusing the reelin-neutralizing CR-50 antibody for 1 week into the hippocampus of adult control mice. However, infusion of exogenous reelin into animals that previously received an intrahippocampal injection of

kainate prevented the development dentate granule cell layer dispersion providing a pivotal role for reelin in maintaining normal granule cell lamination in the adult brain. Finally, a recent study demonstrated that reelin expression was significantly lower in the adult DG of animals treated with pilocarpine (Gong et al., 2007). These authors also demonstrated that the loss of reelin immunoreactivity in the DG can be associated with ectopic migration of newly born granule cells into the hilus.

3.2. DISC1 and Reelin: Converging Signaling Pathways

The above findings suggest that the loss of reelin expression in the epileptic hippocampus might contribute aberrant migration of new neurons and cytoarchitectural abnormalities of the dentate gyrus frequently seen after kindling. However, it is presently uncertain how the loss in the reelin positional signal translates into an intracellular event that alters the behaviour of migrating neuroblasts. Analysis of the signaling cascades affected by reelin indicates that it can indirectly activate DISC1, a protein known to be critically involved in modulating intracellular changes important for nuclear translocation and neuronal migration. This brings up the possibility that reelin deficiency may result in impaired DISC1 signaling events that in turn affect cell migration.

Human and mouse genetic studies have revealed that molecular factors – such as doublecortin (Reiner et al., 2004), phosphoinositol kinase 3 (PI3K) (Bock et al., 2003; Jossin and Goffinet, 2007), lissencephaly 1 (Lis1) (Assadi et al., 2003; Wang and Baraban, 2008) and nuclear distribution gene E homolog-like 1 (Nudel or Ndel1) (Lambert de Rouvroit and Goffinet, 2001) – are activated either directly or indirectly during the reelin signaling cascade. Of particular importance is the interaction between reelin signaling and Lis1. Dab1 (the cytoplasmic adaptor protein of the reelin receptor)

and Lis1 bind together in a reelin-induced phosphorylation-dependent manner during brain development (Assadi et al., 2003). Studies have shown that Lis1 directly interacts with Ndel1 and that this interaction regulates dynein-mediated nucleokinesis (Niethammer et al., 2000), a cellular event that is critical in migration and axonal formation during neuronal development (Tsai and Gleeson, 2005). Mutations in the gene encoding Lis1 causes disordered neuronal migration that leads to classical lissencephaly, in which the cortex is absent of convolutions. This condition is associated with epilepsy (Guerrini and Filippi, 2005) and is a risk factor for the development of schizophrenia (Reiner et al., 2006; Tabares-Seisdedos et al., 2006; Tabares-Seisdedos et al., 2008). Thus, the interaction between phospho-Dab1 and Lis1 allows a cross-talk between reelin signaling and Lis1-dependent molecular events that are important in normal neuronal migration.

Strong evidence has accumulated to show that DISC1 forms an extremely important binding complex with Lis1 and Ndel1 (Kamiya et al., 2006; Morris et al., 2003; Taya et al., 2007). This interaction is critical for mediating proper neuronal migration and neurite expansion in both the adult and developing brain (Kamiya et al., 2005; Meyer and Morris, 2008; Meyer and Morris, 2009). Interestingly, selective downregulation of DISC1 in adult generated dentate granule cells results in a series of changes that are extremely similar to the effects of epileptic seizures, including ectopic cell migration, increased dendritic outgrowth and rapid integration into preexisting neural networks (Duan et al., 2007; Overstreet-Wadiche et al., 2006; Parent et al., 2006). This led us to determine whether seizure activity could also directly impact DISC1 signaling. Indeed, we show here that long-term kindling selectively decreases DISC1 expression in the

dentate granule cell layer and SGZ (Chapters 3 and 4). Importantly, the combined decrease in reelin and DISC1 expression within the granule cell layer/SGZ on the side of stimulation was associated with ectopic migration of dentate granule cells into the hilus; decreased DISC1 expression alone in these regions on the opposite side of stimulation was not associated with displaced granule cells (Chapter 3). These findings further strengthen our argument that both reelin and DISC1 signals converge on a singular network that is critical in mediating intact neuronal migration. However, our findings also hint at the possibility that reelin signaling may directly modulate the activity of DISC1. This type of interaction remains to be demonstrated experimentally, but the analysis of DISC1 expression in the reelin-deficient mouse (reeler) may serve as an important starting point for such an investigation.

Recently it has been shown that DISC1 inhibits glycogen synthase kinase (GSK) 3β activity through direct physical interaction and that decreased DISC1 expression increases both *in vitro* and *in vivo* GSK 3β activity (Mao et al., 2009). Because GSK 3β is known to be a significant inhibitor of hippocampal neurogenesis through its regulation of the canonical β-catenin/Wnt pathway (Gulacsi and Anderson, 2008; He and Shen, 2009; Madsen et al., 2003), it is expected that suppression of DISC1 signaling should reduce progenitor proliferation. Indeed this was what Mao and colleagues recently reported (Mao et al., 2009). These findings are interesting because they may account for our observation of a transient enhancement in hippocampal neurogenesis after short-term kindling, but not after long-term kindling (Chapter 3). Such bidirectional changes in seizure-induced neurogenesis have been observed in other models of epilepsy, suggesting

that the initial enhancement in the proliferative response of progenitor cells to seizures dissipates with time (Hattiangady et al., 2004).

A possible explanation for these observations is that reduced DISC1 expression in chronic epilepsy may lead to increased GSK 3β-mediated phosphorylation of β-catenin and thus suppression of hippocampal neurogenesis. There is evidence of elevated GSK 3β in Fragile X mice, a genetic condition associated with mental retardation and increased seizure susceptibility (Min et al., 2009). If these findings can be confirmed in chronic models of TLE, then there is a possibility that prophylactic treatment with compounds that selectively inhibit GSK-3 activity, such as lithium carbonate, may have beneficial effects for the treatment of seizure disorders. Indeed chronic treatment with lithium has been shown to prevent mossy fiber sprouting after pilocarpine seizures (Cadotte et al., 2003) as well as to reduce seizure susceptibility in some models of epilepsy (Minabe et al., 1988; Post et al., 1984). Therefore, it might be interesting to determine if chronic treatment with lithium ameliorates the aberrant neurogenesis that accompanies seizure activity and if the potential pharmacological action of lithium's effect might be mediated through alterations in DISC1 pathway.

4. Possible Implications of Altered Dentate Gyrus Structure

A cytoarchitectural modification that is frequently found in specimens with mesial temporal lobe sclerosis involves the dispersion of the dentate granule cell layer, with dentate granule cells extending into the molecular layer (Lurton et al., 1997; Lurton et al., 1998). As a result, the outer border of the dentate granule cell layer is less sharply defined in specimens with hippocampal sclerosis. Granule cell dispersion is typically

observed in about half of hippocampal tissue specimens from patients surgically treated with intractable TLE (Houser, 1990). However, in some cases, there is evidence of bilamination or doubling of the granule cell layer (~20% of granule cell layer dispersion samples) (Blumcke et al., 2009). Granule cell layer dispersion has been frequently associated with an early seizure onset (Houser et al., 1992; Sagar and Oxbury, 1987).

Even subtle evidence of granule cell dispersion can be associated with marked changes in hippocampal excitability. For instance, the p35 (a cyclin-dependent kinase important in brain development) knock-out mutant mouse displays an abnormal morphological organization of the hippocampus, including ectopic positioning of dentate granule cells and cell layer dispersion (Wenzel et al., 2001). p35 knock-out mice also suffer from spontaneous recurrent seizures and field potential recordings indicate that the heterotopic granule cells are activated by incoming perforant path fibers, a finding that supports the idea that alterations in dentate gyrus morphology can produce pro-epileptogenic effects. However, in our experiments (Chapter 3 and 4), we failed to find a relationship between long-term kindling and enlargement of the dentate granule cell layer. We did note, however, an association between DISC1 levels and the width of the dentate granule cell layer. For example, lower levels of DISC1 expression in the granule cell layer and SGZ was associated with a greater increase in the width of the upper granule cell layer and dentate crest. In other words, we found an association between subtle changes in the expansion of the granule cell layer and differences in DISC1 expression levels. These findings became apparent only when non-kindled and kindled animals were both included in the analysis suggesting that intrinsic variation in DISC1 levels in the DG is associated with subtle but statistically significant differences in

granule cell layer width. It is important to note that although these effects are subtle, the variation in DISC1 expression in the DG accommodated a significant proportion of the variance in granule layer width measurements (between 21-34% of the explained variance in dentate granule cell width within the upper blade and crest could be accounted for by differences in DISC1 expression).

As discussed earlier, mesial temporal lobe sclerosis is the most common lesion observed with refractory TLE across all age groups and is consistently found in 60-70% of cases referred for surgical resection. Neuropathologically, mesial temporal lobe sclerosis is frequently characterized by the loss of hippocampal pyramidal and dentate hilar neurons, the presence of dentate granule cell layer dispersion, and extensive gliosis with varying involvement of the amygdala and other mesial temporal lobe structures (Bote et al., 2008; Bouchet and Cazauvielh, 1825; Bratz, 1899; Fisher et al., 1998; Mintzer and Sperling, 2008; Sommer, 1880; Thom et al., 2009; Yilmazer-Hanke et al., 2000). Several retrospective studies have demonstrated that patients with mesial temporal lobe sclerosis frequently have a history of an initial precipitating injury, such as febrile, CNS infection, head trauma, or birth trauma (Fig. 6-1) (French et al., 1993). This had led to the formulation of a “two-hit” hypothesis in which preexisting conditions such as genetic factors, perinatal or postnatal injuries or infections, or developmental CNS anomalies may be responsible for the development of intractable seizures or render the brain more susceptible to permanent injury after an incidental event that otherwise would have had minimal consequences (Velisek and Moshe, 2003).

The "two-hit" hypothesis assumes that two successive events must work together to induce brain injury. In other words, although each of the two events individually are

incapable of producing injury, the occurrence of the first event may prime the brain for the deleterious effects induced by the second event. Some indirect evidence supports this hypothesis. For instance, kindling in postnatal day 15 rats persists into adulthood, so that only one kindling stimulus is necessary to provoke a fully generalized class 5 kindled seizure (Moshe and Albala, 1982). In addition, infant rats subjected to multiple flurothyl seizures and then to a single injection of kainate acid or perforant path stimulation in adulthood displayed more hippocampal damage compared to rats that only received kainate or perforant-path stimulation as adults (Huang et al., 1999; Liu et al., 1999; Schmid et al., 1999). These results suggest that selective injuries at key points in development may increase the risk for developing seizures or aggravate the extent of cellular injury when the organism is exposed to an additional secondary insult.

Although the relationship between early injury and development of TLE is extremely interesting, an important point is that not every person with evidence of granule cell dispersion or a history of meningitis, head trauma, or complex febrile seizures goes on to develop recurrent seizures as adults. The question is why some patients do. Because the vast majority of dentate granule cells are born during the first three weeks after birth (Frotscher et al., 2007), the dentate gyrus may be particularly susceptible to the effects of early injury. Increased hippocampal neurogenesis and abnormal architectural organization of the dentate granule cell layer are frequently found in young patients with early hippocampal seizures (Blumcke et al., 2001). Indeed, recent molecular neuropathological evidence had reported the persistence of Cajal-Retzius cells in the dentate molecular layer in resected hippocampal samples from TLE patients as well as evidence for alterations in the reelin signaling pathway suggesting that an early

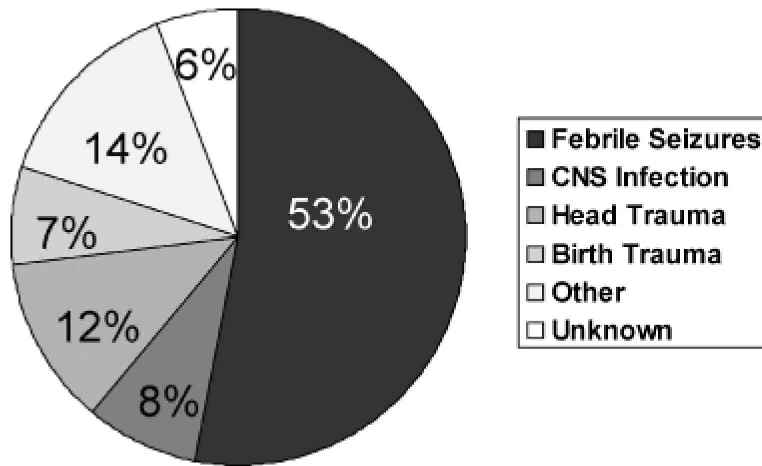


Figure 6-1: The frequencies of initial precipitating injuries associated with intractable TLE in 60 patients (Modified from (French et al., 1993).

injury during development may contribute to the later risk of developing epilepsy as an adult (Blumcke et al., 1996; Blumcke et al., 1999).

It is likely that the statistical variation in dentate granule cell layer width in our study (Chapter 5) most likely reflects subtle individual differences in the expression of various molecular factors, such as DISC1, during key points in development. Although the functional significance of dentate morphological variation within the normal population is not known, it is reasonable to speculate that subtle differences in dentate morphology impacted by DISC1 levels might increase the likelihood for developing epileptic seizures after brain injury later in life. In addition, considering the importance of DISC1 in the laminar organization of the brain during development (Brandon et al., 2009; Ishizuka et al., 2006; Jaaro-Peled et al., 2009; Meyer and Morris, 2008; Schurov et al., 2004), slight variations in DISC1 levels might promote the subtle neuronal migratory and laminar disorganization specifically within the hippocampus. Interestingly, individuals with mild granule cell dispersion but no clear hippocampal damage show increased incidences of subtle hippocampal malformations including ectopic white matter neurons in the mesial temporal lobes and low-grade papillary glioneuronal tumors (Fisher et al., 1998). For most conditions, these modifications would have little qualitative clinical impact. However, they could render the hippocampus more vulnerable to injury and perhaps the development of epilepsy under conditions of trauma. The condition described above would be analogous to the situation where individuals with mild brain impairment do not typically show evidence for neuropsychological impairment unless they are considerably fatigued or are required to perform tests under time-pressure situations. In other words, the statistical variation in dentate granule cell layer width may possess

functionally meaningful consequences only if a unique set of conditions are present at the time of observation. At present, the specific set of conditions that would be necessary to unmask this latent dentate gyrus abnormality is not known.

To date, there has been no experimental attempt to address the question of whether DISC1-induced dentate morphological variation has a clinical impact in the normal population. Unpublished results from our laboratory indicate that there may be a relationship between levels of dentate DISC1 expression and the rate of kindling. For example, a weak but statistically significant positive correlation was found between DISC1 expression in the dentate granule cell layer and the number of stimulations required to evoke the first class 2 and class 3 motor seizures ($r = -.36$, $P < .043$, $n = 9$). However, there was no relationship between the total number of generalized class 5 motor seizures and DISC1 expression. The transition from a limbic partial seizure semiology (Class 2 seizures) to more generalized motor seizure responses (Class 3 seizures) marks an important point in the process of epileptogenesis, in which the propagation of epileptiform activity is initially confined to circuits involving the ipsilateral hippocampus (between stages 1 and 2) and then begins to spread to the contralateral hippocampus and descending motor circuits (between stages 2 and 3) (Hewapathirane and Burnham, 2005). Importantly, this transition point is also marked by the sudden appearance of bilateral maximal dentate activation in dentate granule cells, which is associated with a compromise in the "gating" function of the DG and spread of epileptiform activity into the hippocampus proper. (Lothman et al., 1992) (see Chapter 1). Although the analysis described is exploratory in nature and thus direct causal inferences

should not be made, it does raise the interesting idea that DISC1 may be a regulator of seizure susceptibility by indirectly impacting dentate excitability.

Finally, it is also important to note that statistical variation in dentate layer morphology in the normal population may have minimal functional consequence. This possibility of course cannot be ruled out without direct experimental manipulation. However, there is evidence that subtle hippocampal malformations involving mild dentate granule cell layer dispersion have been reported in relatives of patients with mesial temporal lobe sclerosis (compared to age-matched control) most of whom did not have a history of febrile convulsions or seizures (Fernandez et al., 2001). Interestingly, many of these individuals also showed mild clinical indicators of neuropsychological dysfunction in psychometric measures involving memory. It would be interesting for future studies to determine whether single nucleotide polymorphism of the DISC1 gene, which can also produce similar cognitive deficits (Callicott et al., 2005), might be found in subjects that display evidence for mild dentate morphological abnormalities or have family members that have a genetic propensity for developing epilepsy.

5. Implications of Abnormal Hippocampal Neurogenesis on Learning and Memory In the Epileptic Brain

The fact that hippocampal neurogenesis is a phenomena that can be readily observed in a large variety of species (Gould, 2007) has hinted that newly generated neurons may have a distinct effect on hippocampal functioning. Because of the well-known role of the hippocampal formation in learning and memory (Eichenbaum, 1997; Squire, 1986), it has been speculated that newly formed neurons may play a role in this process. Indeed young granule cells in the DG show a greater propensity for synaptic

plasticity than fully mature granule cells (Schmidt-Hieber et al., 2004). In fact, when considering the limited spontaneous activity of dentate granule cells and the extensive powerful inhibitory modulation that they receive from local interneurons, increased hyperexcitability among new neurons might serve as a means to amplify information-relevant activity and improve integrated network function in the hippocampus.

The question that remains open is whether or not the addition of a large number of new neurons directly affects hippocampal memory function in the epileptic brain. As shown previously (see Chapter 3, 4, 5), many seizure-generated neurons display abnormal morphological characteristics, such as exaggerated dendritic outgrowth and maintenance of hilar-basal dendrites, which can have a substantial effect on functional integration into dentate networks (Zhao and Overstreet-Wadiche, 2008). In addition, a small proportion of seizure-generated neurons also migrate abnormally into regions that are usually devoid of granule cells. These abnormal features undoubtedly affect normal hippocampal functioning and memory formation. However, the maintenance of hippocampal-dependent learning and formation of long-term episodic memories also requires the continuous addition of newly functional granule cells into the granule cell layer circuitry, a finding that is supported by both animal and computational studies (Aimone et al., 2009; Jessberger and Kempermann, 2003; Shors et al., 2002; van Praag et al., 2002). From this perspective, the decreased addition of new functional granule cells into the granule cell layer during chronic epilepsy would likely impair hippocampal-dependent learning and memory function and may contribute to the etiology of cognitive and behavioural deficits often found in TLE patients (Kuruba and Shetty, 2007). Supporting this view, clinical studies have shown that patients with longer durations of

refractory TLE typically display more severe cognitive impairments over time (Bjornaes et al., 2005; Carreno et al., 2008; Jokeit and Schacher, 2004; Porter, 2008; Sass et al., 1990; Sass et al., 1995). Thus, it is possible that the cognitive deterioration observed in patients with refractory TLE is at least partially linked to diminished DG neurogenesis. Indeed, we have observed that long-term kindled rats display the greatest level of cognitive impairments at a point when levels of hippocampal neurogenesis is diminished (Chapter 5) (Kalynchuk and Wintink, 2005).

The model that we have proposed to account for kindling-induced impairment in hippocampal-dependent memory formation and function is presented in Fig. 6-2. This model is based on the general observation that it requires at least two to four weeks for newly generated neurons to begin fully integrating themselves into preexisting adult hippocampal circuits (van Praag et al., 2002). Because hippocampal cell proliferation and neurogenesis are highest during the early stages of kindling (i.e., around the 30th stimulation) (Chapter 3), it is reasonable to assume that cells born around this point would have had sufficient time to differentiate, migrate, and integrate themselves by the time the 99th kindling stimulation has been delivered. Important to this model is the observation that the degree of kindling-induced cognitive and behavioural impairment displays a monotonic relationship with the number of kindling stimulations, i.e., cognitive impairments are less severe during the early stages of kindling but worsen dramatically as a greater number of stimulations is delivered (Kalynchuk et al., 1997).

How does the addition of a large number of functional neurons impact network function and memory formation in the epileptic hippocampus? One possibility is that the aberrant integration and maintained presence of a large number of newly generated cells

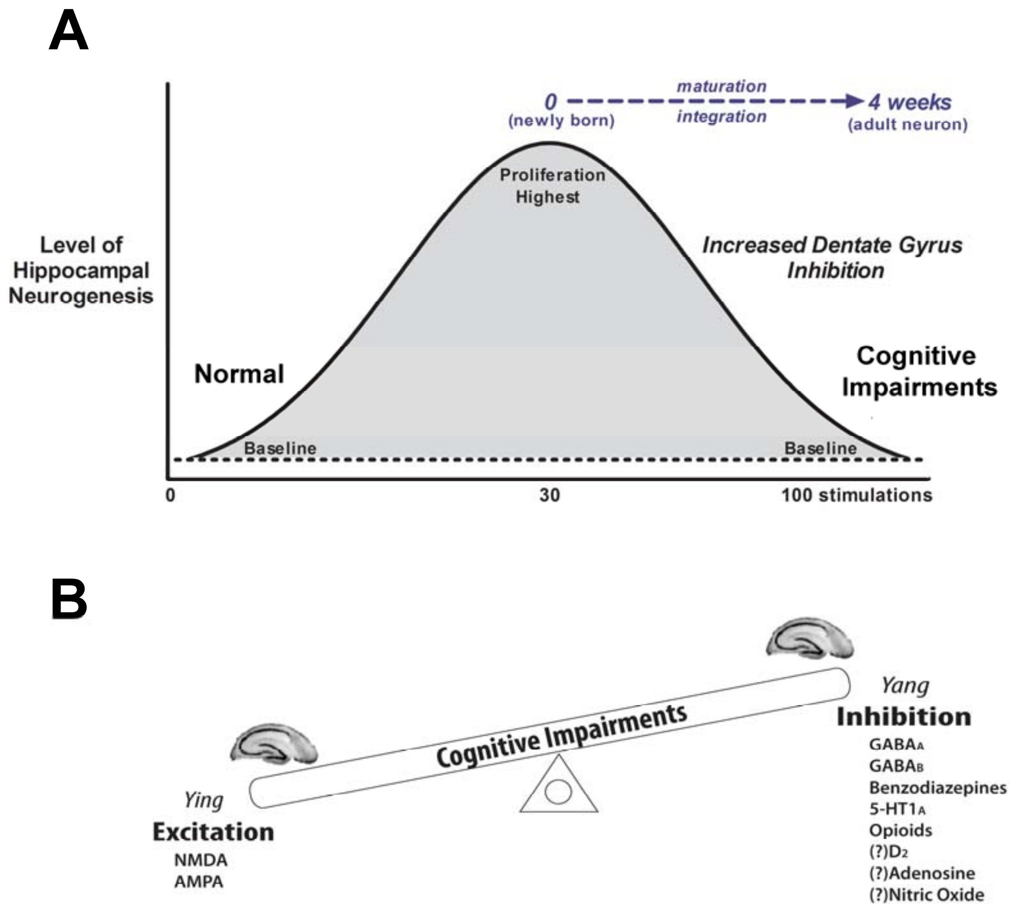


Figure 6-2 Hypothetical model to explain the impact of aberrant hippocampal neurogenesis on interictal cognitive impairment. A) Time course of seizure-induced neurogenesis and interictal cognitive impairments in the amygdaloid kindling model of temporal lobe epilepsy. B) A hypothetical model for explaining the increased cognitive impairments associated with long-term kindling. It is argued that repeated kindled seizures results in a progressive change and weighting towards increased inhibition within the dentate gyrus circuits. This compensatory increase in inhibitory activity within the dentate attempts to reestablish the "functional gating" mechanism and limit the spread of epileptiform activity within the hippocampal network. However, because the increased inhibition suppresses the activity of both mature and recently generated dentate granule cells, it will result in a loss in overall hippocampal-dependent information processing. This in turn contributes to the learning impairments and cognitive impairments displayed by long-term kindled animals. (?) denotes possible changes in receptor subtypes or neurochemical systems after kindling. Adapted from : (Kalynchuk and Fournier, 2009).

impairs behavioural function by degrading network performance. Modeling studies have offered some indirect insight into this problem by analyzing the effect of adult neurogenesis on learning and memory in neural networks with a constant neuronal turnover, where the addition of new neurons with randomly imposed connections is compensated for by the death of randomly chosen pre-existing neurons (Becker, 2005; Chambers et al., 2004; Deisseroth et al., 2004; Garthe et al., 2009; Wiskott et al., 2006). Under such conditions, slight beneficial effects on new learning accompany the clearance of pre-existing neuronal connections, i.e., old memories. In support of this idea, an interesting behavioural study using the forebrain-specific presenilin-1 gene knockout mice revealed a striking deficiency in environmental enrichment-induced neurogenesis (Feng et al., 2001). In addition, they also found that these transgenic mice with deficient hippocampal neurogenesis exhibited longer retention of contextual fear memory. This led them to hypothesize that the addition of new neurons into the hippocampal network via neurogenesis may participate in the periodic clearance of outdated hippocampal memory traces, thereby making room for the acquisition and temporary storage of new memories. Because a large number of functional neurons are incorporated into the hippocampal network after seizures (Overstreet-Wadiche et al., 2006), it is tempting to suggest that a similar situation might occur in the epileptic DG in which the inadequate clearance of seizure-generated neurons could diminish the capacity for the network to form and store new memories.

Based on our findings from Chapter 5, another intriguing possibility is that although seizure-generated neurons may be integrated into adult dentate circuits, they may not adequately respond to physiological levels of stimulation associated with

learning. In this study, long-term kindled rats were trained in a trace fear conditioning paradigm at a point when cells generated during the early stages of kindling would be characterized by heightened plasticity (Ge et al., 2007; Piatti et al., 2006; Tashiro et al., 2007; Wang et al., 2000) and enhanced responsiveness to learning stimuli by expressing immediate early genes (Kee et al., 2007). However, despite showing no evidence of differences in post-shock freezing behaviour during training, we were surprised to find that after testing long-term kindled rats in the original training context, few BrdU-labeled cells expressed the cellular activation marker c-fos, whereas short-term kindled and non-kindled rats both showed a small proportion of new cells (~2%) that were activated (BrdU+/c-fos+ cells) during the testing conditions. Importantly, the long-term kindled rats showed a significant deficit in freezing behaviour, whereas the other groups did not suggesting that impaired activation of seizure-generated cells might be associated with diminished memory recall.

5.1. They are not too excited: Factors Leadings to Impaired Activation of Adult-Born Neurons in the Epileptic Hippocampus

What could account for the reduced activation of seizure-generated neurons during both training and memory retrieval? One possibility is that seizure-induced remodeling of previously formed synaptic connections and the induction of various growth factors (such as FGF-2, BDNF) might alter the local environmental milieu encountered by new neurons preventing them from easily assimilating into the existing circuitry (Morimoto et al., 2004; Racine et al., 1998). Another possibility that is not mutually exclusive, is that these new cells are being actively inhibited by surrounding cells and thus prevented from responding during the learning period (the "active

inhibition hypothesis"). In support of this idea, we found that in addition to diminished activity of the newly born cells after testing, the total extent of Fos-immunoreactivity in the DG of long-term kindled rats was also significantly lower than controls, suggesting that there may have been a general reduction in the activation of the DG. In addition, several studies have shown that inhibitory receptor expression and binding (e.g., for $GABA_A$/benzodiazepine, and 5-$HT1_A$ receptors) is significantly increased within the DG after kindling, whereas excitatory receptor expression and binding (e.g., for NMDA and AMPA receptors) is generally decreased (*see* Fig. 6-2B) (Kalynchuk et al., 1999; Kalynchuk et al., 2001; Kalynchuk et al., 2006; Lehmann et al., 1996; Liu et al., 2009; Tuff et al., 1983a; Tuff et al., 1983b). In line with these molecular studies, it has been shown that kindling increases feedback and feedforward inhibition in the DG (Gutierrez and Heinemann, 2001; Masukawa et al., 1999). (Although there is evidence that feedback and feedforward inhibition is reduced other areas of the hippocampus, such as in the CA1 subfield (Alger and Teyler, 1976; Michelson et al., 1989).

Support for the "active inhibition hypothesis" is provided by a recent study from Jakubs and colleagues (2006), in which they showed that after electrically-induced status epilepticus, the excitatory drive on seizure-generated neurons was reduced, whereas inhibitory input to these same cells was increased. These findings could only be accounted for by seizure-induced changes in the local microenvironment encountered by these cells at the time of development because cells generated after a period of wheel running in non-epileptic animals did not show similar changes. Therefore, it is conceivable that the reduced activation of newly generated neurons after behavioural testing might not be due to a failure of seizure-generated cells to correctly integrate

within preexisting circuits. Rather it likely that the reduced activation of these cells reflects a compensatory increase in local inhibitory input that functions to maintain network homeostasis or a seizure-free state at the expense of inhibiting all other forms of dynamic network states.

There is evidence that GAD67, a major GABA-synthesizing enzyme that is strongly modulated by activity (Esclapez et al., 1994; Liang et al., 2000), is significantly upregulated in the dentate granule cell layer after long-term kindling (see Figure 6-3). Interestingly, preliminary experiments using double immunolabeling of GAD67 and BrdU have revealed that there is a significant increase in GAD67 punctate staining surrounding the somatic compartment of BrdU-labeled cells in long-term kindled rats (Fournier, Botteril, & Kalynchuk unpublished observations 2009). Although GABA has depolarizing effects on new neurons early in their maturational development (Overstreet et al., 2005), it is unlikely that GABA input to our BrdU-labeled cells would be excitatory as new neurons of this age (i.e., ~ 4 weeks) typically receive phasic inhibitory input (Ge et al., 2006). Therefore, new cells born during the early stages of kindling might receive increased inhibitory input from surrounding cells during their maturation as seizure stimulation continues.

At present, the source of the increased GABAergic innervation after long-term kindling is not known. However, there are two possibilities. One possibility is that increased sprouting from local inhibitory neurons might contribute to increased innervation of dentate granule targets. Although there is loss of parvalbumin-containing inhibitory interneurons in the hilus in epileptic patients, there is evidence that perisomatic inhibitory input is preserved or actually increased on dentate granule cells (Wittner et al.,

2001). This hyperinnervation might be a compensatory change in the inhibitory network that serves to prevent the occurrence of future seizures, however, it cannot be excluded that these conditions could also promote more effective synchronization of granule cell firing once a seizure has occurred (Freund and Katona, 2007). The second possibility is based on the fact that dentate granule cells can transiently express a GABAergic phenotype in an activity- and protein synthesis-dependent manner (Gutierrez, 2000; Gutierrez, 2002; Gutierrez et al., 2003; Jaffe and Gutierrez, 2007; Lamas et al., 2001; Lehmann et al., 1996; Ramirez and Gutierrez, 2001; Schwarzer and Sperk, 1995), giving rise to GABAergic transmission from mossy fibers onto their CA3 targets. For example, after seizures, dentate granule cells show an increase in GAD67 expression (Ramirez and Gutierrez, 2001). Because of the considerable evidence of mossy fiber sprouting after seizures (Blaabjerg and Zimmer, 2007; Danzer et al., 2009; Santhakumar et al., 2005; Walker et al., 2002), it is possible that some of these sprouted terminals, which innervate the inner molecular layer and hence the dendrites of dentate granule cells, might also contain and release GABA. These mossy fiber terminals will likely provide inhibitory input onto both mature and newly generated dentate granule cells, thus limiting their activity.

It is unclear whether the increased inhibition within the DG actually contributes to the epileptic condition. However, considering that the DG is known to regulate hippocampal excitability and serves as a major gateway through which excitation from the entorhinal cortex enters the hippocampus (Lothman et al., 1992), it is conceivable that the development of inhibitory processes and other seizure suppressing mechanisms following repeated seizure activity might reflect a homeostatic response that serves to

maintain the interictal state. As a result, the learning and cognitive impairments experienced by some epileptic patients might be a by-product of the brain's attempt to limit the future occurrence of seizures.

If the above suggestion holds true, then prophylactic treatments that serve to increase GABAergic transmission may inadvertently worsen the cognitive impairments experienced by some patients. Indeed there is data available showing that pharmacological treatments that reduce seizure frequency can actually aggravate interictal behavioural complaints in some patients (Chkhenkeli and Geladze, 1982; Flor-Henry, 1983; Kanner, 2001; Krishnamoorthy et al., 2002; Schiffer, 1987; Wolf, 1991). Although the mechanism(s) responsible for producing these effects are not well understood, it is likely that potentiation in GABA-mediated inhibition will play a role (Kalynchuk and Fournier, 2009). For example, there is evidence that treatment with flumazenil (a benzodiazepine antagonist) can decrease interictal emotionality in partially kindled rats (Adamec, 1993).

One intriguing possibility might be that if the elevated levels of GABAergic inhibition within the epileptic DG could be slightly reduced before training, then it might be possible that kindled rats would show normal learning and memory performance. Of course the major limitation with such a proposal is that decreased GABAergic inhibition might actually facilitate too much excitation, thus seizure generation. However, a dose-response study involving focal administration of a selective GABA antagonist, such as bicuculline, into the DG, would allow for the determination of an optimal concentration that can reduce inhibitory activity but not trigger spontaneous epileptiform activity. In fact, there is evidence that focal administration of bicuculline into the CA1 subfield of the

hippocampus can enhance memory retention on a one-trial step-down avoidance task (Luft et al., 2004). Further study in this area may help identify novel pharmacological as well as potential non-pharmacological approaches that selectively target impaired hippocampal neurogenesis in an attempt to provide effective seizure relief and decrease the cognitive impairments associated with chronic epilepsy.

It is should be acknowledged that seizure-induced disruption in neuronal circuits in addition to those involving the DG will likely contribute to impaired performance on this task. Preliminary data has shown that kindling-induced changes in the perirhinal cortices might be an important site mediating the learning impairments in trace fear conditioning after long-term amygdaloid kindling (Fournier, Botteril, & Kalynchuk unpublished observations 2009). For instance, when a step-wise multiple regression was used to determine if the number of c-fos+ neurons in various brain structures (e.g., basolateral amygdala, central amygdala, periaqueductal gray, cingulate, CA3, CA1, hilus, dentate gyrus) could predict freezing behaviour during the context text, c-fos expression in the left perirhinal cortex was the first structure to enter into the regression equation and it accounted for approximately 20% in freezing behaviour. Importantly, c-fos expression was markedly reduced in the left perirhinal cortex after long-term kindling. Previous work has found that perirhinal kindling impairs anxiety-like behaviour and object recognition in rats (Hannesson et al., 2005). Moreover, the perirhinal cortex is also known to play an important role in auditory trace fear conditioning (Kholodar-Smith et al., 2008). Therefore, it is likely that in addition to disruption of the dentate gyrus circuits kindling-induced changes in a variety of other brain regions, such as the perirhinal cortex, will also contribute to the deficits found in the trace-fear conditioning paradigm.

6. Limitations

6.1. Clinical Relevance of Kindling

Kindling has been established in every species systematically studied to date, including frogs, reptiles, mice, rats, rabbits, dogs, cats, rhesus monkeys, and baboons (McNamara et al., 1980). In fact, the behavioural patterns of amygdaloid kindled seizures are remarkably similar in many species, yet some differences do exist. For example, stimulation of rhesus monkeys for periods of approximately 5 to 9 months is required to induce partial motor and secondarily generalized seizures, whereas stimulation of Senegalese baboons (*Papio papio*) for only 11 to 22 days is required to induce partial motor and secondarily generalized seizures in this species (Wada et al., 1978; Wada and Osawa, 1976).

Despite the fact that kindling is one of the most widely used models of seizures and epilepsy, it remains unclear how the results from kindling experiments further our understanding of the neurobiology of human epilepsy (Bertram, 2007). In addition, arguments have been raised that kindling may not be an optimal model of epilepsy because kindling does not result in a state of recurring spontaneous seizures, the common definition of epilepsy. Although spontaneous seizures after kindling have been reported (Brandt et al., 2004; Michalakis et al., 1998; Pinel and Rovner, 1978), rodents typically require on the order of 150 to 300+ stimulations before they exhibit spontaneous seizures. However, there is some evidence that the development of spontaneous seizures may occur sooner during the process of kindling for some species (i.e., baboon, gerbils, canines, felines, etc.) It is also unclear whether the kindling process actually occurs in humans. In fact, the ingravescent progression of epileptic events observed with kindling have only been witnessed in clinical practice occasionally (Monroe, 1982). The incidence

of developing epilepsy in patients who receive repeated episodes of electroconvulsive therapy (ECT) to treat severe depression is in fact quite low (Fink et al., 2008; Krueger et al., 1993) but also see (Rasmussen and Lunde, 2007). Moreover, there is a report of a patient who underwent over 1200 separate ECT treatments and never developed spontaneous seizures (Lippman et al., 1985).

If kindling does occur in humans, what aspects of human epilepsy might be due to a kindling-like mechanism? It is known that focal hyperexcitability can develop near a vascular anomaly or slowly growing neoplasm. In both situations, micro-bleeding could occur (Chaskis and Brotchi, 1998). Over time, micro-bleeding into brain parenchyma that results in the deposition of iron into the extracellular space could lead to the development of focal seizure activity in a process analogous to kindling (Willmore et al., 1978). Other evidence that a kindling-like process might occur in human patients is provided by the direct inspection of patient histories. For example, many patients report a prolonged history of "strange feelings" and other aspects of experiential symptomology that are later recognized to be part of the seizure when patients begin developing more obvious complex partial seizures with alterations in consciousness or awareness (Glaser, 1987). In addition, there are some situations in which patients who have a long history of stable partial seizures for many years only begin seeking further medical help once these seizures start to evolve and manifest as motor convulsions (Engel, 1996). Although it is impossible to ascertain with certainty that these events represent a clinical progression in the patient's seizure disorder, the fact that resolution of these symptoms occurs following surgery or aggressive pharmacological management provides at least partial support for the hypothesis that seizure disorders might "kindle" in patients over time. Of course it is

possible that a kindling-like process may be more common in human patients. However, the worry of legal retribution and accusation of medical misconduct may have prevented wide-spread report of this phenomena in the clinical literature (McIntyre et al., 2002).

6.2. Additional Molecular Markers that May Contribute to Aberrant Neurogenesis after Seizures

It is premature to assume that reelin is the only guidance cue affected in TLE or that deficits reelin and DISC1 are the only systems that alter somatic locations of granule cells (Chapters 3 and 4). Other potential mechanisms might exist. One possibility involves the direct action of GABA proliferating progenitor cells. It is known that complex GABAergic networks are found in the adult neurogenic zones and that GABA-mediated signaling plays an important role in instructing the proliferation and differentiation of progenitor cells (Platel et al., 2008; Wang et al., 2003). In addition, GABA signaling between astrocytes and newly proliferated cells have been shown to control the speed of neuronal precursor migration (Bolteus and Bordey, 2004). For example, when ambient GABA levels are high, there is a significant reduction in the rate of migration with many cells migrating ectopically. In contrast, administration of the $GABA_A$ receptor antagonist bicuculline enhances the migration rate of these cells suggesting that neuronal precursor cells "sense" endogenous levels of GABA via $GABA_A$ receptors and ultimately reduce their speed of migration in regions of high GABA concentration. In fact, administration of $GABA_A$ receptor antagonists in developing animals or human infants is known to elicit heterotopic brain malformations suggesting that increased $GABA_A$-mediating signaling can disrupt normal cell migration in the developing brain (Frieder and Grimm, 1985; Stefovska et al., 2008; Weisz and

Sarkozi, 1989). As mentioned earlier, GABA markers, such as GAD67 and $GABA_A$ receptors, show significant upregulation in the dentate after kindling. It is conceivable that increased GABAergic signaling after a seizure, especially in the SGZ, may inadvertently affect migrational behavior of neuroblasts causing these cells to become displaced from the granule cell layer into the hilus.

Another molecular candidate for producing aberrant hippocampal neurogenesis might be brain-derived neurotrophic factor (BDNF). BDNF expression is increased by seizures (Isackson et al., 1991) and BDNF infusions into the intact adult rat brain stimulated hippocampal neurogenesis and led to the appearance of ectopic dentate granule cells in the hilus (Scharfman et al., 2005). It is important to note that none of these suggested mechanisms are mutually exclusive and all of them could contribute to the aberrant integration of adult-born neurons after seizure-induced injury.

6.3. Sex-specific effects on hippocampal plasticity and neurogenesis

With the exception of Chapter 2, which compared both intact females and males on kindling-induced changes in synapsin I expression, we have limited data examining potential sex-specific effects after kindling. We acknowledge that this is a limitation of the present work. There is considerable evidence that changes in hippocampal excitability occur at different points throughout the female estrus cycle (Warren et al., 1995) and that this may confer differential risk for seizure susceptibility (Scharfman and Maclusky, 2006). It is also known that limbic seizures can directly arrest ovarian hormone cyclicity in kindled animals (Edwards et al., 1999; Edwards et al., 2000). For instance, amygdaloid kindling thresholds are lowest with estradiol replacement and highest with progesterone

replacement in ovariectomized females (Edwards et al., 2000). Moreover, it well known that intact females typically show fewer neurodegenerative effects after seizures and that this effect is directly related to the circulation of ovarian hormones, such as progesterone, that are known to have neuroprotective properties (Scharfman et al., 2008; Scharfman and Maclusky, 2008). Finally, many of the oestrogens are known to increase hippocampal cell proliferation in female rats (Barha et al., 2009). Whether these specific differences impact the degree of functional neurogenesis after seizures is presently not understood and could provide an important area for future experiments.

7. Future Directions

7.1. Does Kindling Affect Neurogenesis in Other Brain Regions?

There is evidence that seizures up-regulate neurogenesis in other brain regions. For instance, it has been shown that pilocarpine seizures increase the proliferation of neuroblasts within the subventricular zone and their migration along the rostral migratory stream toward the olfactory bulbs (Parent et al., 2002). Interestingly, these prolonged seizures also induced a portion of these neuronal precursor cells to exit the rostral migratory stream prematurely and migrate into forebrain regions such as the striatum and neocortex.

Another region that has shown evidence for adult neurogenesis is the piriform cortices (Pekcec et al., 2006). The piriform cortex is especially interesting considering its capacity for seizure generation (Loscher and Ebert, 1996). The site of generation for the cells that populate the piriform in the adult brain appear to be derived deep in the caudal subventricular zone, where neuroblasts transverse a ventrocaudal migratory stream to enter the rostral piriform cortex (Shapiro et al., 2007). There is evidence that pilocarpine-

induced seizures can increase the birth of new neurons in this region (L. Shapiro, personal communication). However, these types of seizures also produce significant degeneration, especially within this brain region (Peredery et al., 2000) making it unclear whether the increased proliferation in this region occurs in response to increased cellular injury and neuronal dropout or due to seizure-induced alterations in migratory guidance proteins that cause some cells to migrate astray.

We have preliminary data suggesting that long-term kindling can also increase neurogenesis in this region as inferred by BrdU and doublecortin immunohistochemistry (Fournier, Botteril, & Kalynchuk unpublished observations, 2009). The majority of the cells are located in the principal cell layer, layer II, although some pyramidal-semilunar transitional neurons are stained in layer III. Interestingly, we were able to find that a few of the BrdU labeled cells also co-expressed reelin. Reelin is known to be abundantly expressed in this region; however, it has not been documented that reelin-synthesizing neurons are generated in the adult brain. Although the functional relevance of this finding is not known, it does provide an interesting starting point to begin addressing whether seizures may influence functional neurogenesis in other regions besides the hippocampus.

7.2. Does the Decrease in Reelin Expression After Kindling Actually Reflect the Loss of Reelin-Expressing Neurons?

In Chapter 3, we showed that there was a decrease in the number of reelin-expressing cells in the dentate SGZ after long-term kindling. Importantly, it appears that these cells are lost at some point in the process of long-term kindling because the number of reelin-expressing cells is similar to that of non-kindled controls during the early stages of kindling. It is known that a reduction in a number various types of inhibitory neurons

reduces the inhibitory drive on excitatory neurons. This leads to an enhancement of the effects of excitatory afferent input onto these cells. In support of this hypothesis, a wide range of morphological and physiological studies from many laboratories using human tissue and animal models have shown that some specific subtypes of GABAergic interneurons are lost in TLE (Buckmaster and Dudek, 1997; Houser and Esclapez, 1996; Obenaus et al., 1993; Sloviter, 1987; Zhang and Buckmaster, 2009). In addition, kindling-induced decreases in paired-pulse inhibition have been consistently reported in the CA1 region (Kamphuis et al., 1986; Kamphuis et al., 1992; Kapur et al., 1989; King et al., 1985; Michelson et al., 1989). It has been proposed that a persistent reduction in GABA immunoreactivity in the CA1 region after kindling may account for some of these observations (Kamphuis et al., 1987; but *see* Lehmann et al., 1996). It also appears that the loss of inhibitory neurons may be highly specific to subpopulations of GABAergic neurons. For instance, Sloviter (1987; 1991) reported that after electrically-induced status epilepticus, an almost complete loss of somatostatin-immunoreactive neurons in the DG is found, whereas GABA- and cholecystokinin-immunoreactive neurons remain intact. In addition, Magloczky and Freund (Magloczky and Freund, 1993) compared the vulnerability of specific subpopulations of hilar interneurons in the hippocampus following unilateral injection of kainate into CA3. They found that the most vulnerable cell types were the hilar somatostatin and calretinin-positive inhibitory neurons, as well as the mossy cells. It is known that reelin is expressed in the adult DG by specific subsets of hilar interneurons that are known to be vulnerable to seizure-induced injury (Alcantara et al., 1998). Interestingly, reelin is rarely expressed in hippocampal subpopulations of parvalbumin-, cholecystokinin-, and vasoactive intestinal polypeptide-immunoreactive

neurons (all < 6% colocalization). However, variable numbers of calretinin- (i.e., 58% colocalization), calbindin- (14%), and somatostatin- (75%) and neuropeptide Y- (30%) immunoreactive neurons express reelin. Thus, reelin appears to be synthesized and released by distinct populations of GABAergic neurons that may be lost in the epileptic brain.

An alternative explanation is that the decreased reelin expression does not reflect the actual loss of reelin-expressing neurons, but instead reflects a decrease in reelin synthesis. There are some data supporting the idea that inhibitory transmission may be preserved or even enhanced after kindling. For example, paired-pulse inhibition is typically increased in the DG after kindling (Stringer and Lothman, 1989; Tuff et al., 1983a; Zhao and Leung, 1992) suggesting that functional alterations within inhibitory circuits of the DG after seizures may represent compensatory changes that attempt to balance the enhanced excitatory input into this region (Wittner et al., 2001). Furthermore, there is evidence that the reelin gene promoter can show epigenetic regulation (Chen et al., 2002; Dong et al., 2005). In neuropsychiatric disorders, such as schizophrenia, it has been found that hypermethylation of the reelin promoter is associated with decreased transcriptional activity of the reelin gene, a process that ultimately leads to a reduction in reelin mRNA and protein expression through chromatin remodeling mechanisms (Grayson et al., 2005). Interestingly, these effects can be reversed with the use of valproic acid (Costa et al., 2003; Tremolizzo et al., 2002), a compound that possesses active DNA dimethylation properties and is known to possess anticonvulsant properties.

There has been limited work examining whether the epigenetic regulatory machinery of the cell can be directly affected by epileptic seizures; however, one recent

study showed epigenetic changes in the reelin promoter of hippocampal samples from temporal lobe epileptic patients (Kobow et al., 2009). They found that there was an increase in DNA methylation of the reelin promoter that resulted in negative transcriptional regulation of reelin expression. One intriguing possibility is that although some reelin-expressing interneurons might be lost after long-term kindling, many cells may still be present but are actively inhibited from producing reelin because of hypermethylation of the reelin promoter. If true, then treatments that specifically target dysfunctional epigenetic regulatory systems might help reverse the changes in the reelin promoter in the epileptic hippocampus. Recently, Jessberger and colleagues (2007a) showed that treatment with valproic acid can prevent aberrant neurogenesis after kainate-induced seizures. Interestingly, these animals also showed normal performance in an object recognition test. Although these results suggest that seizures might directly impact the epigenetic regulation of specific genes, further study will be necessary in order to identify which specific genes show direct seizure-induced epigenetic modulation.

7.3. Changes in DISC1 Expression in Other Models of Epilepsy?

Outside of the experiments described in this dissertation, there has been no systematic study of the effects of seizures on DISC1 expression. DISC1 is expressed in additional hippocampal regions, including CA3 and CA1. Therefore, , it might be interesting to determine whether seizures affect DISC1 expression in these regions. Preliminary evidence suggests that long-term kindling of the amygdala, but not long-term kindling of the caudate nucleus, is associated with a reduction of DISC1 expression in the dentate granule cell layer/SGZ and a slight decrease in DISC1 expression within the CA1

hippocampal subfield (Fournier, Botteril, Johnson, & Kalynchuk, 2009). At the present moment, we are not certain if long-term kindling of the caudate impacts functional hippocampal neurogenesis. However, considering that caudate-kindled rats do not show cognitive or behavioural impairments after long-term kindling, it is likely that caudate kindling does not significantly disrupt hippocampal and amygdalar circuitry (Kalynchuk et al., 1998).

We have also examined the effects of pilocarpine seizures on DISC1 expression. Animals were sacrificed 6 weeks after injection with pilocarpine. In contrast to long-term kindling (which incidentally also lasts for ~6 weeks), we observed no change in DISC1 expression within the dentate SGZ/granule cell layer. However, there was evidence for a significant decrease DISC1 expression in parts of the CA3 and CA1 subfields. We were extremely surprised by these findings. One possibility is that because the period of active neurogenesis and accelerated dendritic growth of new neurons in the DG occurs approximately 2 weeks after pilocarpine seizures (Parent et al., 2006), it was possible that 6 weeks reflected a time-point in which any previous change in DISC1 expression had returned to normal. Indeed there is evidence that hippocampal cell proliferation decreases in chronically epileptic animals (Hattiangady et al., 2004). Future studies examining potential alterations in DISC1 expression at various time-points after the induction of pilocarpine may help clarify this issue and determine a specific role for DISC1 in mediating aberrant seizure-induced neurogenesis in other models of limbic epilepsy.

7.4. Does Impaired DISC1 Expression Mediate Aberrant Structural Growth of New Neurons After Seizures?

Considering the evidence that selective DISC1 knockout produces cellular changes in newly born cells that are similar to the effects of epileptic seizures, there is a strong possibility that seizures may affect DISC1 signaling in dentate progenitor cells. To examine this possibility further, additional studies may consider utilizing retroviral-mediated cell labeling to identify the cells born in response to seizures. Correlations can then be performed in which intrinsic levels of DISC1 expression at the single cell level are calculated and compared with a variety of dendritic features (such as apical dendrite length, branching order, spine density etc.) in the new neurons. Retroviral procedures have selective advantages over BrdU methodology in that the entire structure of neurons can be easily identified using standard fluorescent microscopy, whereas BrdU labeling is only specific to the nuclear structure of the proliferating cell and necessitates multiple labeling techniques. Finally, selective knockdown of DISC1 expression in new dentate granule cells using an RNA-interference approach will permit a more direct experimental test of the consequences that loss of DISC1 expression has on the proliferation, maturation, and migration of new neurons after epileptic seizures. Such approaches may help identify specific molecular pathways mediated by DISC1 that could be critical in producing aberrant seizure-induced changes in neuronal development and maturation.

7.5. Impact of Altered Reelin Signaling Outside of the Dentate Gyrus

It has been become increasingly clear that reelin not only controls migration during embryogenesis but also promotes neuronal maturation and synaptic development in the adult brain (Costa et al., 2002; Fatemi, 2005; Rogers and Weeber, 2009; Weeber et

al., 2002). Neuroanatomical studies have found that in the absence of reelin or Dab1, neurons show stunted dendritic growth, decreased branching , and fewer dendritic spines compared to wild type mice (Costa et al., 2001; Pappas et al., 2001). Several studies have also shown that reelin plays a role in promoting long-lasting changes in synaptic responsiveness or plasticity in the adult brain. For instance, the addition of recombinant reelin to hippocampal slices dramatically elevates the magnitude of hippocampal LTP at Schaffer collateral-CA1 synapses—a process that requires the combined activity of both ApoEr2 and VLDLR receptors, as well as Dab1 and Src kinase-induced phosphorylation of NMDA receptors at tyrosine residues (Beffert et al., 2004; Weeber et al., 2002). Considering that CA1 pyramidal cells are more vulnerable to recruitment into seizure networks and sensitive to excitotoxic damage, it would be interesting to determine what effect seizures may have on reelin expression in this region. However, it is presently unclear if reelin expression in the CA1 region is also affected in the chronically epileptic brain. To begin addressing this question, a combined molecular and electrophysiology study could be performed in which the effects of applied recombinant reelin on CA1 excitability on hippocampal slices from epileptic animals is examined. In addition, microdissection of the CA1 subfield will allow for examination of the distribution of reelin protein and mRNA, in order to determine if reelin expression is compromised in this region.

7.6. Contribution of Reelin and DISC1 to Neuropsychiatric Deficits Associated With Temporal Lobe Epilepsy

Multiple studies have confirmed a high comorbidity of epilepsy with depression, anxiety disorders, bipolar disorder, and schizophrenia (Gaitatzis et al., 2004; Hermann et

al., 2000; Kanner, 2008; Marsh and Rao, 2002b). Although the association between epilepsy and neuropsychiatric disturbances has been controversial, (Cornaggia et al., 2007; Whitman et al., 1984), several studies have found a higher frequency of behavioural problems and neuropsychiatric illness in patients with epilepsy than would be expected on the basis of a chronic illness alone (Hermann and Jones, 2006; LaFrance, Jr. et al., 2008; Marsh and Rao, 2002a; Swinkels et al., 2005; Tellez-Zenteno et al., 2007). Estimates have further shown that the prevalence of interictal emotional and moods disorders among TLE patients may be as high as 50% (Vazquez and Devinsky, 2003) suggesting that in a large percentage of patients these conditions are a source of significant behavioural disability.

The molecular mechanisms responsible in elevating the risk for developing neuropsychiatric illness in TLE patients are not known. However, considering that impairments in reelin (Costa et al., 2002; Fatemi et al., 2000; Fatemi et al., 2005) and DISC1 (Burdick et al., 2005; Ishizuka et al., 2006; Nakata et al., 2009) signaling contribute to the etiology of mood and thought disorders in other neuropsychiatric illnesses, it is possible that these signaling pathways may be dysfunctional in TLE patients suffering from neurocognitive and neurobehavioural disorders. Therefore, future genetic linkage studies directed at studying the relationship between variants in the DISC1 and reelin gene and their distribution in patients with temporal lobe epilepsy with or without neuropsychiatric illness may help shed light on novel molecular candidates that are important in producing cognitive and behavioural impairment in these patients.

8. Conclusions

In this dissertation, evidence was provided that the loss of reelin and DISC1 after kindling may have contributed to the abnormal migration, development, and integration of newly generated neurons in the adult hippocampus. In addition, we revealed that many seizure-generated neurons are functionally inactive during learning and as such, that they may be directly involved in the behavioural and cognitive impairments frequently found in chronically epileptic patients. Finally, we showed that repeated epileptic seizures also alter the structural plasticity and synaptic wiring in many brain regions outside of the dentate gyrus, including the CA3 and CA1 subfields, as well as the amygdala. These important observations provide a starting point for understanding how changes in neurogenesis and structural plasticity within the hippocampus contributes to the development of temporal lobe epilepsy and the cognitive impairments associated with this condition. By understanding the molecular mechanisms that govern adult neurogenesis in the epileptic brain, more selective means of manipulating neurogenesis might emerge that allow for the positive aspects of neurogenesis, such as enhanced neural production (and repair), to proceed in the absence of potential negative aspects such as pathological integration and function. Further examination of these mechanisms may lead to more effective treatments or interventions that serve to limit the deleterious consequences of seizures on the brain and behaviour.

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