THE SITE-SPECIFIC EFFECTS OF KINDLING ON COGNITION AND ADULT HIPPOCAMPAL NEUROGENESIS

A Thesis submitted to the College of Graduate and Postdoctoral studies
in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Department

of Medicine at the University of Saskatchewan

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«До тех пор, пока вы предмет не постигли, он для вас представляется сложным и туманным. Но как только истина уловлена, все становится простым. Признак истины - простота, и все гении просты своими истинами.»

Иван Петрович Павлов

(Лекция 1918)

“As long as you don’t understand the subject, the picture seems to be cloudy and perplexing. But once the truth is caught – everything becomes simple. Simplicity is the ultimate sign of the truth, and all geniuses are simple in their truths.”

Ivan Pavlov

(Lecture notes 1918)

ABSTRACT

Nearly 1.5% of the general population is affected by epilepsy. Despite a long history of research and clinical endeavors to combat the disease, in 1 of 3 cases, epilepsy is intractable and resistant to medication treatment. Current pharmacological strategies target seizure onset as the primary manifestation of illness; however, patients with advanced stages of the disease suffer from a wide array of psychiatric comorbidities. Anxiety, depression and memory deterioration are the chief complaints of epileptic patients delegated from neurologic to psychiatric care.

Temporal lobe epilepsy is the most resistant form of epilepsy and the form that is most complicated by the presence of psychiatric and cognitive comorbidities that are increasingly recognized as critical factors in long-term patient care. These comorbidities are independent risk factors for poor quality of life. In fact, studies have shown that in patients with epilepsy, co-morbid factors correlate more strongly with poor quality of life than does seizure frequency. At present, the behavioural comorbidities associated with temporal lobe epilepsy are poorly understood, and their management is difficult because many commonly prescribed anticonvulsant drugs make them worse. Therefore, even patients with some degree of seizure control often continue to experience debilitating behavioural problems that impair their daily living.

This thesis attempts to understand the impact of seizures originating from different brain sites. The research described in this thesis was conducted using an animal model called kindling to investigate the hypothesis that seizure-induced alterations in hippocampal neurogenesis play a significant role in the cognitive deficits associated with epilepsy. The experiment used 25 adult male rats. Rats were divided into four group: hippocampal kindled (n = 6), amygdala kindled (n = 7), caudate nucleus kindled (n = 6) and control rats (n = 6). Kindled

rats received 99 electrical stimulations delivered to the appropriate brain region through an implanted bilateral electrode. At the end of the kindling phase, rats were subjected to a fear conditioning paradigm to assess cognitive behavior. After the fear conditioning, rats were sacrificed and immunohistochemical analyses were done to assess the impact of seizures on hippocampal neurogenesis and neuronal activation. The results indicated that there is a significant deterioration of cognitive performance following long-term limbic kindling (i.e., hippocampal and amygdala kindling only). This was accompanied by an increase in hippocampal neurogenesis that was paralleled with low expression of immediate early genes. Collectively, these findings enhance our understanding of mechanisms underlying the behavioral co-morbidities associated with temporal lobe epilepsy and **demonstrate the link between adult neurogenesis and cognitive impairments following long-term kindling of limbic brain regions.**

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LIST OF ABBREVIATIONS

dHip – dorsal hippocampus
BLA – basolateral amygdala
BrdU – bromodeoxyuridine
CS – conditioned stimulus
TBS –tris-buffered saline
PBS - phosphate-buffered saline
IHC – immunohistochemistry
ARC – activity-regulated cytoskeleton-associated protein, also known as Arg3.
DCX – doublecortin
NHS – normal horse serum
NGS – normal goat serum
BSA – bovine serum albumin
GCL – granule cell layer
DG – dentate gyrus
OB – olfactory bulb
AN – adult neurogenesis
FTC – fear trace conditioning
IEG – immediate early gene
TLE – temporal lobe epilepsy
BDNF – brain neurotrophic factor
CN – caudate nucleus
DS – dorsal striatum
SN – substancia nigra (lat.)
CeA – central amygdala

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Epilepsy

Epilepsy is a chronic neurological disease affecting approximately 50 million people worldwide (Birbeck et., 2010). It is also estimated that an additional 2.4 million people will be diagnosed with epilepsy every year. The majority of diagnoses (80%) fall onto patients from developing countries and importantly, it affects age groups during their most productive years of life (Birbeck et al., 2010). One in ten people would experience at least one epileptic seizure within the normal lifespan and one third of them will develop epilepsy.

The current International Statistical Classification of Diseases (ICD – 10) provides following definition of epilepsy:

'*Epilepsy is a brain disorder characterized by episodes of abnormally increased neuronal discharge resulting in transient episodes of sensory or motor neurological dysfunction, or psychic dysfunction* (ICD – 10: G-40).' ICD – 10 dedicates nearly 70 diagnostic chapters under the umbrella of 'epilepsy'. This is a heterogeneous disease complicated by a wide variety of clinical manifestations that depend upon many factors such as brain site, etiology, duration and time pattern of the onset. Such wide array of factors may have synergetic effects and complicate the process of diagnosis and treatment.

There are numerous causes of epilepsy, as both genetic and acquired factors may contribute and interact, suggesting that the pathophysiology of this disease is quite complex. Spontaneous seizures, which are the definitive feature of epilepsy leading to a diagnosis, are thought to be the result of a disbalance between inhibition and excitation shifting the system towards hyperexcitability. This process may occur in a restricted brain site and manifest as a

partial epilepsy, and/or subsequently ramify into a generalized epilepsy when seizure activity synchronizes and hyperpolarizes several brain sites simultaneously. Genetic causes are thought to be the most widespread causative effect for epileptic seizure development. Brain tumors, either benign or malignant are another common cause for epilepsy, accounting for nearly 30% of epilepsy cases (Liigant et al., 2001). Brain injury is another risk factor that may unfold into epilepsy in 6% of cases (Hausser et al., 1993) and generalized seizures are associated with traumatic brain injuries within the onset of the seizure (Lowenstein et al., 2009). Stroke is a major risk factor for elderly patients and may explain epilepsy occurrence in one-third of patients (Cleary et al., 2004). The list of epilepsy etiology may be associated with infections like toxoplasmosis or meningitis, febrile seizures in newborns or those seizures occurring in obstetric patients under the condition of severe eclampsia. Despite such variability, there appears to be a common pathophysiological mechanism. All epileptic seizures arise from excessively synchronous activity and sustained discharge of a selected neuronal population (Fisher et al., 2005). Therefore, the unifying feature of all epileptic syndromes is a persistent increase of excitatory systems. Albeit, because we do not have a full and clear picture of the mechanisms that underlie this excitation, further scientific investigation is necessary.

As mentioned above, spontaneous and unprovoked seizure onset is the definitive diagnostic feature of epilepsy. These seizures may be presented as brief episodes of a loss of consciousness accompanied by involuntary movement involving a part of the body (partial) or the entire body (generalized). Patients suffering from epilepsy also demonstrate increased risk for other psychiatric disorders that are paralleled with cognitive disturbances. Pharmacological resistance is a common phenomenon among patients with full-blown epilepsy converting them into candidates for surgery. However surgical resection of affected brain sites does not alleviate

cognitive deficits. Further, surgical patients often have more than one brain lesion within the affected area, a phenomenon called ‘dual pathology’. This appears to be common in younger patients. Additionally, current pharmacological treatment has a variety of side effects that contribute towards cognitive deterioration in patients with epilepsy. Alas, **nowadays treatment strategies leave patients with no effective treatment against the cognitive and emotional comorbidities of epilepsy**.

Temporal lobe epilepsy (TLE) is the most common form of epilepsy in adults. It is also one of the most pharmacoresistant types and appears to be hard to access for surgical treatment. The diagnosis of TLE may be further divided into partial with or without consciousness impairments and complex partial subtypes. Detection of epileptic foci is an additional diagnostic step that may label TLE with limbic or neocortical subtype, which is a rare clinical case scenario. Low amplitude with rapid activity rhythmic spikes, either synchronous or asynchronous, in the electroencephalogram (EEG) is a defining feature of this diagnostic group. The specific anatomic position of the hippocampus puts it into a crucial role contributing towards the progression of limbic TLE. Post-mortem analysis of patients suffering from TLE indicates total cellular reorganization in the hippocampal formation as sclerotic changes take place (Parent et al., 1997). Besides possible consciousness impairments, seizures can be accompanied by a variety of sensory phenomena such as auras, hallucinations or persistent Deja vu feelings. Epileptic onset frequently involves repetitive behavioural patterns or orofacial automatisms (head nodding, tremor of the limbs, lip smacking). The seizure itself is a relatively short event that leaves the patient in a post-ictal period. Interestingly, to date, there is no clear understanding about how and why seizures are terminated. However, the onset itself is just a visible manifestation of

progressive changes happening within the whole system. The chronic progression of behavioural changes in epileptic patients leads to a lower quality of life and stigmatization.

1.1.1. Epilepsy comorbidities

A comorbid condition is one that occurs during the progression of a main disease such as epilepsy. Thus, psychiatric and social comorbidities are diagnostic satellites of epilepsy as an index disease. Nearly 50% of patients suffering from epilepsy report at least one psychiatric or somatic comorbid disorder (Keezer et al., 2015; Forsgren et al., 1992). Among them, 20% of patients suffering from TLE demonstrate psychiatric complaints (Gaitatzis et al., 2004). Mood disorders, anxiety, psychosis and personality changes are the most common conditions in patients with epilepsy (Bragatti et al., 2004). Importantly, depression has a much more debilitating effect on the quality of life in patients with TLE than seizure frequency itself (Boylan et al., 2004; Perrine et al., 1995). Subsequently depressive states are the strongest risk factors for suicide in patients with epilepsy (Schmitz et al., 2005). Fear and anxiety are also common complaints as they are the most frequent feelings induced by seizures. In fact, rapid onset of fear and anxiety may present as panic disorder, leading to misdiagnosis (Sazgar et al., 2003).

Patients with TLE also suffer from cognitive impairments because the medial temporal lobes are involved in learning and memory processes. Patients with TLE often demonstrate deficits in declarative and episodic memory (Butterbaugh et al., 2004; Schwarcz et al., 2002), as well as impairments in long-term memory consolidation and spatial memory (Cataldi et al., 2013; Haag et al., 2010).

Any comorbid state can affect therapeutic decisions in people with epilepsy. Migraines, hepatic insufficiency or depression could be factors influencing the decision toward a type of antiepileptic drug as a treatment. Cognitive deficits may also question the suitability of surgical intervention in patients with TLE because of potential severe memory impairments from temporal resections.

Taking into consideration the above discussion, epilepsy may present not only as a neurological disease but also as a psychiatric condition. The clinician may be deceived by depressive or affective symptoms that mask epilepsy, thus complicating diagnostic procedures. The patient, on the other side, may face therapeutic 'Zugzwang' in attempt to combat epileptic comorbidity because neither pharmacological nor surgical intervention are able to reduce psychiatric impairments. Future scientific efforts are needed to understand the underlying mechanisms that govern epilepsy progression. It is evident and clear that current progress in the treatment of epileptic patients is not satisfactory, and specific need exists to better understand the mechanisms underlying behavioral co-morbidities in epilepsy and potentially bring novel treatment strategies to the clinic.

1.2. Kindling

In 1969, Graham V Goddard and colleagues released a publication titled "A permanent change in brain function resulting from daily electrical stimulation" where they provided a detailed description of a possible animal model of epilepsy. 'Kindling' is a term coined by those pioneering researchers and it refers to the gradual development and intensification of motor seizures resulting from daily electrical stimulation of a specific brain region. To target a selected brain site, animals (typically rats) are first subjected to stereotaxic surgery aiming to implant

permanent electrodes to deliver electrical stimulations. Intensification of seizure activity that is paralleled with behavioural convulsions resulting from electrical stimulation is a reliable hallmark of a successful kindling procedure. The kindling phenomenon proved to be a valid animal model of epilepsy once it was demonstrated that long-term electrical stimulation may elicit spontaneous convulsions (Pinel et al., 1978). A variety of anticonvulsant drugs may decrease and even reverse epileptic activity in limbic-kindled rats (Adamec et al., 1986) – that was another property of kindling that confirmed its relevance to kindling as a model of epilepsy. Much like current epileptogenic dogma, kindling decreases protective brain mechanisms (i.e., the GABA system) in response to focal discharge (Kokaia et al., 1994). This brief description clearly demonstrates that kindling initially models focal partial seizures. With the daily progression of electrical stimulation and the recruitment of other brain regions into seizure discharges, kindling comes to model complex partial seizures with secondary generalization (McIntyre et al., 1979). Finally, animals become undeniably epileptic once seizures become spontaneous after many days of electrical stimulations (Pinel et al., 1975; Wada et al., 1976).

Kindling creates a "pathology" on multiple levels. Goddard, as pioneer of kindling, argued that kindling induces structural alterations within or between neurons (Goddard et al., 1969). However, nowadays kindling is thought to systematically induce a sequence of long-term functional and structural changes, initiated by perturbations in synaptic transmission that eventually lead to morphological changes within neurons and neuronal circuits (Sutula et al., 1991). The initial episode of network synchronization during kindling upregulate NMDA receptor dependent components (Sayin et al., 1989). Then, NMDA receptor dependent elements induce intracellular Ca^{2+} influx. A high intracellular Ca^{2+} state activates the transcription of early immediate genes, which results in an avalanche of gene alterations (Hughes et al., 1998; Shin et

al., 1990; Sutula et al., 1996). Kindling also affects neurotrophins such as BDNF, NT3, NT4 (Xiao-Ping et al., 2004). In sum, these 'subneuronal' alterations contribute towards cellular pathology, and finally neuronal cell loss and apoptosis emerge.

Even a single seizure induced by kindling can induce apoptosis (Bengzon et al., 1997). Systematically repeated electric stimulations result in a measureable neuronal loss (Kavazos et al., 1994; Kolotski et al., 2002). Neuronal cell death is followed by a compensatory mechanism of 'tissue replacement', thus glial cells replace previously functional neurons. And finally, prolonged and systematic cellular death results in a pathomorphological phenomenon called hippocampal sclerosis (Thom et al., 2014). Therefore, seizure-induced neuronal injury and subsequent neuronal loss with hippocampal sclerosis initially starts from excitotoxicity, excessive Na+ and Ca^{2+}, osmolytic stress and free radical production (Henshall et al., 2007).

Once intrinsic compensatory mechanisms approach exhaustion, functional dysregulation can occur. This can be seen in kindling as a change in multiple domains of behaviour. For example, partial amygdala or hippocampal kindling in cats increases defensive responding to rats (Adamec et al., 1976). There are also studies showing that long-term-kindling produces anxiogenic effect in rats (Adamec 1990, Kalynchuk et al., 2000). Further, kindled animals develop long-lasting deficits in declarative memory (Hannesson et al., 2003). With kindling progression, there is a gradual development of memory deficits assessed in a radial maze task (Sutula et al., 1995). Kindled rats also demonstrate retention deficits in the Morris water maze (Gilbert et al., 2000). Therefore, depending on the kindling protocol, electrical stimulations can disrupt a wide spectrum of memory functioning, from spatial memory and navigation to declarative and associative memory.

Cognitive deficits observed in animal models of TLE were confirmed in human patients. For instance, a recent longitudinal neuropsychological study documented that patients with TLE have pronounced intellectual decline after 15-30 years compared to age-matched controls tested with IQ (Jokeit et al., 2002; Oyegbile et al., 2004).

There is general agreement that cognitive dysfunctions produced by kindling are most likely caused by cumulative alterations in hippocampal structures with progressive reorganization. Hippocampal sclerosis, neuronal cell death, and mossy fiber sprouting are detectable morphologic consequences of the kindling application that eventually may lead to cognitive deficits. However, the exact mechanisms that control and mediate these cognitive deficits remain unknown.

Taking collectively, kindling can produce pathological changes on multiple levels, from cellular and morphological aberrations to behavioural outcomes. In the present experiment, we chose kindling as a model of epileptic seizures because of its advantageous opportunity to control the number and frequency of seizures over a given period of time. Importantly, kindling provides an opportunity to compare how seizures originating from different brain regions affect behaviour and cellular organization.

1.3. Neurogenesis

During the past century, most of the neuroscientific community believed that adult neurogenesis was not possible. It was believed that once the mammalian brain reached maturity, the creation of new neuronal cells ceased. It has been nearly 50 years since the first report of neurogenesis in a mature rodent brain (Altman et al.1963). It is now widely accepted that neurogenesis occurs in the human adult brain as well as in other vertebrates (Eriksson et al.,

1998; Spalding et al., 2013). Recent scientific progress provided compelling evidence that new cells are continuously added to the mammalian brain throughout adulthood (Dery et al., 2013, Spalding et al., 2013). The term "neurogenesis" was defined as a process of cell division and development from a stem cell into a mature fully functional neuronal cell (Ming et al., 2011). It should also be mentioned that developmental neurogenesis and the notion of adult neurogenesis significantly differ. This research line focused on the scope of adult neurogenesis.

Although it is true that most neurons are generated before birth and never regenerate, two brain regions in human brain play an avant-garde role creating new neuronal cells on a daily basis. They are the dentate gyrus (DG) of the hippocampus and subventricular zone (SVZ) of the lateral ventricles (LV). The rodent brain also demonstrates such activity in the olfactory bulb (Ernst et al. 2015). There are reports about additional sites involved in adult neurogenesis; however, we do not focus on them.

Adult neurogenesis involved in a broad spectrum of functions that are important for higher cognition, learning and memory (Cameron et al. 2015). Spatial coding, short-term memory and long-term memory formation, including its subdivisions of declarative and non-declarative memory, are supported by adult neurogenesis. Interestingly, studies also show an upregulation of neurogenesis following antidepressant treatment, indirectly, this may link depression and emotional regulation to adult neurogenesis (Hanson et al., 2011). We centre our research question on the connection between adult adult-born neurons and hippocampal-mediated memory functions (Aimone et al.2011; Deng et al.2010). There is a positive correlation between increased neurogenesis rates and enhanced memory performance on a Morris Water-Maze task; rat strains with low rates of neurogenesis appear to be slow learners (Kempermann et al., 2002). Conversely, ablation of neurogenesis may deteriorate performance on hippocampal-

dependent memory tasks (Clelland et al., 2009; Park et al., 2015). A recent study applying fast x-ray technology to arrest neurogenesis confirmed that adult-born hippocampal cells are required for novel object recognition, indicating that immature neurons get selectively recruited into hippocampal circuits during the update of stored information (Suárez-Pereira et al., 2015). Possibly, these processes provide a platform for pattern separation, which is critically important for learning processes. Pattern separation is a computational model explaining the ability to discriminate one memory from other stored memories. Importantly, ablation of neurogenesis affects cognitive abilities differently depending on the timeframe when the arrest of neurogenesis occurred. Selective inactivation of 4-week old cells, excluding those of other ages, significantly impairs retrieval of hippocampal memory (Gu et al., 2012). Possibly, this may indicate different functional roles of hippocampal cells during maturation. Clearly, the phenomenon of adult neurogenesis is a critical player in many domains of brain functioning.

Following the classic Pavlovian conditioning school, a trace fear conditioning paradigm may be considered as one of the manifestations of memory (Pavlov et al., 1927; Beylin et al., 2001). Mounting evidence demonstrates the active involvement of adult neurogenesis during all stages of memory process – formation, consolidation and storage of fear memories (Seo et al., 2015; Zhao et al., 2008; Snyder et al., 2005). There is a clear connection between trace conditioning tasks and neurogenesis. Several types of learning that depend on the hippocampal formation increase the number of newly generated neurons in the dentate gyrus. For example, learning a trace eyeblink conditioning task and spatial navigation in the Morris water maze increases the number of cells in dentate gyrus by enhancing their survival and proliferation (Gould et al.,1999a; Lemaire et al.,2000). Those tasks that do not require hippocampus do not influence on the process of cell growth (Gould et al., 1999). However, the connection between

neurogenesis and fear trace conditioning is not transparent. Published studies have shown that the suppression of neurogenesis can impair, enhance or even have no effect on trace conditioning tasks (Cuppini et al., 2006; Achanta et al., 2009; Jahol- Kowalski et al., 2009; Guo et al., 2011). Possibly, such obscurity may emerge due to methodological differences in neurogenesis suppression protocols. Taking into consideration the recent hypothesis that adult-born neurons may buffer hormonal and behavioural responses to stress, emotional factors may also modulate the results of such manipulations (Hayashi et al., 2008; Castilla-Ortega et al., 2011). Depending upon the type of conditioning protocol, the arrest of neurogenesis may deteriorate or improve performance on fear trace conditioning task (Seo et al. 2015). In those tasks that implement non-associative changes in fear expression, neurogenesis-arrested mice demonstrate elevation of fear behaviour. But in those procedures with minimal non-associative plasticity, neurogenesis-arrested mice show a decrease of fear expression (Seo et al., 2015). Therefore, it is important to emphasize that our study includes a trace fear conditioning protocol involving associative mechanisms.

Typically, any continuous process consists of multiple phases with its critical time-points and developmental stages, and neurogenesis is no exception to this principle. Adult neurogenesis follows a multifactorial pathway driving the stem cell into a mature neuron that eventually integrates into existing memory circuits. A cell generated from a basket cell flourishes into a mature neuronal cell, with its full functional capacity, within 4-6 weeks (Lledo et al., 2006). The process of cell growth goes through 3 consecutive stages: proliferation, maturation and survival. The adult rat brain creates thousands of cells every day, but most of them die within a few weeks (Brown et al., 2014). There are also data suggesting that adult neurogenesis may persist across the full lifespan from birth to physiological death (Knoth et al., 2010). Despite such promising

findings, it is clear that adult neurogenesis declines exponentially with age (McDonald et al.2005). As mentioned above, the SVZ of the lateral ventricles and SGZ of the dentate gyrus as well as the olfactory bulb (OB) are restricted regions of the rat brain involved in processes of adult neurogenesis. In both places, dormant neuronal stem cells (NSC) give rise to neuronal progenitor cells (NPC) that have limitations in their proliferation. At this point, the fate of the new cell may be to become a glial cell or a neuron (Gage et al., 2000). The next phase of cell development is migration. Those cells designated to function in the olfactory bulb move from the SGZ via the migratory rostral stream to reach the olfactory bulb. The migration pathway of DG neurons is significantly shorter because it only involves having the cells move from the SGZ to the GCL. Finally, once a neuron migrates, it creates ramifications into the upper layers of the DG and connects to existing memory circuits.

Adult neurogenesis is regulated by numerous intrinsic and extrinsic factors. Among latter ones, well known are environmental enrichment, socialization and diet (Aimone et al., 2014). Voluntary running, as well as resistance training, increase the birth of new neuronal cells (Guerrieri et al., 2015), possibly by increasing vascularization and neurotrophic factors, providing more blood flow and oxygenation to hippocampal structures (Yak et al., 2014; Yarrow et al., 2010; Hötting et al., 2013). Interestingly, intermittent fasting in male rates also facilitates the proliferation of new neuronal cells (Manzanero et al., 2014; Lee et al., 2002). The above mentioned environmental factors coincide with memory enhancements tested on various behavioural paradigms (Kobilo et al., 2014).

Intrinsic factors also play an indispensable role in the regulation of adult neurogenesis. As mentioned, the local vasculature provides a platform for supplementing the process of cell growth. Internal layers of vascular tissue secrete factors that induce self-renewal of stem cells

and progenitor cell proliferation (Shen et al., 2004). Brain derived neurotrophic factor (BDNF) is another important contributing factor that has drawn much scientific attention recently. Studies indicate that high expression of BDNF is found within dentate gyrus in granule cells (Katoh-Semba, Takeuchi, Semba, & Kato, 1997). Further, local infusions of BDNF into the hippocampus increases the proliferation of progenitor cells (Scharfman et al., 2005).

We also should denote the semantic difference between the words 'regulation' and 'control' because such a difference may create a misleading picture. The word 'regulation' in the literature is used to describe fluctuations from the baseline of neurogenesis. However, 'controlling' factors may produce a higher impact on the system because they are also involved in early embryogenic stages. The sequence of transcription factors such as Pax6-Neurog2-Tbr2-Neurod1-Tbr1 are a prominent example of a controlling factor (Kemperman et al., 2011). Collectively, all factors influencing adult neurogenesis may act in a synergetic fashion increasing or suppressing the process of cell maturation.

Using carbon method dating, there are data suggesting that the adult human brain can produce up to 700 new neurons every day. This represents 1.7% of neuronal cell turnover for every year of human life (Spalding et al., 2013). This is a significant event in human physiology and any alterations in the normal progression of neurogenesis may negatively contribute to many pathological conditions. Gaining a detailed understanding of the processes regulating adult neurogenesis may potentially bring new therapies to combat neurological and psychiatric diseases.

1.4 Neurogenesis and pathology

Adult neurogenesis has been associated with a number of psychiatric and neurological

diseases. Major depression, epilepsy, schizophrenia, anxiety and Huntington disease are well-described examples (Zhao et al., 2008; Schoenfeld et al., 2015; Gil-Mohapel et al., 2011). Indeed, depression and chronic stress are associated with depletion of hippocampal neurogenesis (Jacobs, van, & Gage, 2000; Reif, Schmitt, Fritzen, & Lesch, 2007). It has been established that epileptic seizures increase the number of new neuronal cells (Parent et al., 2002). Different animal models of epilepsy confirm this phenomenon: pilocarpine induction, kainic acid and kindling all result in a higher number of proliferating cells. Our laboratory has shown that limbic kindling increases cell proliferation and promote dendritic hypertrophy (Botterill et al., 2015; Fournier et al., 2010). Indeed, there are numerous studies showing that seizures can distort adult neurogenesis through all stages of cell growth including proliferation, maturation, migration and integration (Parent et al., 2008). Thus, seizure-generated neurons display a significant difference in differentiation, migration, morphological maturation in contrast to neurons born under normal conditions. Seizures not only increase the number of new neuronal cells but accelerate the speed of their maturation (Overstreet-Wadiche et al., 2006). Additionally, to accelerated maturation, granule cells born after seizures also extend abnormal dendritic processes in the opposite direction – to the hilus (Shapiro et al., 2005; Ribak et al., 2006). These abnormal dendritic processes are known as hilar basal dendrites, and they are thought to contribute to hyperexcitability and may be considered as an epileptogenic hallmark (Pekcec et al., 2006). Further structural reorganization includes mossy fiber sprouting, granule cell dispersion and hilar ectopic granule cell distortion, leading to a pathomorphological constellation that could facilitate hyper-excitation (Ribak et al., 2012). Despite such active scientific interest within the past few years, it is not clear yet what causes the acceleration in cell growth seen under conditions of repeated seizures. It is not clear what the functional relevance of increased neurogenesis might

be in the epileptic brain.

On a related topic, there appears to be a tight connection between adult hippocampal neurogenesis and status epilepticus, in that ablation of hippocampal neurogenesis before hippocampal-induced acute seizures in mice leads to a reduction in chronic seizure frequency (Cho et al., 2014) This study also shows a normalization of cognitive deficits associated with epilepsy. Further, these ameliorating effects are long-lasting and persist for up to 1 year. Another recent study suggests that after kainic-acid induced seizures in rats, swimming exercise may ameliorate cognitive deficits, even if the exercise is delayed (Geranial et al., 2016). Importantly, increased neurogenesis in TLE animal models is observed only in the acute phase. With the chronic progression of the disease, there may be a significant decline in hippocampal neurogenesis (Hattiangady et al., 2008; Danzer et al., 2012; Danzer et al., 2016).

Our laboratory previously showed that rats received 99 stimulations in the BLA region display a significant decrease in memory performance that coincides with significant increase of neurogenesis (Fournier et al., 2013). Thus, there is a link between aberrant neurogenesis and memory impairments in an animal model of epilepsy.

1.5. Markers of neurogenesis and cell activity

For the past 20 years, significant technological advances opened the possibility of tracing the 'private' life of a neuronal cell and describing the factors influencing its birth, maturation and development. The new era of confocal imaging coincided with the discovery of histological markers such as BrdU, doublecortin and NeuN. These two events helped to further our understanding of factors influencing cell fate. During the cell growth process, different cell lineages create different cell types. They express exclusive cell surface proteins serving as a

marker to discriminate specific cell types and detect its stage of development. Here I selectively list and describe markers we applied in this study.

1.6 Neurogenesis markers

Neuronal migration cell protein, also known as doublecortin (DCX), is a microtubule-associated protein expressed by neuronal cell precursors and immature neurons. Neuronal cell precursors express DCX continuously during the first 2-3 weeks of cell maturation. The decrease of its expression starts from the 2nd week (Brown et al., 2004). Adult-born neurons expressing DCX also display functional relevance despite their immaturity. A Recent study by Suarez-Pereira suggests that DCX-expressing neurons are necessary for updating memory in object-recognition tasks (Suarez-Pereira et al., 2015).

Bromodeoxyuridine (BrdU) is a synthetic nucleotide that mimics a thymidine structure. This unique molecular structure may substitute for thymidine during DNA replication and halt cell division in the S-phase. BrdU is a powerful instrument for monitoring the process of cell proliferation at any given time point. It is important to acknowledge that BrdU is a toxic and mutagenic substance. Thus, the dosage fluctuations may negatively affect the experimental procedure. BrdU is deemed to be a gold standard marker of cell proliferation and may help to visualize the process of cell mitosis at any given time point.

Nerve cells possess unique morphological structure allowing for the reception, transmission and storage of information. These operations may happen within a timeframe of milliseconds (neurotransmitter-mediated communication) or minutes like second messenger-mediated events. Trans-synaptic activation evokes a cascade of events including induction of new programs of gene expression (Black et al., 1987). Thus, numerous extracellular and

intracellular factors may modulate neuronal gene expression: membrane electrical activity, and neurotrophic growth factors like IGF-1 (Insulin Growth Factor -1) (Hu et al., 1994). Indeed, activation of immediate early gene (IEG) expression by extracellular stimuli is not a unique property of a neuronal cell. IEG's were first discovered in non-neuronal cells as a response to growth factors (Sheng et al., 1990). Expression of IEG's is transient and does not require protein synthesis, which may explain why their expression is so variable and fast. Nowadays, IEG expression is deemed to be a relevant molecular marker for a neuronal population that undergoes plastic changes that mediate learning processes (Kubik et al., 2007; Minatohara et al., 2016). It is also believed that IEG's are involved in transitioning the neuronal activity into long-term memories (Lanahan et al., 1998; Jones et al. 2001). As an example, pharmacologically induced convulsive and sensory stimuli provoke an upregulation of Arc, zif268 and Fos expression (Lyford et al., 1995). Behavioural tasks also induce significant IEG increases that are detectable in a variety of brain regions (Ramirez-Amaya et al., 2005).

1.6.1. FOS

c-Fos is a proto-oncogene with the structure of an amino acid protein that acts inside the cell nucleus. Historically, the *c-Fos* family of genes were first discovered in rat fibroblasts as the transforming gene of the osteogenic sarcoma virus (Milde-Langosch 2005). The *c-fos* gene family was initially identified as an important factor in carcinogenesis (Mahner et al., 2008; Tulchinsky 2000). However, later is was established that Fos protein expression correlates with the level of a recent neuronal activity (Chung et al., 2005; Hoffman et al., 1991). For example, Fos protein expression in behaving rats reflects a summation of integration of activity-dependent calcium influx (Cruz et al., 2013).

C-fos acts rapidly in altering gene transcription in response to cell surface signaling (Dragoon et al., 1990) Expression of the *c-fos* gene peaks about 30-60 min after stimulation, and following a repression phase, it returns to its basal activity level (Greenberg et al., 1984). Fos is the protein product of *c-fos* and it may be induced by a variety of factors such as cytokines, tumor promoters, UV radiation and growth factors. FOS plays an important role in a variety of intracellular processes such as cell proliferation, differentiation and cell survival. Other studies suggest that Fos may also be involved in regulating the expression of BDNF and mediating mechanisms of neuronal excitability (Zhang et al., 2002).

1.6.2. ARC

Activity regulated cytoskeleton-associated protein, or ARC (also known as Argo 3.1) is a protein that belongs to the immediate early gene family. Initially Arc was identified as the immediate early gene that was regulated by synaptic activity. This protein is involved in a variety of processes including embryogenic development and the regulation of cell morphology, cytoskeletal organization (Liu et al., 2000). ARC may also play a prominent role in the acquisition and maintenance of several forms of hippocampal-dependent learning (Godowsky et al., 2001; Ploski et al., 2008). This immediate early gene is particularly implicated in the stabilization of LTP, LTD and homeostatic plasticity and may also contribute to modifying synaptic strength via postsynaptic density (Messaoudi et al., 2007). Importantly, Arc expression is critical for long term memory formation (LTM) and memory consolidation. Knockout Arc mice fail to demonstrate successful consolidation of fear memory, but acquisition and short-term memory formation were intact. (Plath et al., 2006). In a recent study, Arc knockout mice were presented as a unique animal model to study neural substrates of memory processing as the

animal demonstrates normal basal behavioural level while cognitive processing disrupted (cf. McCurry et al., 2010; Plath et al., 2006). Thus, the role of Arc is indispensable for healthy memory consolidation.

Interestingly, Arc gene expression is also related to functions that extend far beyond neuronal firing. Arc\Arg 3.1 regulates -amyloid generation, which is actively involved in Alzheimer disease (Wegenast-Braun et al., 2009; Wu et al., 2011). In our study, we applied Arc antibody staining to detect recent neuronal activity following fear trace conditioning. This is a widely used effector protein and has maximal induction times of 5 minutes (RNA) or 60 minutes (protein) after behavioural testing (Botterill 2016). Further, there are data indicating that Fos and Arc signaling are necessary for the process of recruitment of newly generated cells for spatial learning and memory formation (Kee et al., 2007b, Trouche et al., 2007).

1.7. Neuroanatomy

The next section explains the three brain regions that were involved in my experimental procedures. Each paragraph briefly describes the anatomical features, functional relevance and the connection to epileptogenesis as the prime focus of my research.

1.7.1. Hippocampus

The hippocampus is a critically important brain structure for a proper memory functioning. It lies at a crossroads of all information processing in the brain receiving inputs from most of the cortex as well as from subcortical regions. Viewed this way, the hippocampus can be thought of as building connections to many brain structures directly or indirectly via the entorhinal cortex (Hebb 1949).

The hippocampal formation is a group of regions that includes the dentate gyrus, hippocampus, subiculum, presubiculum, parasubiculum and enthorinal cortex. The hippocampus anatomically has three pyramidal cell subfields: CA3, CA2, CA1 (CA means cornu ammonis); this organization appears to be similar across all mammalian species (Amaral et al., 2007).

The dentate gyrus is a trilaminate cortical structure that has a characteristic S shape. This is a cortical region representing an internal part of the hippocampal formation (Amaral et al., 2007). The granule cell layer (GCL) is a principal layer of the DG that comprises granule cells which are densely packed. A large portion of the GCL is located between CA1 and CA3. Three types of neurons represent a neuroanatomical organization of the DG: dentate principal granule cells, dentate pyramidal basket cells and mossy cells. The branch of granule cells extends through the molecular layer and ends at the ventricular surface. Granule cells give rise to unmyelinated axons called mossy fibers. The dentate gyrus is a principal anatomical input region for the hippocampal formation (Strien et al., 2009; Danzer et al., 2008; Lopez-Rojaz et al., 2016). It is widely accepted that the dentate gyrus has a primary function in providing a neurobiological platform for short-term memory and spatial orientation. It is deemed to be a pre-processing station for incoming information that most likely transforms the data by the principle of pattern separation. Thus, the dentate gyrus is capable of segregating similar information inputs and creating distinct differential patterns. This mechanism prepares incoming data for long-term storage in the CA3 region (Jonas et al., 2014).

Much of the scientific literature describes the hippocampus as a purely cognitive structure involved in the processes of memory functioning. Another portion of the literature treats the hippocampus as a regulator of emotion. Recent studies indicate different functional relevance within different subregions of the hippocampus (Fanselow et al., 2010; Schumacher et al., 2016)

This concept was initially proposed by anatomists, who observed differences in output\input projections between the dorsal hippocampus and ventral hippocampus (Swanson et al., 1977). Further studies also confirmed functional discrepancy, for instance, the dorsal hippocampus but not ventral hippocampus contributes to spatial memory processes. Thus, there was a rationale to segregate the hippocampus further into three zones: the dorsal, intermediate and ventral zones.

As I discussed in section 1.3, the dentate gyrus is a region that also carries an important function of adult neurogenesis (Piatti et al., 2013; Parent, 2007). Adult hippocampal neurogenesis considered to be a neuronal substrate for cognition, normal memory function and the process of memory 'update' (Suárez-Pereira et al., 2015; Anacker and Hen, 2017; Oomen et al., 2007). Apart from the memory processes, it has been already hypothesized that seizures may alter normal hippocampal neurogenesis (Parent 2002), which in turn may contribute towards epileptogenesis and cognitive impairments (Benardo, 2002; Cho et al., 2015).

A large body of epilepsy research has focused on the dentate gyrus because of its pivotal role in epileptogenesis (Dudek et al., 2007). The term epileptogenesis refers to the process by which previously normal brain is functionally altered and inclined towards the development of abnormal electrical activity that results in spontaneous recurrent seizures (Goldberg et al., 2013). There are several reasons why the dentate gyrus has generated a high level of interest for epilepsy research. The dentate gyrus is considered to be a critical structure in TLE because it is often presented as an epileptic focus and epileptogenic zone (Dudek et al., 2007). As previously discussed (Section 1.2), there is often neuronal cell loss following recurrent seizure attacks. Several concepts support the hypothesis that neuronal cell loss facilitates epileptic activity (Houser et al., 1996; Babb et al., 1991). Therefore, a reduction in the number of hilar neurons in

the epileptic brain may invoke network hyperexcitability because of the decrease in excitatory input to interneurons (Sloviter et al., 1987; Sloviter et al., 2003).

Another reason for scientific interest in the dentate gyrus stems from its unique trilaminar structure. Any synchronous activity in the laminar organization of the dentate gyrus generates large field potentials in the extracellular space (Dudek et al., 2007). A seizure may start in a nearby structure and it would be harder to detect abnormal electrophysiological activity because of the small field potential, but once synchronous activity involves the dentate gyrus, large amplitude field potentials or 'fast ripples' will appear on the electroencephalogram. These high-frequency electrographic oscillations serve as a hallmark of the epileptic activity (Bragin et al., 2002).

Collectively, the dentate gyrus is a highly important brain structure that is involved in processes such as memory, cognition, neurogenesis and epileptogenesis. Given my interests in the effect of kindling on cognition, I targeted the dorsal hippocampus as a kindling site to determine the impact of seizures on behaviour and neurogenesis.

1.7.2. Basolateral Amygdala

The amygdala is an almond-shaped group of nuclei located deep and medially within the temporal lobes. This is a complex structure comprising about 10 different nuclei. The sensory input from various emotional states, predominantly fear and anxiety, enters the amygdala and transfers towards the basolateral complex (Kandel et al., 2000). The amygdala includes two major divisions that differ in their function and connectivity: the basolateral amygdala (BLA) and central amygdala (CeA). The basolateral complex (BLA) is further subdivided into the lateral (LA), basolateral (BL), and basomedial nuclei (BM). The central nucleus (CeA) is divided

into the lateral nucleus (CeL), the medial nucleus (CeM), and the intercalated cell masses (ICMs) regions (Duvarci et al., 2014). Glutamatergic neurons are the principal cellular component of the BLA and approximately 20% of the total cellular structure belongs to GABAergic interneurons (Pitkanen et al., 1995; Spampanato et al., 2011).

The amygdala has always been associated with the processing of fearful and aversive stimuli (Morris et al., 1996). In the early 1990's, a series of experiments with inactivation and surgical lesions of the BLA region suggested that the BLA plays a critical role in synaptic plasticity for the acquisition of Pavlovian fear learning and memory (Clugnet et al., 1990; LeDoux et al., 1990). Further, damage to the amygdala produces deficits in fear conditioning in humans (LaBar et al., 1995; Bechara et al., 1995). Conversely, fear conditioning is linked to increases in amygdala functional activity (Buchel et al., 1998; LaBar et al., 1998). Interestingly, there is also evidence suggesting that in addition to its role in fear and other negative reactions, the amygdala may also play a role in pleasurable reactions. For instance, lesions of the basolateral nuclei leave intact the learned association between the tone and the non-rewarding attributes of the food but disrupt the association between the tone and positive attributives of the food (Blundell et al., 2001; Kandel et al., 2000).

The amygdala has long been connected to the symptomatology of TLE. Stimulation of the amygdaloid complex may elicit the full spectrum of symptoms experienced by patients with intractable TLE (Gloor et al., 1982). The term 'dysphoric disorder of epilepsy' has also been proposed to describe a constellation of symptoms such as emotional instability, dysphoria, aggression and rage attacks accompanied by decreased volume of the amygdalar complex that suggests a causal link to the amylagdoid nucleus (Elst et al., 2009). There is also data indicating that selected populations of amylagdoid cell types demonstrate increased vulnerability to

epileptogenesis (Pitkanen et al., 1998). In fact, the amygdala of several species (including rats) is exquisitely susceptible to kindling (Goddard et al., 1969). Therefore, the basolateral amygdala is of great interest in relation to epileptogenesis and its influence on cellular and behavioural outcomes.

1.7.3. Caudate Nucleus

The caudate nucleus (CN) is a distinct anatomical structure that acts in conjunction with the putamen (oval-shaped formation at the base of the forebrain) to form the dorsal striatum. The dorsal striatum in turn belongs to a larger structure called the basal ganglia. The CN receives projections from various structures; input projections are coming predominantly from all areas of the neocortex and one-tenth of afferent connectivity comes from the substantia nigra (SN) (Hokfelt et al., 1969). The functional role of the CN is usually described in the context of a functional relevance of basal ganglia pathways to which it belongs. Broadly speaking, it is implicated in the modulation of motor functions, sensorimotor coordination including response selection and initiation. However, the CN specifically has a function on its own, independently from the structures it is merged with. The CN plays an important role in stabilizing body posture and limb movement as well as regulating the speed and accuracy of directed movements (Villablanca et al., 2010). The frontal cortex acting with the CN are the functional part of the telencephalic inhibitory system; balancing posterior ventral-diencephalic and brain stem arousal sites (Villablanca et al., 2004).

The basal ganglia have never been associated with epileptogenic activity that leads to epilepsy. To date, there are no documented clinical reports that indicate a specific epileptiform

activity in this region and there are no specific changes following electrical activity in the after-discharge period.

However, it has been shown that the CN may cause a modulatory effect on hippocampal epilepsy (Vella et al., 1990). Possibly, the basal ganglia play an inhibitory role in TLE (Rektor et al., 2012). Some lines of research also demonstrated a significant reduction of seizure activity following caudate electrical stimulation. This effect was observed in different epilepsy models, cats suffering penicillin-induced hippocampal seizures (Rektor et al., 2012) as well as in cobalt-induced neocortical seizures (Grutta et al., 1988) demonstrated a significant reduction of seizure activity. Early clinical trials confirmed these initial observations and two patients had complete elimination of seizure activity following electrical stimulation of the CN; another four patients reported a significant reduction in seizure frequency (Sramka et al., 1976). Furthermore, intracaudate NMDA injections were capable of inhibiting pilocarpine-induced epileptic activity in rats (Turski et al., 1987). This study suggested that the caudate-putamen GABA-dependent efferent pathways may regulate the excitatory threshold of the limbic system. Thus, the CN can modify spiking activity of hippocampal structures and contribute to protective mechanisms against epileptiform activity. However, future research necessary to understand exact underlying mechanisms influencing electrical activity on the limbic structure.

1.8. Pavlovian Fear Trace Conditioning

Classical Pavlovian fear conditioning involves learning an association between specific environmental stimuli and an aversive event. An animal learns to anticipate the emotionally significant event and mounts a physiological response to that event. The association of a conditioned stimulus with an unconditioned stimulus involves subcortical structures (Curzon et

al., 2009). However, if a context is the predictor of the aversive stimulus, then the hippocampus is involved in learning the association (Rudy et al.,2004). There are two types of fear conditioning protocols: delay and trace conditioning. In delay conditioning, the aversive conditioned stimulus (tone) co-terminates with the unconditioned stimulus (foot shock). In this scenario, the amygdala is primarily involved. Fear trace conditioning differs procedurally from 'delay' with the presence of an empty interval called a 'trace', which segregates the termination of a tone as a CS and the beginning of an aversive stimulus such as a footshock. There is additional complexity to trace conditioning due to the involvement of the hippocampus.
Fear conditioning represents a behavioural paradigm that allows neuroscientists to study the neuronal circuitry underlying emotion, learning and memory. For decades, these paradigms have been extensively studied due to their simple experimental design and robust effects.

Several lines of research have demonstrated that the hippocampus and amygdala are critical for the acquisition of auditory fear trace conditioning. Lesions of the basal amygdalar nuclei produced deficits in auditory fear conditioning and contextual conditioning (Goosens et al., 2001). Further, rats with BLA lesions demonstrate a significant decrease in freezing behaviour in the presence of cues previously paired with footshock (Vazdarzhanova et al., 1998). The hippocampus, especially the dorsal hippocampus and CA3 region are critically important for the acquisition of trace fear conditioning (Curzon et al., 2009). Indeed, humans with damage to the hippocampus are able to acquire delay conditioning but not trace conditioning (Clark et al., 1998). Collectively, these studies provide support for the idea that auditory fear trace conditioning is both hippocampal-dependent and amygdala-dependent.

1.9. Experimental goal and the hypothesis

The exact mechanisms that lead to cognitive deficits following persistent seizure activity remain unknown. One possible explanation is that aberrant adult hippocampal neurogenesis contributes to the memory impairments associated with epilepsy (Cho et al., 2015). Indeed, hippocampal neurogenesis plays important role in learning and memory (Eichenbaum, 1997), as discussed in the previous sections of Chapter 1. Newly generated granule cells demonstrate high structural plasticity in comparison to mature cells (Schmidt-Hiebe et al., 2004). It has been also demonstrated that seizures induce high rates of neurogenesis (Parent et al., 2002). Seizures distort not only the quantity, but also the quality of newly generated granule cells, because neurons born under seizure conditions display pathomorphological characteristics including exaggerated dendritic outgrowth and the maintenance of hilar-basal dendrites (Zhao et al., 2008). However, it is yet unclear whether seizure-induced changes in neurogenesis contribute to seizure-induced cognitive deficits. It is important to clarify this research question, because the research outcomes may potentially suggest a novel treatment approach for patients suffering from cognitive decline associated with TLE.

1.9.1. The specific aims and goals

The specific aim of my thesis is to examine site-specific kindling outcomes on memory function, neurogenesis and rates of neuronal cell activation.

Research question: Do kindling induced changes in neurogenesis play a role in the cognitive deficits? To address this question, I will subject rats to long term kindling of three specific brain areas—the dorsal hippocampus, basolateral amygdala, and caudate nucleus. To examine

kindling-induced alterations in neurogenesis, I will inject BrdU at the time-point when electric stimulations impact hippocampal cell proliferation (30^{th} electric stimulation). Kindling will continue after BrdU injections to the 99^{th} stimulation, so that newborn cells can mature under conditions of repeated seizures. Then, the rats will be trained in a trace fear conditioning paradigm to examine the impact of site-specific kindling on fear memory retrieval. Shortly after behaviour testing, the rats will be sacrificed for immunohistochemical assessment of neurogenesis and neuronal cell activation via immediate early gene expression such as ARC and FOS.

Hypothesis: I hypothesize that kindling in different brain sites has differential effects on cognition, neurogenesis and levels of cellular activation. In particular, limbic but not non-limbic kindling would exaggerate neurogenesis and impair cognitive function of rats. If kindling induced changes in neurogenesis contribute towards cognitive deficits, then I expect that limbic, but not non-limbic kindling would alter neurogenesis and the expression of IEGs. Specifically, I expect that limbic kindled rats would have fewer newborn neurons expressing IEG's than non limbic or control rats, which would indicate impaired integration of newborn neurons after limbic kindling.

CHAPTER 2

Seizures originating in different brain regions have differential effects on fear memory and the functional integration of seizure-generated granule neurons

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2.1. Abstract

Epilepsy is known to instigate cognitive deficits. Acute seizures lead to an increase in the number of adult born neurons in the hippocampus (Botterill et al., 2015; Kokala et al., 2011). Recent findings demonstrate that there is a possible link between memory impairments and aberrant neurogenesis (Cho et al., 2015; Jessberger et al, 2015). Our previous studies established that the location where seizures originate may play critical role in the development of cognitive deficits (Botterill et al, 2017; Fournier et al., 2013). However, it is not known how specific seizure location impacts the rate of adult hippocampal neurogenesis. In present study, we subjected 25 rats to long-term kindling of various brain sites, then examined hippocampal neurogenesis and rates of neuronal activation following behavioural tasks. We labeled seizure-generated hippocampal neurons with BrdU after 30 stimulations and continued kindling until rats received a total of 99 stimulations. After the kindling procedure was complete, we tested rats on a trace fear conditioning task to examine the impact of seizures that originate from different brain sites on a fear memory retrieval. After fear conditioning, we sacrificed the rats and processed hippocampal tissue for immunohistochemitry to analyze neuronal activity in response to memory retrieval through immediate early gene expression. We found that long term kindling of limbic, but not non-limbic brain sites led to significant impairments in fear memory retrieval. Low rates of IEG activation in response to behaviour tasks were accompanied by exaggerated neurogenesis. These results suggest that seizures prevent the normal process of integration of newborn neuron into memory circuits. We also found that kindling of the dorsal hippocampus produced an extreme number of neuronal cells that corresponded to the lowest activation of neuronal cells and significant deficits in memory retrieval. Collectively, these data indicate that

seizure origin plays critical role in adult neurogenesis rates and provides more support for the idea that neurogenesis contributes to the cognitive deterioration that accompanies TLE.

2.2. Introduction

Epilepsy is a group of neurological disorders encompassed by recurrent unprovoked seizures (Bernard et al., 2003). Temporal lobe epilepsy (TLE) is the most common form of epilepsy that is also complicated by pronounced cognitive deficits (Engel et al., 2001); it is notorious for its resistance to pharmaceutical intervention, thus to date there is no effective therapeutic strategy to target both seizure frequency and comorbid manifestations. Possibly, research endeavors should be directed towards the pathophysiology of epileptogenesis to answer clinical questions in regards to treatment of TLE.

For the past decade, much experimental attention has been directed towards understanding the phenomenon of adult hippocampal neurogenesis. It was established that new neurons are continuously added to hippocampal structures throughout the full life span of the mammalian brain. Adult neurogenesis is a multifactorial phenomenon regulated by a variety of internal and external factors such as diet, environment, stress and inflammation. There is also mounting evidence that acute seizures provoke an increase in the rate of cell proliferation, but the functional consequences of this increase is unclear. Further, there are numerous studies suggesting that neuronal cells produced after acute seizure attack may contribute to both epileptogenesis and cognitive impairments (Cho et al., 2015; Zhong et al., 2016).

Kindling refers to the gradual development and intensification of motor seizures resulting from daily delivery of electrical stimulations to specific brain regions. Kindling as a model has been successfully used to study epileptic seizures and its impact on neuronal plasticity. Our

previous studies have demonstrated that long-term kindling of the basolateral amygdala produces significant deterioration in fear memory retrieval that coincides with increased neurogenesis. Our findings supported the hypothesis that hippocampal neurogenesis is heavily involved in hippocampal-dependent memory tasks, thus perturbations in neuronal cell growth reflect on cognitive deficits. We also observed that seizures elicited through amygdala stimulation impair the normal activation of neurons during memory retrieval (Fournier et al., 2013).

In present experiment, we applied long-term kindling (99-stimulations) to three discrete brain regions to examine the impact of repeated seizures on hippocampal neurogenesis and neuronal activation in response to the retrieval of a fear memory. There were four treatment groups including the control group, caudate nucleus (CN) kindled, basolateral amygdala kindled (BLA), and dorsal hippocampus kindled (dHip). To examine the impact of repeated seizures on neuronal birth, we delivered three injections of BrdU following the 30^{th}, 33^{rd} and 36^{th} kindled or sham stimulation. We continued the stimulations for another 4 weeks until rats received 99 stimulations and new neuronal cells marked with BrdU would reach maturity.

The next day, after the delivery of the last stimulation, rats were subjected to an auditory trace fear conditioning task. We quantified the expression of markers of recent neuronal activity such as Arc and Fos to examine levels of neuronal activity in response to behavioural testing following long-term kindling. To examine levels of activation of seizure-generated neurons that are 4 weeks old we quantified Arc positive cells on immunofluorescent labeling with BrdU marker.

A key aspect of this experiment is that all kindled rats experienced the same number of seizures, but those seizures were initiated in different brain regions. We expected that the BLA and dHip kindled rats would show cognitive deficits but the CN kindled rats would not. If

aberrant neurogenesis and deficient integration of newborn neurons is involved in these cognitive deficits, we should see these effects in the BLA and dHip kindled rat that show memory deficits, and not the CN kindled rats that do not show memory deficits. Our results largely supported this hypothesis, and confirm that seizure-generated neurons are unable to integrate into existing memory circuits.

2.3. Materials and methods

2.3.1. Subjects

We used 25 Male Long-Evans rats that were purchased from Charles River (Canada). Each rat weighted 200-250g upon arrival from the supplier. They were housed individually in a rectangular polypropylene cage with standard laboratory bedding and free access to food and water. The housing room was maintained at a temperature of 20^0C (±1 °C) on a 12:12 light:dark cycle with lights on at 8:00 am. All experimental procedures were performed during the light period of the light:dark cycle. All rats were treated in accordance with the Canadian Council for Animal Care and the University of Saskatchewan Committee on Animal Care and Supply. All efforts were made to minimize the number of animals used.

2.3.2. Surgery

All rats were handled daily to acclimatize them to the animal colony room. After 1 week of acclimation, the rats were individually anesthetized with isoflurane (5%) and received an injection of a preoperative analgesic (Anafen, Ketoprofen, 10mg/kg, s.c.) to reduce pain and inflammation. After secure fixation in a stereotaxic apparatus, a mixture of isoflurane and oxygen was delivered through mouth tube to maintain anaesthesia. Isoflurane gas was

administered in a concentration of a 5% as initial step and 2.5% as a maintenance concentration. Targets of the surgery procedure were 3 discrete brain regions: the dorsal hippocampus (dHip), basolateral amygdala (BLA) or caudate nucleus (CN). A single bipolar electrode (MS-303-2-B-SPC, Plastics One, Roanoke, VA, USA) was implanted into left hemisphere of each rat in one of the relevant brain regions. The following stereotaxic coordinates were used: CN (+ 0.2 mm anteroposterior, + 3.2 mm mediolateral, - 5.7 mm dorsoventral); BLA (- 2.8 mm anteroposterior, + 5.0 mm mediolateral, - 8.5 mm dorsoventral); dHip (- 3.5 mm anteroposterior, + 2.6 mm mediolateral, - 3.1 mm dorsoventral) (Paxinos & Watson, 1998). To secure the electrode to the scull, 4 jeweler screws and dental acrylic were added as a final step of the surgery procedure.

All electrode placements were confirmed at the end of the experiment during the immunohistochemistry procedure. Sections containing electrode tracks were mounted on slides and used to confirm electrode placements. In addition, a Nissl stain was performed on selected brain sections to visualize the precise electrode location (Figure 1).

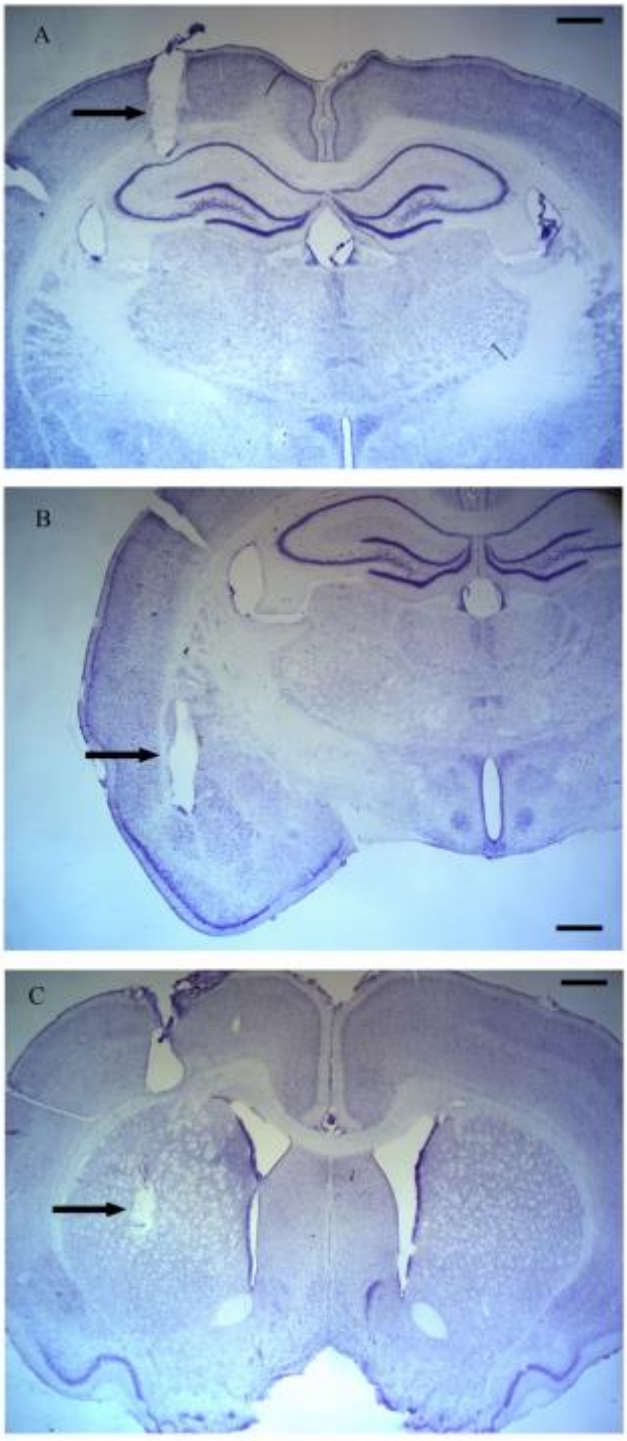

Figure 1. Representative photomicrograph of Nissl stained brain tissue. Electrode trace in dorsal hippocampus region - A; B – basolateral amygdala; C – caudate nucleus electrode mark. 1X magnification, Scale bar 100um.

2.3.3. Kindling

Following the surgical procedure, a 2-week recovery period took place in order to facilitate recovery. The rats were randomly distributed into 4 treatment groups: dHip (n=6), BLA (n=7), CN (n=6) and Control (n=6). The control group included rats who had electrode implants from each region of interest (dHip, BLA, CN).

Histological analysis revealed that 25 rats had correct electrode placements in the dHip, BLA and CN regions respectively. Representative electrode placements for each group are shown in Figure 1. Kindling progressed normally in all rats. Electrical kindling involved the daily application of a 1-second train of biphasic square wave pulses delivered at a frequency of 60Hz, an amplitude of 800 μA (peak-to-peak), and a duration of 1 ms. Rats received 3 stimulations per day, 5 days per week with a minimum 3 hours break between each consequent stimulation. The total number of stimulations for each treatment group was 99-stim that was delivered approximately within 6.5 weeks. Rats assigned to the control group received 99 sham stimulations. This kindling protocol is identical to one used repeatedly in our laboratory (Fournier et al., 2013).

The behavioral convulsions induced by each electrical stimulation were scored using the revised 8 class extension (Pinel & Rovner, 1978) of Racine's original five class convulsion scale (Racine, 1972). Each numerical class of convulsions corresponds with severity of body motor involvement: Class 1: orofacial automatisms, Class 2: orofacial automatisms with head nodding, Class 3: unilateral forelimb clonus, Class 4: rearing with bilateral forelimb clonus, Class 5: rearing with bilateral forelimb clonus followed by falling, Class 6: multiple class 5 convulsions and falling episodes, Class 7: previous classes with running fit, Class 8: previous classes with intermittent muscle tonus. Based on this scoring scheme, a rat was considered to be "kindled"

when it displayed 3 consecutive class 5 convulsions. (Pinel & Rovner, 1978). There were no spontaneous seizures observed at any point during the experiment.

2.3.4. BrdU injections

One purpose of this experiment was to investigate development and maturation of seizure-generated neurons following the kindling procedure to determine whether pathologically born cells can integrate into memory circuits. Previous studies from our laboratory demonstrated that amygdala kindling increases the rate of cell maturation after 30 stimulations (Fournier et al., 2010). Therefore, in this experiment 3 BrdU injections at the dose of 75 mg/kg, 169 mg/m were delivered intraperitoneally 4 hours after the delivery of 30, 33 and 36 stimulations. All rats including the control group received 3 BrdU injections approximately 2.5 weeks after the first day of kindling procedure. Each injection was separated 24 hours apart. BrdU (BA-5002; Sigma-Aldrich) was dissolved in warm physiological saline solution (50 °C) and sterile filtered. After the last injection occurred, the kindling procedure resumed.

2.3.5. Fear conditioning

After the delivery of last stimulation, a number of behavioural tests took place in order to assess the cognitive consequences of kindling. Behavioural procedures comprised 4 consecutive days of training and testing:

Day 1. Habituation. Rats went through a habituation process to acclimatize them to the operant chambers. Two rats at the same time were brought to the brightly lit experimental room and then placed individually into operant chambers. They were allowed to explore the chamber for 10

min. Acetic acid solution 0.6% (v/v) was applied to eliminate olfactory cues for every rat going through operant chamber.

Day 2. Training. Rats were trained to learn the conditioned association. They were placed into the same room as they were on Day 1. After 180s of acclimatization, each rat received 7 sound tones (conditioned stimuli – CS) paired with a footshock as the unconditioned stimulus (US). Trace conditioning comprised a 16-s tone (85 dB, 2 kHz, 5 ms/rise fall time) followed by trace time of 30-s. A foot shock (0.9 mA) with a duration of 2-s was delivered at the end of the 30 sec trace period. Between every CS-US onset there was 180-s pause. The chambers were cleaned with acetic acid solution after every rat.

Day 3. Tone test. The tone test assessed the rat's ability to recall the association between the tone and the footshock. Rats were exposed to a novel operant chamber and received the same auditory tone from day 2. The tones were delivered 4 times (16 s duration, 85 dB, 2 kHz, 5 ms/rise fall time) with an interval of 198-s. Here we scored freezing time in response to the tone.

Day 4. Context test. The context test assessed the rat's ability to recall the association between the environment and the footshock. Rats were tested in the original environment that they were initially trained and habituated in. Rats were placed into the operant chamber for 480-s, and freezing was assessed during this time.

Freezing was defined as the absence of body movements except for those necessary for breathing. To evaluate defensive behaviour, we expressed freezing time as the percentage of time

spent freezing. The freezing episode of each rats was summed and divided by the number of observations then multiplied by 100.

2.3.6. Perfusions

Two hours after the context test, the rats were deeply anesthetized with sodium pentobarbital (240 mg/kg, i.p.) and perfused transcranially with saline solution and then with cold (-20 °C) formaldehyde fixative (pH = 7.4). The brains were extracted and stored in the same solution until sectioning. Using a vibrating microtome (Vibratome 3000, Vibratome Company, St. Louis, MO, USA), all brains were sectioned at a thickness of 30 μm. Brain sections were securely stored in polycarbonate membrane dishes with a cryoprotectant solution, consisting of 30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, and 30% (v/v) ethylene glycol in 0.1 M PBS (pH = 7.4).

2.3.7. Post-mortem analysis: immunohisochemistry procedures

We used standard immunohistochemistry protocols to assess the effect of kindling on the expression of several markers of neurogenesis and cell activation. All immunostaining procedures were performed with a free-floating technique to reach maximal antibody penetration.

2.3.7.1. BrdU staining

BrdU immunohistochemistry was used to visualize the number of proliferating cells. Sections were treated with 0.3%H_2O_2 for 30 minutes. After TBS rinsing, the sections were gently transferred to 2 N HCl 267 at 45 °C for 1 hour to denature the DNA and expose the BrdU

antigen. 0.1M Borate Buffer was applied for 30 minutes to stop the chemical reaction and the tissue was then placed into blocking solution comprised of 5% NHS, 0.3% Triton-X-100 and TBS. After blocking, the sections were incubated with a primary mouse anti-BrdU monoclonal antibody (1:500, 48 h, 4 °C, Roche 272 Diagnostics) followed by incubation of the secondary antibody (biotinylated horse anti-mouse, 1:500, 2 h, room temperature, Vector Laboratories). Next, sections were placed in an avidin–biotin peroxidase complex for 1 hour (1:200, room temperature, Vectastain ABC Elite, Vector Laboratories). The last step of the immunolabeling procedure was visualization with 0.033% (w/v) 3,3′-diaminobenzidine (DAB) and 0.00786% 278 (v/v) hydrogen peroxide diluted in PBS until brown coloration of the tissue occurred. After the procedure, all sections were mounted onto slides and coverslipped the day after.

2.3.7.2. Fos staining

Fos protein immunohistochemistry was used to visualize neuronal activity after fear memory retrieval. Sections were exposed to hydrogen peroxide for 30 min in a concentration 0.3% at room temperature to stop endogenous peroxidase activity, followed by block-cocktail of 5 % (v/v) normal goat serum, 1 % (w/v) bovine serum albumin, and 0.3 % (v/v) Triton-X-100 in 0.1 M PBS for 1 hour. For the next 72 hours, all sections were incubated in primary Fos anti-rabbit polyclonal antibody at a concentration of 1:15,000 (Calbiochem, La Jolla, CA, USA) diluted in blocking solution. Next, sections were placed into biotinylated goat anti-rabbit IgG secondary antibody for 2 hours (1:500, Vector Laboratories, Burlingame, CA, USA) diluted in 0.3% (v/v) Triton-x-100 with 0.1M PBS, then treated with avidin-biotin-peroxidase (1:500, Vectastain ABC Elite, Vector Laboratories). Nickel-intensified DAB that comprised of 0.02% (w/v) 3,3’-diaminobenzedine, 2.5% nickel ammonium sulfate and 0.000083% (v/v) H_2O_2 in

0.175 M sodium acetate visualized the immunolabeling procedure. After all rinses were done, sections were mounted onto glass slides and coverslipped.

2.3.7.3. Arc staining

Arc protein immunohistochemistry was also used to visualize neuronal activity. Arc immunostaining started with exposing the sections to 0.3 % (v/v) H_2O_2 for 30 minutes then transferring them into a block solution as described above. Primary ARC antibody (1:1000 rabbit anti-arc Synaptic Systems, Goettingen, Germany) diluted in block solution was applied to the sections for 48 hours. Next step was incubation in secondary antibody (1:500, 2 hours, Vector Laboratories) mixed with block solution. Immunoreaction was visualized using the DAB (0.05%) method (Botterill et al., 2015; Shu et al., 1988). Sections were then mounted onto glass slides and coverslipped.

2.3.7.4. Immunofluorescence double labelling

Immunofluorescence double labeling was processed to visualize the presence of BrdU with Arc. This would identify newborn cells that were active during the recall of a fear memory. Following rinsing procedures, sections were treated with 2 N HCL for 30 min under 45 ℃ in a hot bath. Next, borate buffer was applied to stop the reaction and the section were washed in TBS 3 times. Prior to incubation with primary antibody, the sections were immersed in blocking solution (5% NGS, 0.3% Triton-X-100, 1% BSA in 0.1M TBS for 30 min). Following blocking procedure, the sections were incubated simultaneously for 48 hr at 4℃ with Arc (1:500 anti-rabbit arc, Synaptic Systems, Goettingen, Germany) and BrdU (1:500 anti-mouse BrdU, Roche Diagnostics GmbH, Mannheim Germany) diluted in blocking solution. Further, the sections were

incubated in secondary antibodies: Alexa 488 Goat Anti-Mouse (1:500; Invitrogen, CA, USA) and Alexa 568 Goat Anti-Rabbit (1:500; Invitrogen, CA, USA) diluted in 5% NGS, 1% BSA, 0.1% Triton X-100 and TBS. This secondary cocktail was incubated for 3 hours at room temperature under minimal light exposure. After final rinsing, the sections were mounted onto charged glass slides and coverslipped with Citifluor fluorescent mounting medium. The final product of immunofluorescent staining was stored in minimal/no light exposure conditions a 4°C until further quantification analysis.

2.3.7.5. Doublecortin immunohistochemistry

Doublecortin (DCX) immunofluorescent labelling was used to confirm that BrdU labeling reflected proliferating neurons. Immunofluorescence with DCX was processed with the following protocol. Sections were rinsed with TBS solution (3 times for 10 min) and transferred to 5% H_2O_2 diluted in 0.1 TBS for 30 min. Then, the sections were rinsed with TBS and incubated in sodium citrate at 85°C for 30min. Following TBS rinse, the sections were transferred to a blocking solution for 30 min (5% normal donkey serum, 1% Triton-X, 1% BSA dissolved in TBS). Next, the sections were incubated in primary DCX antibody for 48 hours at 4°C (1:250 Goat Anti-doublecortin c-18 sc-8066, Santa Cruz Biotechnology, CA, USA).

Following primary antibody exposure, the brain sections were rinsed with a TBS solution 3 times to prepare for secondary antibody exposure. Finally, sections were incubated in secondary antibody Alexa 488 Donkey anti-goat (1:250; Invitrogen, CA, USA) diluted in 5% NGS, 1% BSA, 0.1% Triton X-100 and TBS. The incubation procedure with secondary antibodies was 3 hr at a room temperature; samples were covered with tin-foil in a room with

ambient light. After a final rinsing procedure, the sections were mounted onto glass slides and coverslipped with citifluor fluorescent mounting medium.

2.3.7.6. Quantification

All quantification procedures were done by researchers blind to the treatment groups. Electrode placements were confirmed on immunostained tissue. Sections were examined using a Nikon Eclipse E800 microscope equipped with stage controller and digital camera (MicroFire, Optronics, Goleta, CA, USA). The quantification procedure involved three different methods: stereology counting, profile counting and manual counting.

Stereology counting. We counted BrdU positive cells that were visualized with DAB using unbiased stereological counting. BrdU positive cells were counted at a magnification of 40X in two distinct regions of the dentate gyrus: the subgranular zone (SGZ) and granule cell layer (GCL). The SGZ was identified as the region within a two-cell width in between the inner granule cell layer and the hilus. All subregions of interest were traced using the stereology software program StereoInvestigator (MicroBrightfield, Williston, VT, USA). Both ipsilateral and contralateral sides were included into the quantification. I counted cells in 5 brain sections per animal. All sections were 30 μm thickness and sampled from every 12^{th} brain section sectioned on the Vibratome. For the counting process, we applied a modified optical fractioning method to reduce oversampling (Kuhn et al., 1997). The total number of BrdU positive cells was estimated with the following formula:

$$\mathrm{Ntotal} = \sum Q- \times 1 / \mathrm{ssf} \times \mathrm{A(x,\ y\ step)} / \mathrm{a(frame)} \times \mathrm{t} / \mathrm{h}.$$

$\sum Q^-$ is the number of counted cells; ssf is the section sampling fraction (1 in 12); A(x, y step) is the area associated with each x,y movement (10,000μm2); a(frame) is the area of the counting frame (3,600 μm2); t is the weighted average section thickness; h is the height of the dissector (12 μm) (Botterill et al., 2015; Fournier et al., 2010; Lussier et al., 2013).

Profile counting was applied to quantify the number of Arc and Fos immunoreactive cells visualized with DAB. Profile counts were conducted bilaterally across 5 sections per brain. We counted Arc and Fos immunoreactive cells in three principal hippocampal subfields including the granule cell layer, subgranular zone, and hilar zone. First, at a magnification of 4X, we traced the boundaries of each hippocampal subfield using the rat brain atlas as a guide (Paxinos & Watson, 1998). Then, at a magnification of 40X, we counted cells using a meander profile counting method (Botterill et al., 2015; Knapska and Maren, 2009). The meander scan function is an integrated module of the StereoInvestigator software. The meander scan is a semi-automated scanning method that allow the user to count detected cells within a previously traced contour. And finally, to assess the density of immunoreactive cells, we divided the total number of immunoreactive cells by the total area measurement of the region of interest. The results for Arc and Fos cell counts are expressed as the average number of cells per mm^2.

Manual counting. All sections were captured using an epifluorescence microscope with a built-in stage controller and digital camera (MicroFire, Optronics, Goleta, CA, USA). Immunofluorescent pictures were captured under ambient light at a magnification of 4X under two distinct light spectrums to visualize BrdU (green light spectrum) and Arc (red light spectrum) positive cells separately. Both ipsilateral and contralateral sides of the brain were included in the quantification. BrdU and Arc positive cells were counted manually within the

SGZ and GCL subregions. Only cells that fluoresced brightly within the appropriate channel were included into the analysis. Manual counting was processed in three stages.

First, all BrdU positive cells within SGZ and GCL were counted and averaged for every animal. Second, all Arc positive cells were counted within the SGZ and GCL regions and then averaged. The total number of cells for every section was averaged for each animal. Importantly, the results of the manual counting for both BrdU and Arc corresponded with the number of cells counted using the profile and stereology counting methods. The third step examined the colocalization of BrdU and Arc. To evaluate the number of BrdU positive cells that also express Arc, I merged images from both fluorescent light spectrums using Photoshop. The brightness and contrast for each merged image was adjusted equally across treatment groups. Colocalized cells appeared as yellow-colored as a result of a convergence of red and green channels. All colocalized cells were counted within the SGZ and GCL regions in both hemispheres of the dentate gyrus. The total number of colocalized cells (yellow) were divided by the total number of BrdU positive cells (green) and then the acquired numbers were multiplied by 100 in order to express the data as a percentage.

2.3.7.7. Statistical analyses

All the data were analyzed using the Statistical Package for Social Sciences v21. Statistical significance was determined by one-way analysis of variance (ANOVA).

For each ANOVA, I considered a number of assumptions including the assumptions of homogeneity of variances and equality of means. All data were tested to confirm/reject the assumption of homogeneity of variances with the Levene test ($p<0.05$). The Welch's test was also applied to test the assumption of equality of means ($p<0.05$). If both assumptions were

confirmed, then the Tukey post hoc test was used to detect statistical differences among the groups. If the Levene or Welch's test indicated a violation of the assumptions ($p<0.05$), then I applied a non-parametric Games-Howell post hoc test (Toothaker, 1993) to detect statistical differences among the groups. The Games-Howell test is an appropriate alternative to the Tukey post hoc, because it does not assume equal variance and sample sizes (Ruxton & Beauchamp, 2008).

The statistical significance for all comparisons was set at $P < 0.05$. All graphs were built via Prism 6.0e where graph bars demonstrate the mean ± standard error.

2.4. Results

2.4.1. Kindling impacts fear trace conditioning

Figure 2 shows the results of the behavioural testing. To evaluate baseline activity during day 1 habituation, we measured the number of midline crosses (see Figure 2A). There was no significant difference across groups in midline crosses ($F(3,21)=1.466$, $p=0.252$), thus kindling in both limbic and non-limbic groups did not impair motor activity.

On the training day (day 2) there were no significant differences between treatment groups, either in freezing time before the conditioned stimuli ($F(3,21)=0.449$, $p=0.617$) or during the trace period ($F(3,21)=0.609$, $p=0.617$) (Figure 2B). There were also no group differences in post-shock freezing time as a reaction to 7 CS-US pairings ($F(3,21)=0.843$, $p=0.486$). Prolonged freezing time across all treatment groups indicates successful learning of the conditioning association. The results of the training day procedures indicate that kindling did not affect the acquisition of fear learning.

Day 3 aimed to assess fear memory via the tone test. Our statistical analyses revealed group differences in the percent of time spent freezing (CS: $F(3,21)=18.401$, $p=0.000$; PS: $F(3,21)=18.401$, $p=0.000$). Post hoc analysis of this main effects further revealed that the BLA and dHip kindled rats froze less than the CN kindled rats or the control rats, as shown in Figure 2C.

Day 4 or the context test day aimed to assess memory for the association between the environment and the footshock. The BLA and dHip-kindled rats had a smaller percentage of freezing than the control or CN-kindled rats ($F(3,21)=11.547$, $p=0.000$) (Figure 2D). In addition, we counted a number of fecal boli upon the completion the context test. Statistical analysis of these data revealed that the limbic kindled rats left significantly fewer fecal boli in the operant chamber room compared to rats in the other two groups ($F(3,21)=20.887$, $p=0.000$) (Figure 2E).

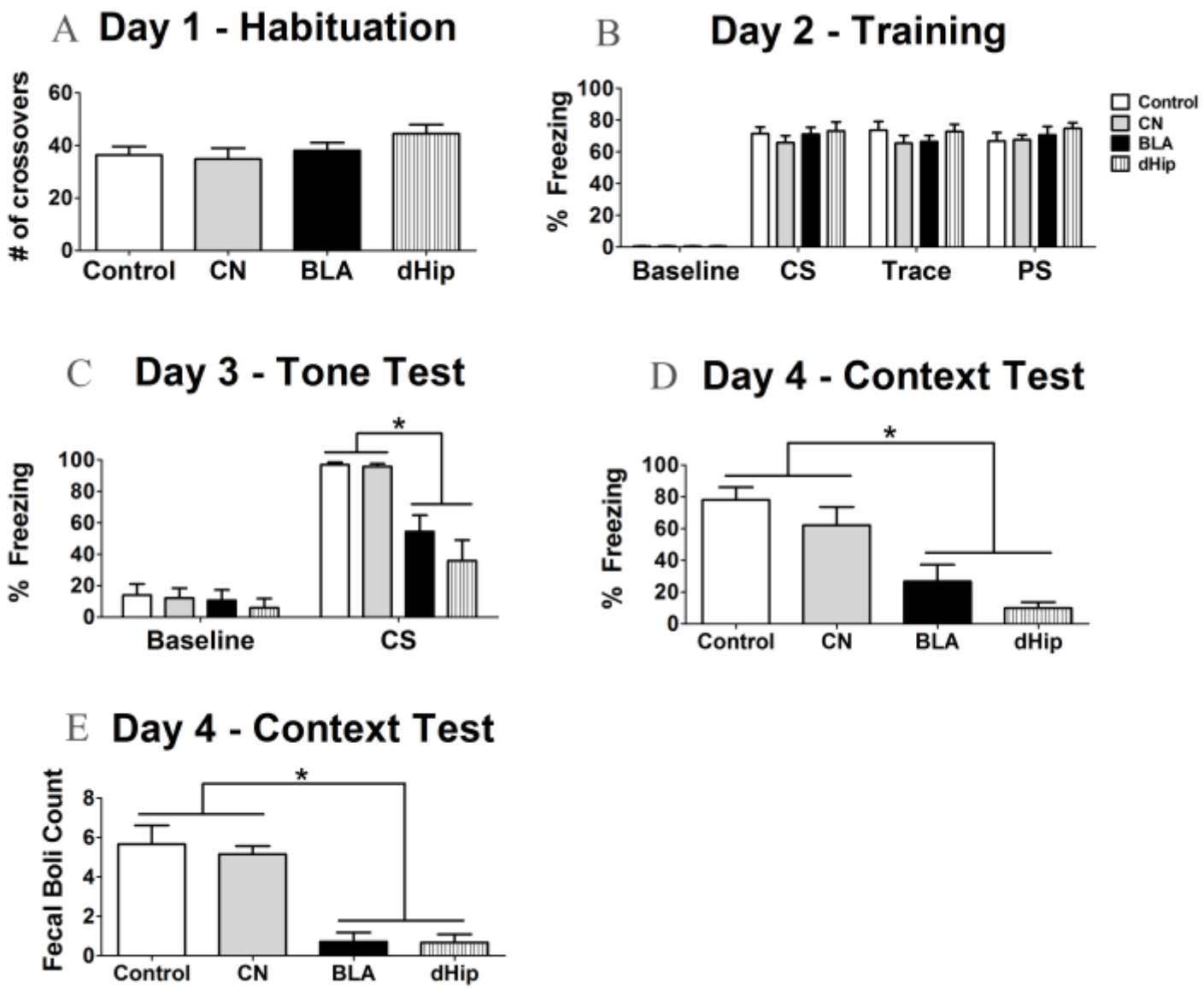

Figure 2. Kindling affects fear trace conditioning. A) Kindling has no effect on habituation. B) Kindling had no effect on acquisition of the hippocampal dependent fear memories. C) Limbic kindled rats freeze significantly less than control and caudate kindled rats. D) First context test evaluated through the time spent freezing and showed a significant decrease of time spent immobile in limbic kindled rats E) Limbic kindled rats confirmed behavioural differences with control and caudate kindled rats, dHip kindled and BLA kindeld found to have lower number of fecal boli in the operant chamber. (CN – caudate nucleus; BLA – basolateral amygdala; dHip – dorsal hippocampus; $*p < 0.05$).

2.4.2. Stereology counting for BrdU as a marker of neurogenesis

Figure 3 shows the results of the BrdU immunohistochemistry. A one-way ANOVA followed by post hoc Tukey test revealed a significant increase in the number of BrdU positive cells in the dHip kindled rats in comparison to the rest of the treatment groups on both the ipsilateral and contralateral sides (ipsilateral $F(3,21)=17.141$, $p=0.000$; contralateral $F(3,21)=11.868$, $p=0.000$; combined $F(3,21)=14.717$, $p=0.000$).

However, caution must be exercised due to the absence of homogeneity of variances assumption; that was confirmed by the Levene Statistic (ipsilateral $F(3,21)=5.179$, $p=0.008$; contralateral $F(3,21)=7.472$, $p=0.001$) and complemented by the Welch equality of means test (combined $F(3,10.686)=10.432$, $p=0.002$). Thus, there is a heterogeneity of variances among the groups. We should also take into consideration the fact that each treatment group comprises small sample sizes and importantly they are unequal ($n=6$; $n=7$; $n=6$; $n=6$;). Collectively, these factors decrease our statistical power making the classical Tukey post hoc test too conservative and incapable to detect potential significant differences between all treatment groups and creating the risk of a Type II error. Therefore, it is appropriate to re-analyze the results using the Games-Howel post-hoc test, which is a rather similar approach to Tukey but does not assume equal variances and equality in sample sizes. Using the Games-Howel test, we found that both the dHip kindled and BLA kindled rats had significantly more BrdU positive cells than the CN kindled and control rats (dHip-control p=0.029; dHip-Caudate p=0.035; dHip-BLA p=0.081) (See Fig. 4). Hence, the present results are congruent with previous reports from our laboratory (Botterill et al., 2015; Fournier et al., 2013).

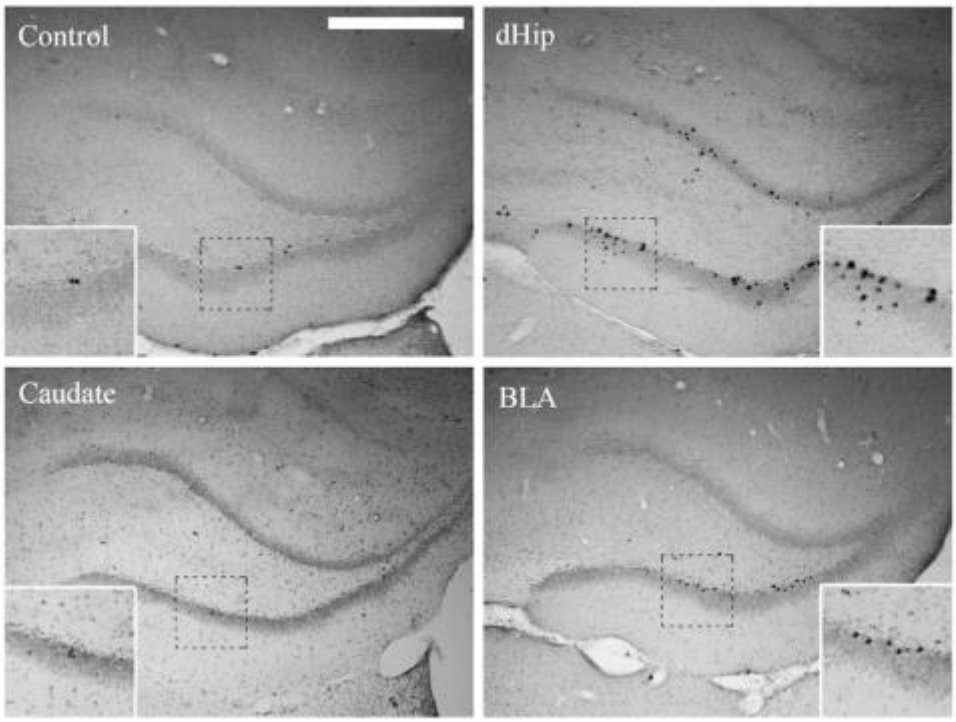

Figure 3. Comparative photomicrograph demonstrates a significant increase of BrdU positive cells in the dHip kindled and BLA kindled rats. Scale bar: 200 μm.

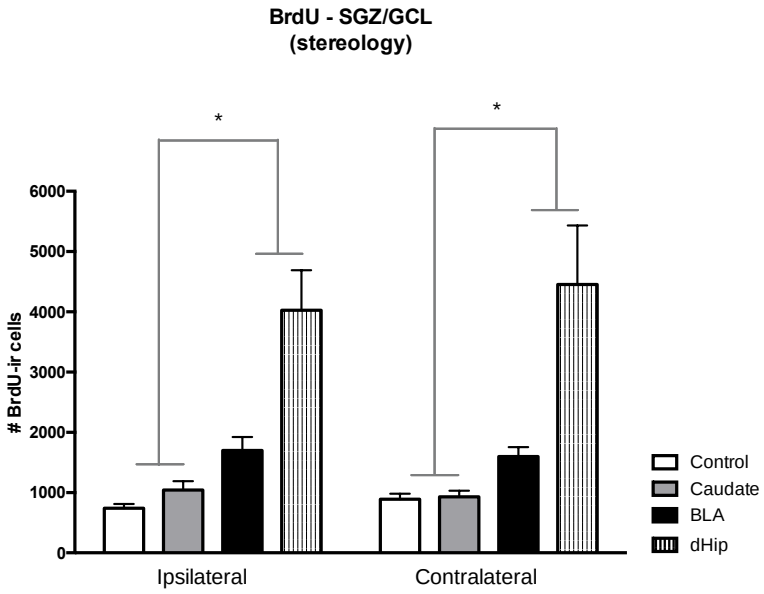

Figure 4. Kindling affected BrdU expression in the SGZ and GCL in both ipsilateral and contralateral brain sites. There was a significant increase of BrdU positive cells in dHip kindled and BLA kindled groups in comparison to the control and caudate kindled groups *(*p=0.01)*.

2.4.3. Doublecortin fluorescence

BrdU is an established method, widely employed by neuroscientists to track the process of cell proliferation. However, BrdU can label any proliferating cell not only neurons. Therefore, I performed immunofluorescent labelling with the immature neuronal marker DCX to confirm that the effects I was seeing with BrdU reflected changes in neurons. I examined the morphologic structure of DCX expressing cells within the SGZ and granule cell layers. Morphological characteristics and the topographic distribution of DCX expressing cells in each group confirmed that the BrdU immunostaining labelled proliferating neurons in the dentate gyrus (See Fig.5).

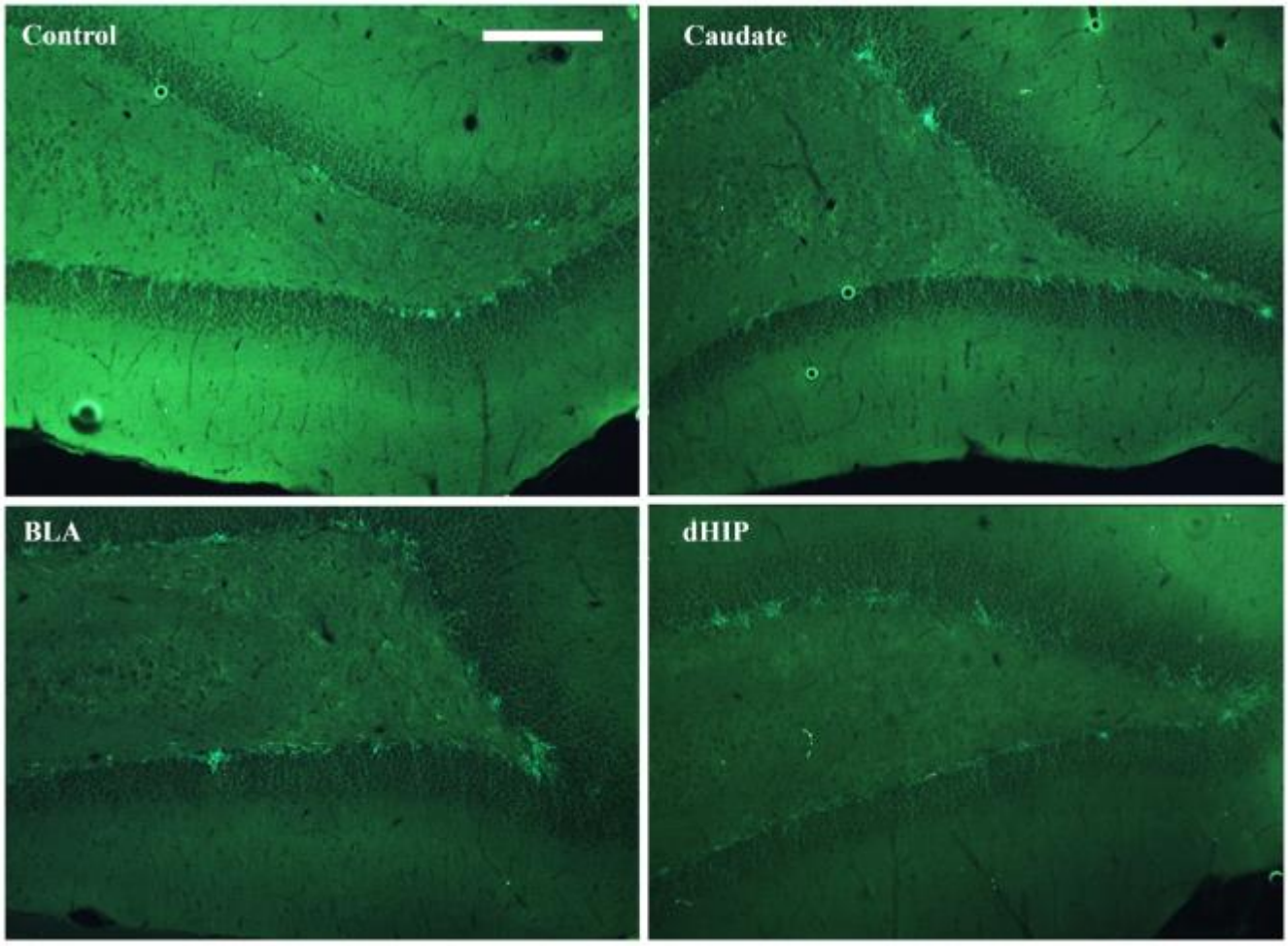

Figure 5. Illustrative example of DCX immunofluorescence across treatment groups. The pattern of neuronal expression within subgranular and granule cell layers corresponds with BrdU immunostaining. DCX expression confirms that BrdU positive cells are proliferating neurons.

2.4.4. Profile counting for Arc immunoreactivity

Figure 6 provides illustrative examples of Arc positive cells among all treatment groups. Tukey host-hoc analysis detected a significant decrease of Arc expression in limbic kindled groups within SGZ/GCL (ipsilateral $F(3,21)=27.225$, $p=0.000$; contralateral $F(3,21)=20.854$, $p=0.000$). Also there was a similar pattern of Arc expression within hilar zone of hippocampus (ipsilateral $F(3,21)=9.772$, $p=0.000$; contralateral $F(3,21)=7.634$, $p=0.001$). We also found that SGZ/GCL expressed more Arc positive cells in comparison to hilar zone (SGZ/GCL ipsilateral total mean =71.942, contralateral=74.803; hilar ipsilateral total mean = 4.05, contralateral total mean =4.720).

This quantification analysis indicates that limbic kindling significantly decreases neuronal activation after retrieval of a fear memory (See Fig. 7).

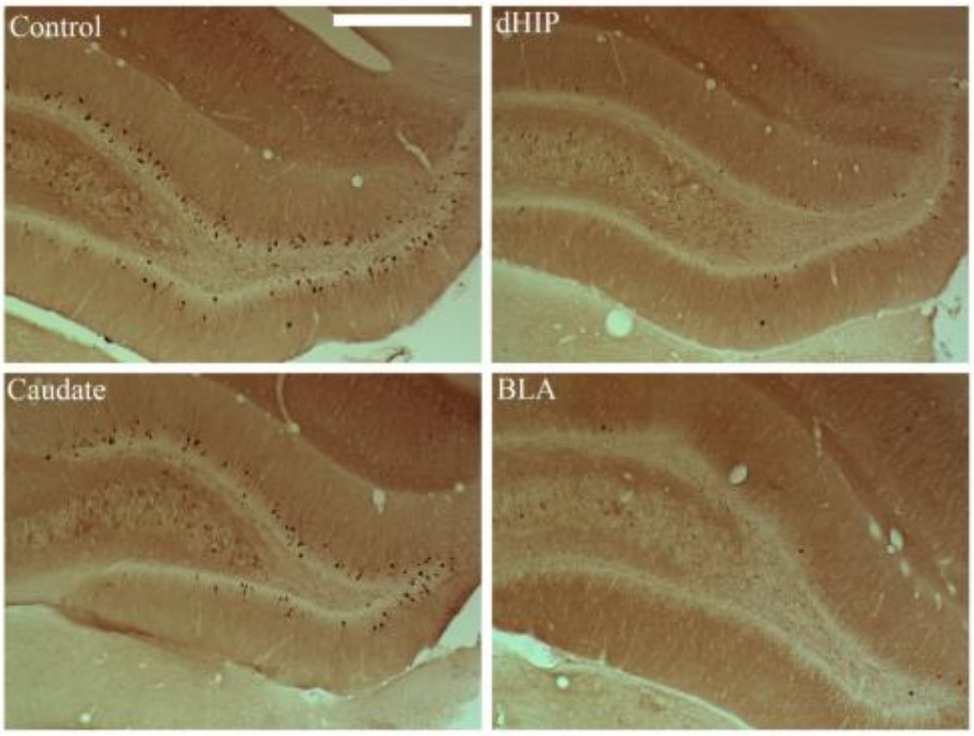

Figure 6. Comparative photomicrograph demonstrates the pattern of Arc expression in the dentate gyrus of four treatment groups. Scale bar: 200 μm

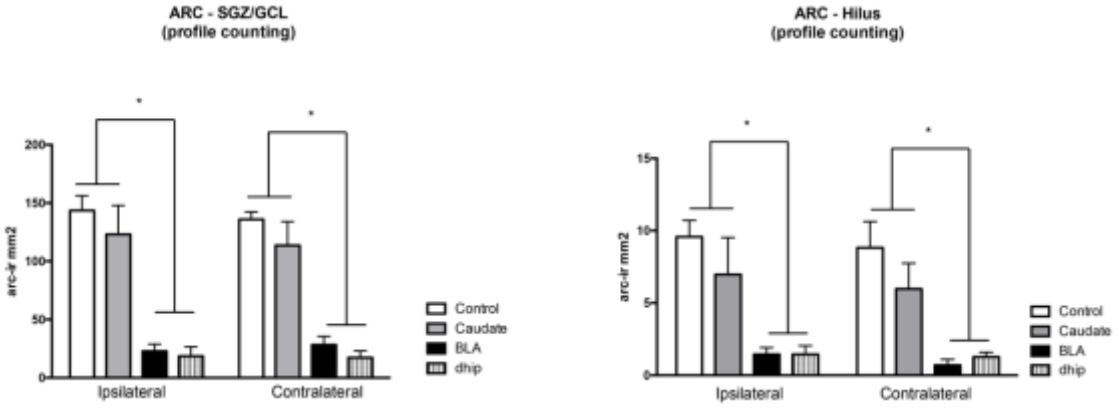

Figure 7. Effect of kindling on Arc expression following context fear memory retrieval. Profile counting was performed in SGZ and GCL layers (left) and hilar zone (right). Both the dHip- and BLA-kindled rats showed a significant decrease in the number of Arc positive cells in comparison to the control and CN-kindled group *[*p ≤ 0.01]*.

2.4.5. Profile counting for Fos immunoreactivity

Figure 8 provides example of Fos expression across all treatment groups. Figure 9 shows the effect of kindling different brain sites on Fos immunoreactivity in the granule cell layer, subgranular zone and the hilus.

Figure 8 reveals a substantial number of Fos positive cells in the control and CN kindled groups within the SGZ and granule cell layer. One-way ANOVA test indicated a significant group difference for both hemispheres in the SGZ/GCL subregions (ipsilateral SGZ/GCL: $F(3,21)=12.517$, $p=0.000$; contralateral SGZ/GCL $F(3,21)=15.659$, $p=0.000$). Tukey post hoc was appropriate because the assumption of heterogeneity of variance was satisfied (Levene test for ipsilateral and contralateral $F(3,21)=2.571$, $p=0.81$ and $F(3,21)=2.805$, $p=0.065$). The Tukey post hoc test indicated that the dHip- and BLA-kindled groups had significantly fewer Fos positive cells within SGZ/GCL compared to the control and CN-kindled groups (all p values <0.006). There were no statistical differences between the limbic kindled groups in either hemisphere ($p>0.5$). Therefore, limbic kindling significantly decreased Fos expression within SGZ/GCL layers (See Fig.9).

One-way ANOVA for Fos expression within the hilus revealed a significant difference between the treatment groups (ipsilateral hilus: $F(3,21)=13.384$, $p=0.000$; contralateral hilus: $F(3,21)=5.409$, $p=0.006$). However, the Levene test indicated a heterogeneity of variance ($p<0.05$). The Games-Howell post hoc test was therefore applied to compare means within the treatment groups. Only one pair had a significant difference within the treatment groups (CN-kindled to BLA-kindled: $p=0.029$; all other pairs - $p>0.064$).

Although the statistical difference was not detected in Fos expression within the hilus in the contralateral hemisphere, there remained an observable difference between CN-kindled compared the rest of the treatment groups. We will discuss this observation later (2.5.).

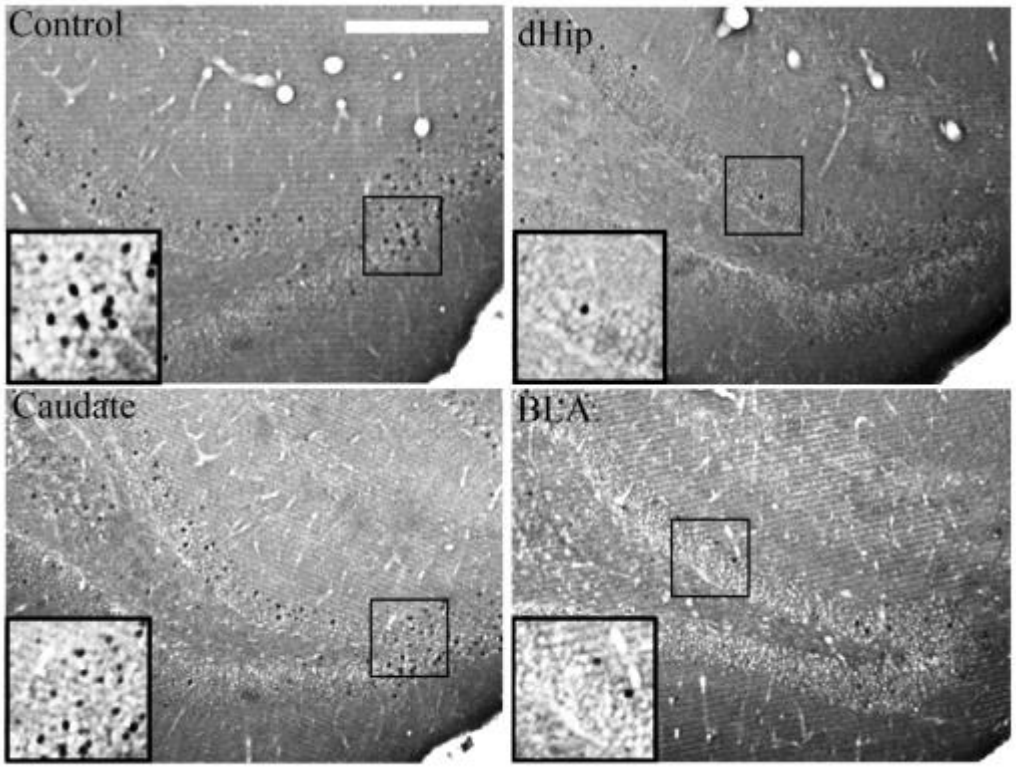

Figure 8. Comparative illustration of FOS expression across the four treatment groups. Photomicrographs at 4X magnification. Scale bar equals 200μm.

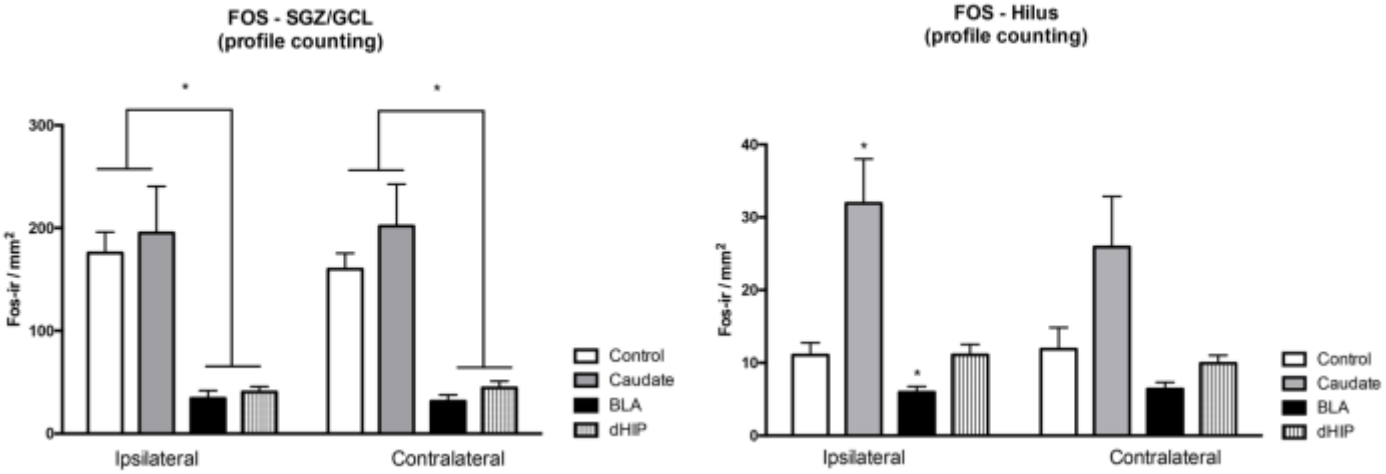

Figure 9. Kindling impacts FOS expression in limbic-kindled rats. There were significantly fewer Fos positive cells in the limbic kindled groups in comparison to the control and caudate groups within SGZ/GCL *[*p ≤ 0.06]*.

2.4.6. Double labelling for BrdU and Arc

Figure 10 shows pattern of BrdU and Arc expression using immunofluorescence. An example of the BrdU+Arc colocalization is provided in Figure 11. I counted BrdU positive cells within the SGZ and granule cell layers. Then, I counted Arc positive cells within the same regions of the dentate gyrus. Finally, I counted colocalized cells (BrdU positive cells that also expressed Arc). Statistical analyses of the BrdU and Arc counts were consistent with my findings reported above. Specifically, limbic kindling significantly elevated BrdU expression within the SGZ/GCL zones in both hemispheres (ipsilateral $F(3,21)=21.795$, $p=0.000$; contralateral $F(3,21)=12.787$, $p=0.000$). Limbic kindling also significantly decreased Arc expression within the SGZ/GCL areas in both hemispheres (ipsilateral $F(3,21)=16.592$, $p=0.000$; contralateral $F(3,21)=20.157$, $p=0.000$). The results from these manual counts demonstrate a similar pattern of BrdU and Arc distribution across all treatment groups (See Fig.12). Therefore, the present dataset corresponds with the results I obtained with profile counting and stereological counting.

Figure 13 shows the results from the colocalization analysis. Statistical analysis using one-way ANOVA followed by Tukey post hoc tests indicated a significant difference between treatment groups (ipsilateral $F(3,21)=7.313$, $p=0.002$; contralateral $F(3,21)=8.374$, $p=0.001$). The CN-kindled and control group had significantly more colocalized cells than the BLA-kindled and dHip-kindled rats. These results demonstrate that limbic-kindling prevented proliferating neurons from Arc activation in response to behavioural testing. The results of my experiment support previous reports from our laboratory (Fournier et al., 2013).

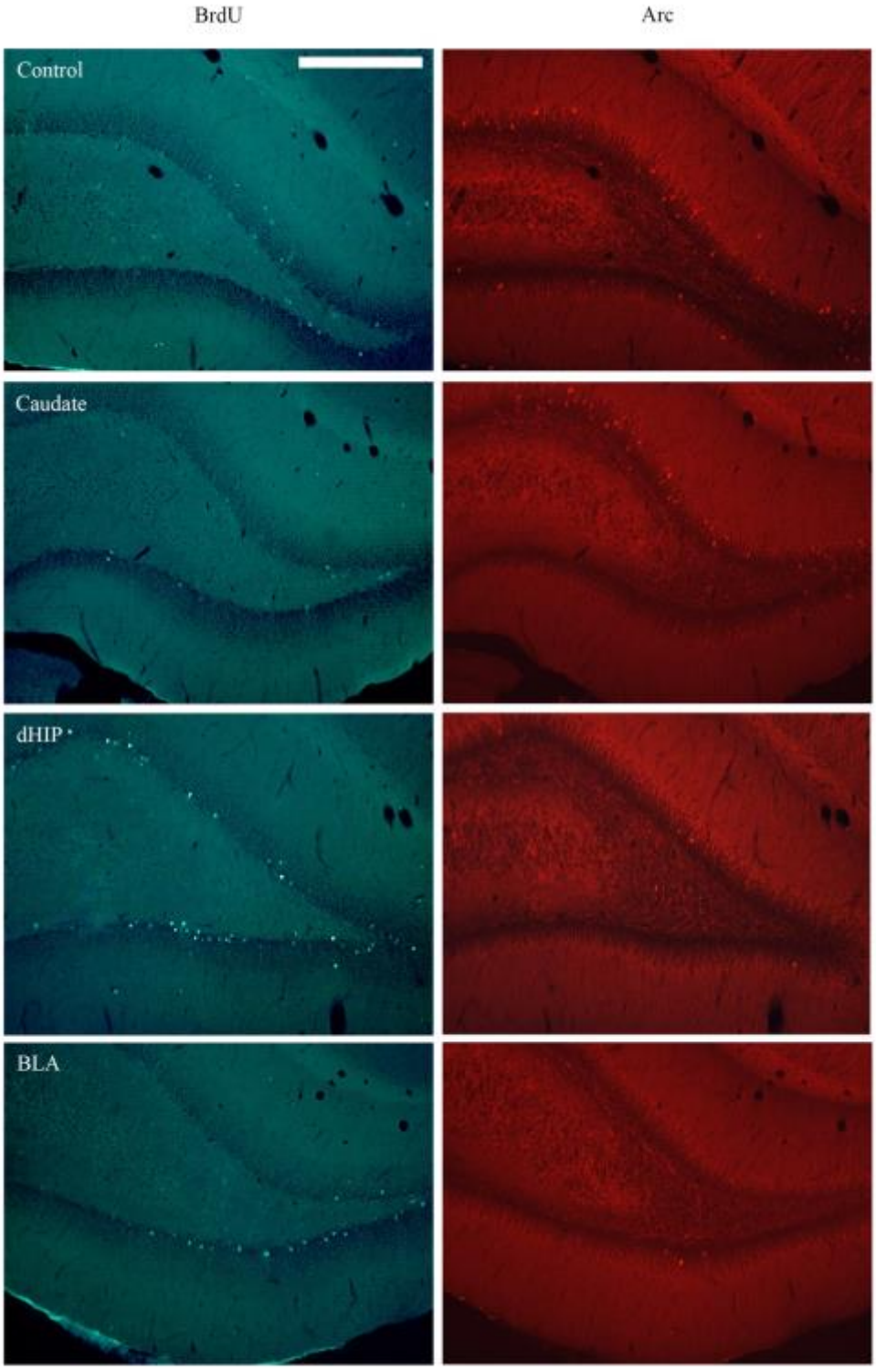

Figure 10. Pattern of fluorescent immunoreactivity of BrdU and Arc in each group. The green spectrum visualizes BrdU positive cells; Red – Arc positive cells. There is a significant induction of BrdU in the limbic –kindled groups. BrdU elevation was paralleled by a significant decrease of Arc expression in limbic-kindled rats. Pictures at 4X magnification. Scale bar equals 200μm.

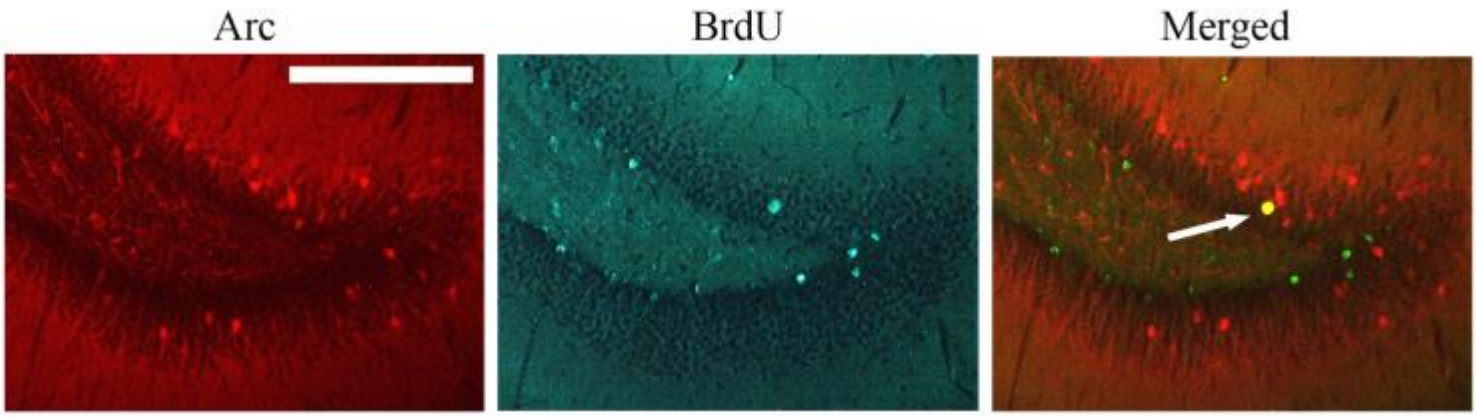

Figure 11. Illustrative example of merged images. The arrow indicates a colocalized cell in the granule cell layer. Pictures at 4X magnification. Scale bar equals 100μm.

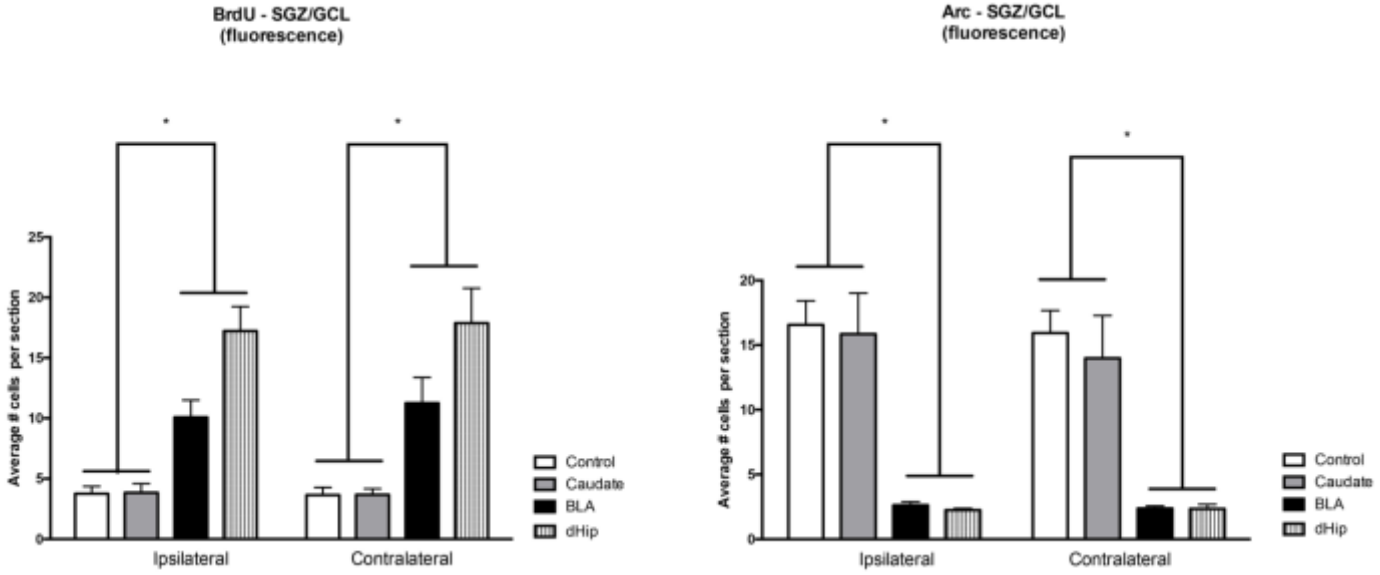

Figure 12. The effect of limbic kindling on BrdU and Arc fluorescent immunostaining following manual counting. Limbic kindling exaggerated neuronal proliferation that is paralleled with decreased Arc expression. **p*=0.000

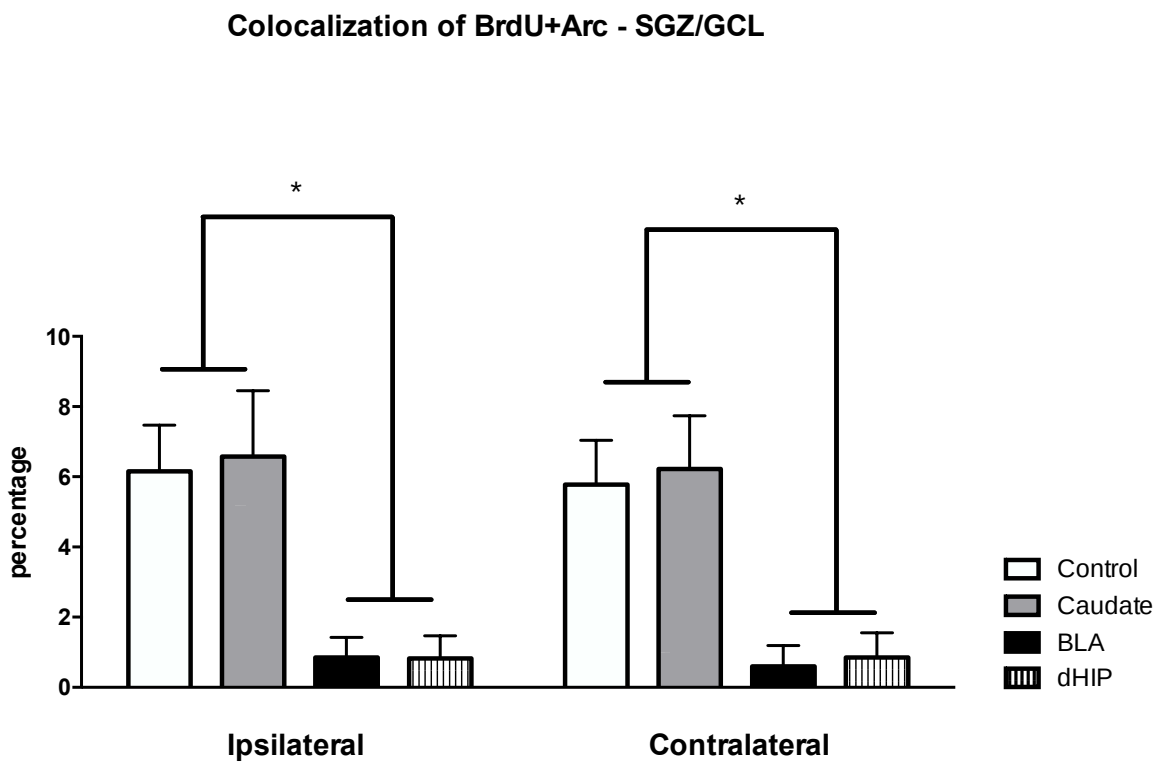

Figure 13. Colocalization analysis of merged images in immunofluorescent double labelling. Caudate and Control subjects demonstrates a significant difference compared to limbic kindled group. *p=0.05

2.5. Discussion

Five principal findings arise from the present study. First, kindling does not impair the acquisition of fear learning. Second, kindling of limbic brain sites (basolateral amygdala, dorsal hippocampus) but not non-limbic brain regions such as the caudate nucleus, significantly impaired the retrieval of fear memories on subsequent behavioural tests. Third, markers of behaviorally relevant neuronal activity markers Fos and Arc were significantly higher in the control group and CN-kindled group compared to the BLA- and dHip-kindled rats. Fourth, limbic-kindled rats demonstrated significantly higher induction of hippocampal neurogenesis, indicated by BrdU and DCX. And finally, exaggerated hippocampal neurogenesis in limbic-kindled rats coincided with cognitive deficits.

In this study, we investigated the impact of long-term kindling in three discrete brain regions on neuronal activation of newborn granule cells. This neuronal activation was measured in response to the recall of a previously acquired fear memory. In line with a previous report from our laboratory (Fournier et al., 2012), we show that seizures increase the survival and accelerate the maturation of newborn granule cells. Limbic kindling has a negative impact on cell migration and its further integration in existing memory circuits.

Temporal lobe epilepsy affects several domains of cognitive functioning. Memory is comprised of several stages: acquisition, consolidation and retrieval. Memory can be further modified by the process of reconsolidation. In the present study, we demonstrated that seizures originating from limbic structures (BLA and dHip) significantly impaired retrieval of fear memories. Previous studies have shown that trace fear conditioning (auditory fear conditioning) is a form of episodic memory that unlike delayed conditioning is sensitive to lesions of the hippocampus. In our experiment, we show that long-term limbic kindling disrupts conditioned

fear in both the tone test and context test. However, the important observation that stems from the behavioural testing is that kindling did not impair the acquisition of hippocampal dependent memory. These results support previous reports from our laboratory (Botterill et al., 2016). Long-term kindling specifically isolated a portion of memory functioning but not all aspect of the memory. This observation may provide a hint towards further understanding of the pathophysiology of memory impairments under the condition of repetitive seizure attacks. Current neurophysiological studies have partially explained the molecular basis of memory acquisition. At the cellular level, trace fear conditioning requires the activation of NMDA receptors (LeDeoux, 2000; Wanisch et al., 2005) that help to detect coinciding events. With the help of AP5 (selective NMDA antagonist) infusions into the amygdala, it was demonstrated that rats fail to acquire fear conditioning (Miserendino et al., 1990). In line with this concept, Langton et al. also performed a series of MK-801 injections (NMDA receptor antagonist) that prevented rats from acquiring fear memories, importantly for both young and old rats (Langton et al., 2007). On the other hand, systematic NMDA injections have been shown to increase cognitive functions (Hlinak and Krejci, 2002; Koek et al., 1990). Both surgical and pharmacological experimental interventions suggest that the NMDA-receptor system is critically important for the formation of memories, but not for the maintenance and/or retrieval of such memories (Constantine-Paton 1994; Izquierdo 1991; Izquierdo et al., 1993; Liang et al., 1993; Quartermain et al., 1994; Rickard et al., 1994). Kindling is known to disrupt the NMDA system (Auzmendi et al., 2009; Shen et al., 2011; Auzmendi et al., 2008). There are eight components of the NMDA system (NR1, NR2A-D, NR3A-B, etc.). NR2A and NR2B are particularly interesting in this context because they are predominantly expressed in the hippocampus (Loftis et al., 2003). Importantly, both NR2A and NR2B subunits return to baseline at the 12-hour time point

after the last electric stimulation (Pratt et al., 1993). However, there are other reports indicating a significant reduction in the NR2A subunit in the amygdala and limbic forebrain in rats subjected to amygdala kindling (Prince et al., 1995). Possibly these controversies could be explained by the fact that these studies had different kindling duration.

Our experimental design involved regular electric stimulations over a prolonged period of time that allowed for adaptive and compensatory mechanisms to come into effect. Given the experimental design of our present study, we may hypothesize that rapid adaptive events, the stabilization of NMDA receptors in particular, within the time-gap between the delivery of the last stimulation and first exposure to the behavioural task may have allowed for a normal acquisition of Pavlovian conditioning. In fact, I conducted a preliminary immunohistochemical analysis of NR2B and PSD-95 labelling in the kindled rats from this experiment but a densitometry analysis revealed no significant differences between the treatment groups. The rats were sacrificed five days after the last stimulation. Given the fact that NMDA receptors are fast acting in nature, it is possible that the five-day gap allowed for the stabilization of the system. This potential stabilization effect may have prevented the detection of changes on the densitometry analysis.

The NDMA receptor is involved in a wide variety of neuronal functions such as synaptic plasticity, learning, memory and epileptogenesis (Li et al., 2009; Newcomer et al., 2000; Fraska et al., 2011; Blair et al., 2008). NR2B-containing NMDA receptors may play a crucial role in the cognitive deterioration observed in epileptic patients. The present discussion of possible neurobiological mechanisms that occur following repetitive seizures only partially covers the complex system that governs the process of memory acquisition, retention and retrieval. Therefore, the neurological basis of this pathology remains to be understood.

The mechanism of seizure-induced cognitive deficits remains unknown. However, there are several possible explanations for our observation that limbic kindling impaired fear memory retrieval in our experiment. In the adult animal, status epilepticus causes neuronal loss in the CA1, CA3 and the dentate gyrus. The pattern of cell loss depends on the animal model being used (Nadler, 1981; Ben-Ari, 2001). In addition to cell loss, the neuronal system is totally reorganized at multiple levels, with mossy fiber sprouting being one example of aberrant neuronal growth of granule cell axons in the epileptic brain (Represa et al., 1987; Sutula et al., 1988).

Our experiment demonstrates that kindling has different effect on neurogenesis depending on the brain site where the seizure originates. This finding may contribute towards the process of clinical evaluation of the severity of the condition in a clinical setting. We found that kindling originating from dorsal hippocampus has the most pronounced effect on the rate of neurogenesis, in that our stereological analysis revealed the highest number of BrdU positive cells in the dHip kindled group. These increases in neurogenesis coincide with poor performance on hippocampal dependent memory tasks.

Although dHip kindling exaggerates neurogenesis and decreases IEG activation, CN kindling induces somewhat opposite effects. Indeed, previous experimental evidence indicated that the caudate nucleus has a modulatory effect on hippocampal epilepsy (Vella et al., 1991). Electrical stimulation or NMDA microinjections has been shown to decrease seizure severity (Turski et al., 1988). In fact, clinical studies confirmed that low frequency brain stimulation of the caudate nucleus was able to suppress epileptic activity and disrupt both focal and generalized epileptic discharges (Šramka et al., 1990). Interestingly, the results of our study following Fos

analysis indicated a slight increase of neuronal activity in comparison to the control group, albeit statistical significance was not observed. It is also worth noting that the ipsilateral hemisphere showed slightly higher Fos activation within the hilus. It is possible that there are hemisphere-specific changes in response to kindling.

Stereological analysis of BrdU immunohistochemistry also showed similar trends. This observation may indirectly confirm that CN kindling may potentially inhibit the negative impact of repetitive seizures on neuronal circuits.

Our observations in CN-kindled rats correspond with reports from other laboratories. For example, central lateral nucleus high-frequency electric stimulations in rats results in cognitive improvement in performance and learning on a visual object recognition task. These improvements also coincide with increased expression of Fos and Zif268 in the caudate, putamen, and hippocampal regions (Shirvalkar et al., 2006). GABAergic system activation is one of the possible mechanisms that modulate behavioural outcomes following caudate stimulation. For instance, high frequency electric stimulation of the CN in vivo in rats demonstrated a significant increase in extracellular GABA expression in the caudate nucleus (Hiller et al., 2007). Therefore, evidence indicates that the CN is involved in inhibitory neural processes (Spehlmann et al., 1977; Rakic et al., 1962; Hiller et al., 2007). These reports may suggest that electric stimulation of the CN may produce potential therapeutic effects for patients suffering from TLE associated cognitive deficits.

In sum, the results of present experiment indicate that kindling of the dHip region and BLA enhance neuronal cell proliferation in the dentate gyrus. Limbic kindling impairs fear memory retrieval and seizure generated neurons demonstrate low neuronal activation following fear memory retrieval. Kindling in the dorsal hippocampus and basolateral amygdala has the

most pronounced negative effects on neuronal cell activation. Kindling in limbic regions prevented newborn neurons from normal integration into existing memory circuits. In conclusion, we provided evidence that aberrant neurogenesis as a result of limbic kindling plays a contributive role towards cognitive deficits.

CHAPTER 3

3.1. General discussion

The general goal of my thesis was to advance the understanding of the mechanisms underlying the behavioural comorbidities associated with TLE. This thesis attempted to gain knowledge about the potential structural and functional changes in the hippocampus following repeated seizure activity.

I hypothesized that kindling in different brain regions would produce differential effects on behavioural outcomes. I further hypothesized that site-specific kindling would produce differential effects on neurogenesis and rates of neuronal cell activation. To that end, I examined the effect of long-term kindling in three distinct brain regions – the basolateral amygdala, dorsal hippocampus and caudate nucleus. As the product of my research, I received three principal sets of data.

First, I examined behavioural outcomes in response to long-term kindling with seizures originating from limbic or non-limbic brain sites. The day after the last kindling stimulation, rats were subjected to a trace fear conditioning paradigm to assess the impact of repeated seizures on the acquisition and retrieval of associative memory.

Second, I examined cell proliferation and neurogenesis following repeated seizures using the markers BrdU and DCX.

Third, I examined the rate of neuronal activation in response to exposure to a pre-conditioned (aversive) environment following behavioural training.

I found that limbic kindling significantly impaired fear memory retrieval. Behavioural outcomes paralleled increased neurogenesis in limbic kindled rats. In contrast, neuronal activation rate was diminished after limbic kindling, and very few BrdU positive cells also

expressed Fos and Arc. These results suggest that limbic, but not non limbic kindling prevent seizure-generated neurons from properly integrating into existing memory circuits in the hippocampus.

3.1.1. Aberrant neurogenesis and cognition

The addition of a large number of neuronal cells generated in response to kindling indeed impaired cognitive performance in rats. Colocalization analysis within my thesis work suggested that limbic kindling prevented newborn neurons from normal integration into the existing memory circuits. This hypothesis is supported by a previous report from our laboratory that seizure generated neurons are less likely to colocalize with the marker of recent neuronal activity (Fournier et al., 2013).

However, other researchers found evidence to support an alternative hypothesis. Granule-like cells produced after pilocarpine induced seizures appear to participate in the underlying neuronal activity that appears during chronic behavioural seizures (Sharfman et al., 2002). Possibly, neurogenesis in the dentate gyrus following pilocarpine seizures can lead to new cells that mature into functional neurons and integrate into the functional circuits (Sharfman et al., 2000, 2002). Importantly, the Sharfman laboratory did not examine the level of neuronal activation of those 'recruited seizure newborn' neurons in response to cognitive tasks. Therefore, although seizure-generated granule cells may mature into functional neurons, it is not clear that these post-seizure neurons can function properly in memory tasks.

There are other interesting perspectives that attempt to explain a connection between aberrant neurogenesis and cognitive deficits. Possibly, aberrant integration and the presence of many newly generated neurons impair memory performance by degrading network performance

(Fournier, 2009; Becker et al., 2005; Wiscott et al., 2006). The constant addition of newly generated neurons into a neuronal network is compensated by randomly chosen pre-existing neurons (Chambers et al., 2004). If indeed, seizure-generated neurons, with their pathomorphological characteristics, still succeed to integrate into a memory circuit, then another possible explanation is a 'wash out' effect. In other words, the permanent accumulation of granule cells simply creates a competition for new neurons to integrate into the network, in addition to this a constant turnover of new neurons create a constantly updating network that may impede normal memory retention (Frankland et al., 2013; Akers et al., 2014). To support this idea, there is evidence that elevated levels of neurogenesis promote forgetting of established memories (Akers et al., 2014).

3.1.2. Why do seizures impede neuronal activation?

The results of my thesis work support concept that seizures dramatically decrease the neuronal activation that is necessary for normal memory functioning. Levels of neuronal activation were severely dampened in the limbic kindled rats. Although aberrant neurogenesis may play an important role in the cognitive deficits that result from kindling, this does not explain the mechanism of neuronal deactivation. It is unknown what factors might mediate this pathological process.

As it has been previously discussed (section 1.2), kindling induces pathological changes on multiple levels. These pathologic changes involve a myriad of events that are connected into the one unified synergetic system. This synergy makes it increasingly difficult to select and understand the leading factors that govern neuronal downregulation in response to recurrent

seizures. However, there are several possible mechanisms that may play dominant role in 'neuronal deactivation' within a memory network.

Recurrent seizures evoke a cascade of compensatory mechanisms. One of them is the observable strengthening of the GABAergic system. For instance, new cells that are born under the condition of status epilepticus receive increased inhibitory activity (Jakubs et al., 2006). Seizures accelerate the process of cell maturation (Overstreet-Wadiche et al., 2006) and at the same time seizure-generated neurons receive inhibitory inputs sooner than the newborn cells created under normal conditions. This pathologic acceleration possibly affects a critical period of neuronal cell development, when cells may exhibit increased plasticity. Therefore, seizure-generated neurons are 'silenced' by the GABAergic system before they unfold their potential of synaptic plasticity. Further, it is well documented that seizures cause mossy fiber sprouting (Blaabjerg et al, 2007; Danzer et al., 2009). It has been hypothesized that the terminals of those sprouting neurons may release GABA into the dendrites of granule cells (Buckmaster, 2012; Santhakumar et al., 2005).Therefore, mossy fiber sprouting may also help to 'silence' both newborn and mature neurons. Viewed this way, the reduced neuronal activation seen in the limbic-kindled rats in this experiment may be the manifestation of a compensatory mechanism. Thus, increased inhibitory input that helps to 'resist' further epilepsy progression comes at the general cost of neuronal deactivation.

Another possible explanation for the reduced neuronal activation following epileptic seizures comes from the perspective of long term potentiation (LTP). Long term synaptic plasticity is a key mechanism that allows for normal memory function and cognition (Malenka, 1994). LTP is associated with increased synaptic connectivity in response to high-frequency stimulation. The induction of LTP requires both GABAergic and glutamatergic systems (Davies

and Collingridge, 1993). Physiological high-frequency stimulation opens NMDA receptor channels allowing the influx of Na^+, K^+, and Ca^{2+}. Inside the post-synaptic neurons, Ca^{2+} activates calmodulin kinase II and protein kinase C. These enzymes induce LTP that serves for the benefit of memory processes (Hamed, 2007). Electrophysiological studies in the rat hippocampus demonstrated that persistent seizure activity has a destructive effect on synaptic plasticity. Seizures cause widespread induction of LTP that reduce overall hippocampal plasticity (Reid et al., 1997; Hamed, 2007). This reduction of hippocampal plasticity following seizure activity may partially explain the limited neuronal activation in the dentate gyrus.

Over the past decade, there was a significant progress in understanding the pathophysiology of epilepsy. However, further scientific efforts are necessary to create a holistic understanding of epileptogenesis and the causes of the cognitive deficits associated with epilepsy.

3.2. Limitations

Kindling is a well-studied animal model of epilepsy. Kindling has been known since the 1960's and has been extensively applied in various experimental situations. Experiments with electric stimulation have been reported in various vertebrate species. Importantly, the behavioural outcomes resulting from amygdaloid kindling are very similar across many species. Local electrical stimulations of the amygdala or the hippocampus are associated with a progressive increase in seizure susceptibility and severity. As it was previously discussed, kindling can induce neuronal degeneration in the limbic region that is very similar to those changes observed in patients with TLE. This observation strengthens the validity of kindling as a model of TLE. Further, kindling has a high predictive validity because anticonvulsant drugs that

successfully demonstrate therapeutic action against kindling are also effective in human patients. Interestingly, kindling also shares many similarities with the behavioural sensitization resulting from psychostimulant drugs (Pierce and Kalivas, 1997; Post and Weiss 1989).

Among other strong advantages of kindling there is a location specificity, thus researchers are able to elicit seizures in a specific brain site and demonstrate high control of the number of seizures and their frequency. This allows for the study of time-sensitive events that parallel epileptic seizures.

However, kindling has received a substantial amount of criticism within the past decade. Spontaneous epileptic seizures are the main feature of the epilepsy in human patients. However, kindling does not elicit spontaneous seizure activity. Although it is possible to elicit spontaneous seizures after more than 280 stimulations, kindling does not successfully mimic this clinical feature in its normal application. Another disadvantage of kindling is that it is still not clear whether kindling can induce epilepsy in a human brain. There are few consistent reports when patients received series of ECT stimulations and later progressed into full-blown epilepsy. In fact, there is a clinical case of a patient that experienced more than 1200 ECT sessions and never developed spontaneous recurrent seizures (Lipman et al., 1985).

Kindling as a model of epilepsy has remained mainstay of epilepsy research for more than 40 years. But the question remains about how the results from kindling experiments advance our understanding of the underlying neurobiology of human epilepsy. Therefore, kindling has a limitation in regards to clinical relevance.

Another limitation of my thesis work stems from sex specificity. Sex differences may play a role in kindling progression and its behavioural outcomes. Sex hormones and their neurosteroid derivatives exert regulatory activity on the excitatory properties of the hippocampus

(Smith et al. 2002). Our experimental procedures were conducted exclusively with male rats. I would like to acknowledge that this is one of the limitations of my present work. Estrogens, androgens and progestins are well-known to alter hippocampal electrophysiology.

Steroid hormones play key role in the neuroendocrine control of neuronal excitability and seizure susceptibility (Herzog et al., 2003; Verotti et al., 2007). Steroid hormones are synthesized in ovarian, gonadal and adrenal structures. In men, the dominant steroid hormones are testosterone and dihydrotestosterone; both exhibit anabolic and androgenic activity. In women, the main circulating reproductive steroid hormones are the estrogens and progesterone. Testosterone has been shown to decrease excitation threshold and increase seizure susceptibility. However, testosterone may exhibit a dual role on neuronal excitability, because it may produce both proconvulsant and anticonvulsant effects depending on the animal model and seizure type (Reddy et al., 2008). The proconvulsant activity of testosterone may be explained by its aromatization to estradiol. Both animal and clinical studies indicate that testosterone metabolites increase seizure activity (El-Khayat et al., 2003; Herzog et al., 2003). Conversely, the androgenic wing of testosterone has a protective role against seizures that were induced by kainic acid (Reddy et al., 2004; Frye et al., 1999). Therefore, testosterone may exert variable effects on seizure activity: proconvulsant at higher doses and anticonvulsant at lower doses. In clinical settings, testosterone injections have not been shown to improve seizures (Herzog et al., 1998). The female estrus cycle also causes fluctuations in hippocampal excitability, which may produce differential risks for seizure susceptibility at different points of the hormonal reporting period (Warren et al., 1995; Scharfman et al., 2006). Unlike testosterone, the estrogens generally facilitate seizure activity (Wooley et al., 2000). Seizures and hormonal activity have a bidirectional connection. Limbic kindling distorts ovarian hormone cyclicity in rats (Edwards et

al., 2000). The effect of the estrogens has been studied using animal model of epilepsy. Epileptic female rats exhibit elevated seizure frequency that coincides with peaking time points of estrogen expression during the ovarian cycle (Sharfman et al., 2008). The estrogens also demonstrate a systemic influence on neuronal functioning. Neurogenesis is no exception, as estradiol may rapidly upregulate neuronal proliferation in the dentate gyrus of female rats (Barha et al., 2009).

Given the limitations of my present work, it is important to note that future studies may further investigate the connection between neurogenesis and site-specific epilepsy under an experimental design that would consider sex differences and kindling limitations. Finally, most of the findings described in the present work are based on animal models, therefore caution must be exercised when extrapolating these findings to the human brain.

3.3. Future directions

The results of my thesis further establish the importance of neurogenesis as a contributing factor in the cognitive deficits associated with TLE. These results may potentially impact a therapeutic strategy of future epilepsy treatment. Due to the importance of normal neurogenesis, it is logical to hypothesize that patients with TLE may benefit from therapeutic interventions that modulate adult hippocampal neurogenesis. For example, rapamycin has been already suggested as one possible option for pharmacologic intervention (Wong 2011; Canpolat et al., 2014). Rapamycin administration can reverse the cognitive deficits produced by status epilepticus in rats (Brewster et al., 2013). These effects of rapamycin may be mediated by a reduction of aberrant neurogenesis and decrease of mossy fiber sprouting (Butler et al., 2015; Zheng et al.,

2009). Therefore, future research should investigate the development of pharmacological modulators of hippocampal neurogenesis.

As mentioned in previous paragraphs, the development, maturation and migration of newborn neurons in the mature brain is mediated by a cascade of intrinsic factors, one of them being reelin (Teixeira et al., 2012). Reelin is a large extracellular matrix protein expressed predominantly by GABAergic interneurons in the hippocampus, and it has been shown to play a critical role in neuronal migration (Teixeira et al., 2012). Reelin-deficient mice have significantly fewer newborn granule neurons as compared to their control counterparts (Won et al., 2006). Conversely, intrahippocampal infusions of reelin enhance the cognitive abilities of mice; this enhancement parallels morphological reorganization of hippocampal structures (Rogers et al., 2011). Intrahippocampal infusions of recombinant reelin have also been shown to reduce neuronal aberrations following epileptic seizures in mice subjected to the kainite model of TLE (Müller et al., 2009). Recently, it was demonstrated that persistent seizure activity leads to a significant decrease in reelin expression, both in the human and animal brain (Frotscher et al., 2016).

Previous reports suggest that that reelin plays an essential role in adult neurogenesis. Considering the relationship between seizure-induced neurogenesis and cognitive decline in TLE, it is logical to hypothesize that reelin in the future reelin may become a therapeutic option for patients suffering from drug-resistant forms of epilepsy and cognitive deficits.
Reelin has already been considered as a potential therapy for depression and mood disorders (Caruncho et al., 2016). Therefore, it is interesting to apply reelin as a therapeutic intervention in an animal model of epilepsy such as kindling. However, there has been criticism regarding safe and effective routes of reelin administration, as reelin is a large protein that has difficulties

crossing the blood brain barrier (Caruncho et al., 2016). Further experimental procedures delineating the pharmacodynamics and pharmacokinetics of reelin are needed. Currently, the field has a knowledge gap in understanding how reelin is affected by pathology, and how the supplementation of reelin could be efficacious in treating pathology. Further studies should apply these questions to animal models of TLE.

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