Retrograde Amnesia of Fear Memories Following Pentylenetetrazol Kindling

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ABSTRACT

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Memories pertaining to fearful events are some of the most salient and long-lasting memories, as they are critical to the survival of an organism. Seizures induce aberrant changes within temporal lobe and limbic brain structures that are critical for supporting fear memories. Seizures can occur at any time; therefore, it is imperative that research address how seizures impact previously learned information. The present series of experiments demonstrate that pentylenetetrazol-kindling induces retention deficits of previously acquired context fear memories in male rats. Kindling induced subsequent fear learning deficits but did not impact spatial learning. Additionally, following kindling, volumetric increase was observed within the hippocampal subfield CA3, as well as increased neural activation within the hippocampal subfield CA1. The results of this work suggests that chronic seizures can alter the function of neural networks important for supporting and retrieving previously acquired memories.

Keywords: retrograde amnesia, anterograde amnesia, context fear conditioning, seizures, pentylenetetrazol, hippocampus, amygdala

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LIST OF ABBREVIATIONS

AED	Antiepileptic Drugs
BLA	Basolateral Amygdala
CA1	Cornu Ammonis hippocampal subfield 1
CA2	Cornu Ammonis hippocampal subfield 2
CA3	Cornu Ammonis hippocampal subfield 3
CeA	Central Amygdala
CFC	Context Fear Conditioning
CT	Computerized Tomography
DG	Dentate Gyrus
DGC	Dentate Granular Cell
EEG	Electroencephalogram
fMRI	Functional Magnetic Resonance Imaging
HPC	Hippocampus
KA	Kainic Acid
LTM	Long Term Memory
MWT	Morris Water Task
MRI	Magnetic Resonance Imaging
NOR	Novel Object Recognition
PTZ	Pentylenetetrazol
RAM	Radial Arm Maze
RAWM	Radial Arm Water Maze
SRS	Spontaneous Recurrent Seizures

STM	Short Term Memory
TBI	Traumatic Brain Injury

CHAPTER I
Introduction

1. Introduction Overview

Our memories not only define who we are, but they are critical to our survival. But what happens when the networks that support these memories become altered? Alterations can occur to the neural networks which support memories and can render these networks incapable of forming new memories, as well as rendering existing memories damaged or lost. Most researchers have examined what happens to the ability to learn and retain new information after alterations to a network have occurred, but it is important to assess what happens to memories that were learned before alterations occur. This chapter will begin with an overview of the structures involved in learning and memory formation as well as the continued support of these memories. I will discuss various ways that alterations can occur, as well as explaining the associated learning and memory deficits they are believed to promote. Then, the focus will shift onto how alterations induced via recurrent seizures impact fear learning and memory. Therefore, I will present an overview of epilepsy in humans and focus on the learning and memory impairments associated Temporal Lobe Epilepsy (TLE), as it is one of the most commonly occurring types of epilepsy and the seizures that occur within the temporal lobe impact structures known to be involved in learning and memory processes (Fisher et al., 1998; National Institute of Neurological Disorders and Stroke, 2020). I will then present various animal models of epilepsy and the related learning and memory deficits reported to date. The chapter will conclude with the presented objectives and proposed hypotheses.

2. Memories Pertaining to Fearful Events

Being able to distinguish between safe versus unsafe environments is crucial to ensure the continued survival of an organism. The information that someone acquires over time is constantly used to navigate oneself throughout the rest of their lives – they guide our behaviours and our decision making, they trigger complex emotions and feelings, and they impact how we perceive the world around us and ourselves. Remembering autobiographical memories, defined as memories pertaining to the self or personal experiences (Conway & Rubin, 1993; Nadel & Moscovitch, 1997), is essential in being able to make judgement calls when presented with similar information at a later timepoint. More specifically, the environment in which one has had negative experiences often serve as a basis for how one will react or what they might expect to happen when returned to a similar place or situation. Therefore, it is not surprising that in comparison to other types of memory, memories pertaining to traumatic or fearful events are unique in that they can be formed almost instantly and can remain intact for the lifetime of the organism (Fendt & Fanselow, 1999; LeDoux, 2014; Nadel & Moscovitch, 1997).

3. Mechanisms Involved in the Process of Fear Learning and Retention

The importance of long-lasting fear memories is evident, so it is not surprising that determining the cellular mechanisms involved in their formation and retrieval is one of the biggest questions in neuroscience. A dominate model suggests that when information is learned, a memory trace is formed and is in a labile state, susceptible to loss or damage (Kandel et al., 2014). However, over time a cascade of cellular and molecular processes within the neuron and at the level of the synapse occur which

stabilize connections and form a memory trace that is less vulnerable to disruption (Kandel et al., 2014; Schafe et al., 2000; Schafe & LeDoux, 2000). This processes, termed cellular consolidation, can take up to 100 hours to complete (Kandel et al., 2014; Schafe et al., 2000). Cellular consolidation is further supported by evidence of memories being impaired when the inhibition of a variety of cellular and molecular processes within the neuron occur during the assumed time period of cellular consolidation (Kandel et al., 2014). Following the stabilisation of a memory trace, it is possible to express this memory at a later time (Alberini & Ledoux, 2013; Lee, 2009). However, when this memory is retrieved, it is believed that the memory trace will re-enter a labile state, followed by a new bout of cellular consolidation. Again, during this labile state the memory can become more vulnerable to disruption (Alberini & Ledoux, 2013; Lee, 2009).

The hippocampus (HPC) is often referred to as the gateway to memory, as it is a structure well known to be involved in fear learning and memory processes (Fanselow & Poulos, 2005). Neuroimaging studies have regularly observed increased hippocampal metabolic activity during the acquisition and retrieval of fear memories (Deng et al., 2010; Frankland & Bontempi, 2005; Tischmeyer & Grimm, 1999). Furthermore, evidence suggests that fear memories can be susceptible to loss when the HPC is damaged following learning (Kim & Fanselow, 1992; Lehmann et al., 2007). Loss of memories acquired before brain injury is referred to as retrograde amnesia (RA) (Lehmann et al., 2007; Squire & Alvarez, 1995), whereas the inability to form new memories after brain injury is referred to as anterograde amnesia (AA) (Elliott et al., 2014; Mayes, 2015; Spiers et al., 2001; Squire & Alvarez, 1995). It is possible to make

fear memories resistant to loss via distributed training, which allows for the prevention of RA from occurring (Lehmann et al., 2009; Lehmann & McNamara, 2011).

The amygdala is largely associated with emotionality (Davis, 1992; J. LeDoux, 2003). It is believed to play a role in both the fear information input and fear expression/output (Davis, 1992). The amygdala links external stimuli to the defense response that an organism expresses (LeDoux, 2003). Therefore the role of the amygdala is not temporary, as activation within the amygdala occurs during both the acquisition and retention of fear memories (Fanselow & Poulos, 2005; Tischmeyer & Grimm, 1999; Tovote et al., 2015). The HPC interacts with the amygdala during the process of memory acquisition and retention as there are projections from the CA1 of the HPC to the BLA of the amygdala (Fanselow & Poulos, 2005; Fendt & Fanselow, 1999; J. LeDoux, 2003; Tovote et al., 2015). Interactions between these structures will occur when the information pertains to that of emotionally charging material – such as fear or anxiety related memories in both humans and non-human animals (Fanselow & Poulos, 2005; Fendt & Fanselow, 1999; Tovote et al., 2015).

4. Alterations to the Memory Network

Damaging a network can provide insight into the degree in which other networks are able to compensate for this functional loss. It can also provide insight into the role of said removed network, by indicating deficits in function. Which is why, of interest is the question: What happens to fear memory when network alterations occur? The reason that this is so important to understand is because alterations to learning and memory networks can occur throughout an organism's life, for example, via physical exercise, Alzheimer's

Disease (AD), strokes, traumatic brain injury (TBI) or seizures (Johnson et al., 2016; O'Leary et al., 2019; Sahyouni et al., 2017; Scarmeas et al., 2009; Stafstrom & Carmant, 2015). Network remodelling can have both beneficial impacts to learning and memory, as well as detrimental effects. By determining what can enhance learning and memory, and what can impair it, we can better understand functioning of these networks to further assess how to remedy impairments.

5. Network Alterations that Enhance Learning and Memory Networks

Exercise has been reported to induce alterations within learning and memory networks (Baek, 2016; O'Leary et al., 2019). These include the induction of neurogenesis, which is the birth of new neurons, as well as increased BDNF (brain-derived neurotrophic factor) expression, which supports synaptic plasticity, within the HPC (Baek, 2016). BDNF supports the survival and growth of new neurons which promote enhanced connectivity between neurons within the HPC (Baek, 2016). These changes can result in the improvement of either one or both acquisition and/or retention of information and have been reported in studies of both human and non-human animals (Baek, 2016; Baruch et al., 2004; Bernardo et al., 2020; Cassilhas et al., 2007; Dao et al., 2013; Hopkins et al., 2012; O'Leary et al., 2019).

5.1. Clinical Research

The effects of regular exercise on memory retention has been observed in younger adult populations (18-36 years of age), where participants who exercised for 4 weeks had enhanced object recognition than those who did not exercise regularly (aka who exercised

intermittently) (Hopkins et al., 2012). Exercise has been found to enhances the cognitive function of elderly people on a variety of tasks: including the Wechsler Adult Intelligence Scale III (which assesses STM), Wechsler Memory Scale–Revised and Toulouse–Pieron's concentration attention test (both of which assess long-term memory (LTM)) (Cassilhas et al., 2007). Exercise is also believed to have a protective effect against AD, which is a neurodegenerative disorder that is characterized by memory loss and typically affects people over the age of 65 (Larson et al., 2006; Radak et al., 2010; Scarmeas et al., 2009). The effects of exercise enhancing learning and memory reported in clinical research have been replicated in non-human animal models.

5.2. Non-Human Animal Models

Exercise has been observed to induce neurogenesis (O'Leary et al., 2019). Following voluntary exercise (via access to a running wheel), rats were found to have a greater freezing response when returned to the context in which they were trained via tone-cued foot shock, but not to the tone itself (Baruch et al., 2004). Similar results have also been observed in adult rats, where adult rats which voluntary exercised were found to freeze more than adult rats which did not voluntarily exercise, in response to both the context alone and the tone alone (O'Leary et al., 2019). Interestingly, adolescent rats did not exhibit this enhanced freezing behaviour (O'Leary et al., 2019). In rat models of AD, physical exercise has been found to improve performance compared to AD rats that do not exercise on spatial tasks such as Morris Water Task (MWT) and Radial Arm Maze (RAM) (Bernardo et al., 2020; Dao et al., 2013). These enhancements in learning and memory following exercise between clinical and non-human animal research are believed

to be promoted by the exercise-induced alterations within the HPC (Baek, 2016; O'Leary et al., 2019).

6. Network Alterations that Disrupt Learning and Memory Networks

There are alterations which can occur to learning and memory networks which have resulted in impairments of either one or both acquisition and/or retention of information. These can include AD, stroke and TBI (traumatic brain injury), and impairments have been reported in both studies of human and non-human animals.

6.1. Clinical Research

As mentioned previously, AD is one example whereby alterations to the neural network (neurodegeneration) result in humans experiencing a decline in cognitive ability such as learning and memory (Larson et al., 2006; Radak et al., 2010; Scarmeas et al., 2009). One study found that fear conditioning in patients with AD, where a visual stimulus was paired with an aversive loud noise, was impaired relative to matched controls (Hamann et al., 2002). Another type of alteration that results in people experiencing learning and memory impairments are through strokes; which is when neurons dies as a result of blood loss, either because of blockages that restrict blood flow or a ruptured artery (Johnson et al., 2016). Research has shown that strokes causing damage within the HPC resulted in patients experiencing RA of both autobiographical memories and general information (ex. facts) learned prior to the stroke (Batchelor et al., 2008). Furthermore, research has found that patients who had an ischemic stroke had lower mean scores across memory tests (subscales of the Wechsler Memory Scale-III

(WMS-III), digit span from the Wechsler Adult Intelligence Scale-R (WAIS-R), and the Corsi span test than controls, and further investigation revealed that visual LTM and visual short-term memory (STM) were the most affected (Karimian et al., 2018). A third type of alteration is TBI (traumatic brain injury), where patients who have experienced a TBI also experience learning and memory impairments (Sahyouni et al., 2017). People with concussion history have longer reaction time relative to controls on a letter variant *n*-back task, indicating that have impaired working memory abilities (Ozen et al., 2013). Additionally, loss of consciousness at the time of the concussion incident can be used as an predictor of later visual working memory (Arciniega et al., 2020). The effects of alterations disrupting learning and memory reported in clinical research have been replicated in non-human animal models.

6.2. Non-Human Animal Research

As explained previously, rodent models of AD consistently reveal cognitive impairments (Bernardo et al., 2020; Dao et al., 2013). In a rat model of ischemic strokes, rats which had a stroke had impaired novel object recognition (NOR) discrimination relative to controls (Pan et al., 2017). Recalling that exercise has been found to enhance learning and memory (Baek, 2016), it is interesting to note that within the rats that had a stroke, exercise was reported to improve NOR discrimination indexes compared to rats that did not exercise (Pan et al., 2017). Mild TBI in rats has been found to impair acquisition of the hidden platform location in the MWT in both male and female rats, despite only female mild TBI rats experiencing a reduction of neurogenesis within the HPC (Wirth et al., 2017). Another method of alteration is by using irradiation to inhibit

neurogenesis, as following a CFC task irradiation-treated-rats freeze less than controls when returned to context (Wojtowicz et al., 2008). However, if rats were trained with tone-conditioned fear (tone co-terminating with a foot shock), there is no difference in freezing behaviour. Voluntary exercise (access to a running wheel) did not rescue this amnesic effect following neurogenesis inhibition (Wojtowicz et al., 2008). Clinical and non-human animal finding taken together suggests that alterations can be induced to a memory network which disrupt learning and memory, and it is interesting that methods which have shown to enhance learning and memory (exercise) are not always capable of preventing or rescuing these deficits.

7. Impact of Synaptic Circuit Remodelling on Previous Memory

As discussed above, memories are not formed instantaneously but rather that neural circuits involved in the encoding and storage of a memory tend to undergo a period of continual remodelling that leads to stability of the memory. Furthermore, network alterations were discussed to present how they can impact (enhance or disrupt) functionality in the ability to acquire and retain subsequent information after the alterations have occurred. These methods largely assess the effects of alterations resulting in anterograde amnesia (AA), the inability to acquire and/or retain information learned after changes to a network (Elliott et al., 2014; Mayes, 2015; Spiers et al., 2001; Squire & Alvarez, 1995). However, it is possible for remodelling to occur after a learning episode has been completed. These changes could result in the inability to retain information which was learned prior to the network alterations, a phenomena termed RA (Elliott et al., 2014; Mayes, 2015; Spiers et al., 2001; Squire & Alvarez, 1995). This raises an

important question: What impact would change to the synaptic architecture of memory networks have on the retrieval of previously acquired memory? Seizures are another method, not yet described, which also alter neural networks (Stafstrom & Carmant, 2015). Within the current experiments (Chapters 2 and 3), seizures will be the method used to induce network alterations, therefore the alterations often incurred, and the resulting behavioural changes are described in detail below.

8. Epilepsy Overview

Epilepsy is a neurological disorder that is characterized by spontaneous recurrent seizures: which are defined as unpredictable synchronous firing of multiple neurons at the same time which disrupt normal brain function (Fisher et al., 2005; Stafstrom & Carmant, 2015). According to the World Health Organization (2019), epilepsy is one of the most common neurological disorders affecting approximately 50 million people of all ages worldwide. Between the years of 2010-2012 roughly 139, 200 people had a diagnosis of epilepsy in Canada (Gilmour et al., 2016). People of lower socioeconomic status have a higher incidence rate of epilepsy diagnoses, and people with epilepsy are also at increased risk of premature death (Duncan et al., 2006; Gilmour et al., 2016; World Health Organization, 2019). Although seizures are considered to be the most debilitating aspect of the disease, it should be recognized that people who have epilepsy also report having comorbidities such as anxiety, depression, psychosis and learning and memory impairments – of which can be just as impactful to the individual's quality of life (Gilmour et al., 2016; Hermann et al., 2000 McConnell & Synder, 1998). The acquired causes of epilepsy can be explained in approximately 40-50 % of patients (Gilmour et al.,

2016; Schmidt & Sillanpää, 2016): they include stroke, TBI, encephalitis, tumors, and genetic factors (Duncan et al., 2006; Schmidt & Sillanpää, 2016; Stafstrom & Carmant, 2015). Since seizures that human patients with epilepsy experience can be caused by a variety of factors, it is not surprising that the age of onset can occur at any point across the lifespan (Gilmour et al., 2016; Hermann et al., 2000; Schmidt & Sillanpää, 2016).

The types of seizures that people experience can be categorized into epileptic seizure types. The first two broad categorizations are between focal versus generalized seizures (Duncan et al., 2006; Engel, 2006; Stafstrom & Carmant, 2015). Focal seizures are those which are localized to one area of the brain (i.e. one lobe, or one hemisphere), whereas generalized seizures typically occur throughout the cortex (Duncan et al., 2006; Stafstrom & Carmant, 2015).

Focal seizures will have varying effects depending on the region in which the seizure is occurring (Stafstrom & Carmant, 2015). For example, if the seizure originates in the occipital lobe, a person might exhibit visual phenomena (Stafstrom & Carmant, 2015). Focal seizures used to be further subdivided into "simple partial seizures" (intact consciousness) and "complex partial seizures" (impaired consciousness) (Berg et al., 2010), however, these terms have been since eliminated and focal seizures and now described in terms of dyscognitive impairments (which still includes the concepts of consciousness as well as awareness or responsivity to stimuli) (Berg et al., 2010; Stafstrom & Carmant, 2015).

In some cases, focal seizures can result in generalized seizures (Berg et al., 2010; Duncan et al., 2006; Engel, 2006). This has been commonly termed "secondarily generalized seizure" (Berg et al., 2010; Engel, 2006), however it has been argued that a

focal seizure "evolving to a bilateral, convulsive seizure" is a more appropriate expression (Berg et al., 2010). In addition to this, generalized seizures are also subdivided into other types of seizures (Berg et al., 2010; Duncan et al., 2006; Engel, 2006; Stafstrom & Carmant, 2015), the most frequent subdivisions are as follows. Absence seizures (formerly call petit mal seizures) appear as though the person has "spaced out" because they stare into space/nothing and are unresponsive to verbal stimuli – sometimes people will also experience eye blinking and/or head nodding (Berg et al., 2010; Duncan et al., 2006; Engel, 2006; Stafstrom & Carmant, 2015). These absence seizures are also classified as typical (where there is little or no cognitive impairment) or atypical (where there is severe cognitive impairment) (Velazquez et al., 2007). Atonic seizures involves the relaxation of muscles throughout the body, which often results in the person's head dropping or the person completely falling (Berg et al., 2010; Duncan et al., 2006; Engel, 2006; Stafstrom & Carmant, 2015). Myoclonic seizures are multiple jerks that are sudden, brief movements which appear to occur involuntarily (without conscious thought), and can affect one or several muscles (Berg et al., 2010; Duncan et al., 2006; Engel, 2006; Stafstrom & Carmant, 2015). Myoclonic seizures are not associated with any obvious changes to consciousness (Stafstrom & Carmant, 2015). Clonic seizures are also jerking of muscles throughout the body, but they are stronger jerks which are longer lasting and can result in the person falling over (Berg et al., 2010; Engel, 2006). Tonic seizures involve the stiffening of muscles throughout the body which can also result in the person falling over (Berg et al., 2010; Duncan et al., 2006; Engel, 2006). Tonic-clonic seizures (formerly called grand mal seizures) are the stiffening of muscles followed immediately by the jerking of muscles which result in the person falling over (Berg et al.,

2010; Duncan et al., 2006; Engel, 2006; Stafstrom & Carmant, 2015). Clonic, tonic and tonic-clonic seizures are all associated with obvious impairments to consciousness (Stafstrom & Carmant, 2015).

The third broad categorization of epileptic seizures is status epilepticus (SE) (Engel, 2006). Both focal and generalized seizures are typically brief in durations as they last only a few minutes before spontaneously resolving (Betjemann & Lowenstein, 2015; Lowenstein, 1998), whereas when a person experiences a convulsive seizure that persists for approximately 30 minutes or longer they are considered to be experiencing SE (Betjemann & Lowenstein, 2015; Lowenstein, 1998; Lowenstein et al., 1999). SE will not spontaneously resolve, and medical intervention (anti-convulsant medication administration) must take place to prevent death and other serious side effects (Betjemann & Lowenstein, 2015; Lowenstein, 1998; Lowenstein et al., 1999).

Seizure experiences and/or development of epilepsy can occur at any age (Berg et al., 2010; Lah et al., 2006). Patients are typically diagnosed as having a seizure experience through an EEG (electroencephalogram) or fMRI (functional magnetic resonance imaging) (Duncan et al., 2006; Fisher et al., 2005; Maydell et al., 2001; Stafstrom & Carmant, 2015). An EEG is used to record electrical activity within the brain (S. J. M. Smith, 2005). It can detect interictal spikes (and/or sharp waves) which reveal cumulative excitatory and inhibitory postsynaptic potentials that are associated with the synchronise firing of neurons – indicating seizure activity (Pillai & Sperling, 2006). An fMRI can be used to assess levels of blood oxygenation throughout the brain to determine where a seizure propagates, and where it spreads to other areas of the brain (Bookheimer,

1996; Stafstrom & Carmant, 2015). Increasingly, EEG and fMRI are used in conjunction to assess seizure activity (Bénar et al., 2003; Gotman, 2008; Stafstrom & Carmant, 2015).

Upon confirmation that seizures are occurring, the focus will shift to determining potential acquired causes. Investigations can be performed through CT (computerized tomography) and MRI (magnetic resonance imaging) (Duncan et al., 2006; Maydell et al., 2001; Stafstrom & Carmant, 2015). Both CT and/or MRI can be used to determine if there are structural abnormalities within the brain that are causing the seizures to occur (Stafstrom & Carmant, 2015). A CT scan can reveal hemorrhage, calcification, tumor, malformation, and lesions (Roy & Pandit, 2011; Stafstrom & Carmant, 2015). The MRI is also able to detect the same abnormalities as a CT, however, it is actually the preferred method as it is more sensitive to detecting these potential causes (Roy & Pandit, 2011; Stafstrom & Carmant, 2015) and can also be used to monitor treatment outcomes within these patients (Rüber et al., 2018).

Treatment of epilepsy varies depending on the patient and their circumstance. There are currently over 20 antiepileptic drugs (AED) that can be given to patients to supress or prevent the recurrence of seizure episodes (Duncan et al., 2006). Despite the efficacy of convulsant interventions, approximately 30% of all patients exhibit drug refractory epilepsy and continue to experience seizures (Duncan et al., 2006; Stafstrom & Carmant, 2015). Patients can also attempt other forms of treatment such as: resection of the affected brain area, vagal nerve stimulation, or changing their diet to a ketogenic diet (high fat, low protein, low carbohydrate) (Bookheimer, 1996; Duncan et al., 2006; Maydell et al., 2001; Stafstrom & Carmant, 2015).

9. Temporal Lobe Epilepsy

According to the National Institute of Neurological Disorders and Stroke (2020), TLE is the most common diagnosed form of epilepsy. Seizures which occur within the temporal lobe of patients with TLE result in alterations to structures within this network. Two structures within the temporal lobe that are impacted by TLE are the HPC and the amygdala (Fisher et al., 1998). Hippocampal sclerosis, which is characterized by tissue loss/damage often reported in the CA1, CA3 (Berkovic et al., 1990; Fisher et al., 1998; Stafstrom & Carmant, 2015), is commonly reported in people with TLE (Berkovic et al., 1990; Fisher et al., 1998; Stafstrom & Carmant, 2015), and there have also been reports of decreased volume of the amygdala in patients with TLE (Fisher et al., 1998).

As mentioned previously, focal seizures will have varying overt symptoms/seizure behaviour depending on the region of the brain that the seizures occur (Stafstrom & Carmant, 2015). Since the temporal lobe is comprised of structures associated with cognition and emotionality, such as the HPC and the amygdala (Fendt & Fanselow, 1999; Murray & Bussey, 2001), it is not surprising that a seizure which occurs within the temporal lobe will cause people to experience unresponsiveness during the seizure and amnesia of the seizure afterwards (Blumenfeld et al., 2004). Following seizures, people with TLE experience learning and memory impairments (G. L. Holmes, 2015; Tailby et al., 2018).

9.1. Difficulties in Examining Learning, AA, and RA in TLE Patients

To be diagnosed with TLE, patients must have experienced seizures (Fisher et al., 2005; Stafstrom & Carmant, 2015). This means that neural networks are already in an

altered state prior to any measures of cognitive function taking place (Biton et al., 1990; Ohira et al., 2019). It is easy to compare learning between TLE patients and controls because you can assess differences while the learning experience is taking place. However, when it comes to retention of this information that is learned, it is difficult to determine if the difference is truly a result of AA or RA. It is possible for patients to have experienced seizures between testing, since the seizures are unpredictable and unprovoked. (Fisher et al., 2005; Stafstrom & Carmant, 2015). If there has been a seizure occurrence between the time of learning and retention testing, then any difference in performance could be a result of RA rather than of AA (Elliott et al., 2014; Lehmann et al., 2007; Mayes, 2015; Spiers et al., 2001; Squire & Alvarez, 1995a, 1995b). On the other hand, the same can be said about interpreting the results as RA research. Since learning in TLE patients occurs in a network that has already been altered by seizures, even if a seizure occurred between the time of learning and retention testing, one cannot say for certain that the results are indicative of RA alone (Elliott et al., 2014; Lehmann et al., 2007; Mayes, 2015; Spiers et al., 2001; Squire & Alvarez, 1995a, 1995b). Therefore, researchers can easily examine for differences in initial learning between TLE patients and controls, however, examining the effects of network changes inducing AA and RA can pose a challenge.

9.2. Learning and AA in TLE

Assessing learning between patients with TLE and controls is relatively simple as measures of performance during the learning task will provide sufficient evidence of deficits (Bell, 2012). Patients with TLE have been reported to have spatial learning

impairments as they were found to make more errors when learning a route through a building than a non-epileptic control sample (Bell, 2012). Since the measure occurs during the learning trail where no seizures have been experienced in that time, it is appropriate to conclude that differences in learning are a result of the seizure-induced altered network that TLE patients have (Bell, 2012). Additionally, when researchers try to examine for potential AA, they perform retention testing relatively soon after the learning takes place (minutes or hours) as to avoid seizure occurrence between learning testing (Helmstaedter et al., 1991). For example, participants will be asked to remember random symbols, during immediate recall of reconstructing the random symbols with the images no longer in front of them, and TLE patients exhibited retention deficits as they made more errors in their drawings relative to non-epileptic controls (Helmstaedter et al., 1991).

9.3. RA in TLE Patients

In respect to RA researchers will test patients with TLE regarding information that is believed to have been learned prior to them experiencing seizures or throughout their lives (Lah et al., 2006). For example, some researchers found that left TLE patients have impairments recalling facts about public material (ex. famous people and events) and autobiographical memories relative to controls, whereas people with right TLE will have impairments only in autobiographical memory content (Lah et al., 2006). Other researchers have also found that patients with TLE have been reported to have deficits in autobiographical episodic memory (details), but have intact autobiographical semantic memory (gist like, facts) relative to controls (Viskontas et al., 2000). This method of

examining for RA can still pose the problem as knowing exactly when seizure onset occurred relative to the learning experiences can remain unknown.

One way of assessing RA is through accelerated forgetting. Accelerated forgetting occurs when the forgetting of a memory over time in which the rate of decay following learning is greater over days or weeks relative to another group (i.e. controls) (Butler & Zeman, 2008). For example, patients with TLE will demonstrate accelerated forgetting during cued recall of verbal and visuospatial material when tested overtime: comparing 30 seconds, 10 minutes, 1 day and 1 week retention after the learning experience took place, as well as comparing retention 30 minutes versus 4 weeks after the learning experience took place (Cassel et al., 2016; Narayanan et al., 2012). Patients will also experience accelerated forgetting of autobiographical event memory information when comparing retention 30 minutes versus 4 weeks after the learning experience took place (Narayanan et al., 2012).

In the study conducted by Cassel et al. (2016), where participants conducted retention tests at 1-day and 1-week intervals, five of the thirteen participants were reported to have experienced seizures between the learning and retention tests: two during the 1-day interval and all five during the 1-week interval. Although not all participants experienced a seizure after the learning experience, all patients with TLE forgot the information faster than controls (Cassel et al., 2016). This further emphasizes how challenging it can be to determine if these amnesic effects are a result of RA or AA, as the group which did not experience seizures between training and testing still exhibited a deficit relative to controls, indicating AA, yet one could still argue that those who did experience a seizure are experiencing RA.

In the study conducted by Narayanan et al. (2012), where patients were assessed 4 weeks after learning, researchers report the mean number of partial seizures per month as 5 and generalised seizures as 3-5 per year in the TLE patients. However, it is not clear whether this data was collected between the learning and testing interval or in the months/years leading up to participation in the research study (Narayanan et al., 2012). One might assume that TLE patients experienced seizures during this 4-week inter-test period, but without overt explanation of this, it remains difficult to confirm that the results are in fact representative of RA.

Another example can be seen where patients with TLE were found to exhibit accelerated forgetting between a 30-minute and 3-week interval from learning relative to controls and to people with generalised epilepsy (where the location of seizure propagation is not known) (Muhlert et al., 2011). However, people with TLE did not exhibit accelerated forgetting relative to controls and generalized seizure groups between a 40-second to 30-minute interval – this is not entirely surprising as people with TLE are reported to have adequate learning and retention immediately and shortly after, but it is over time that the forgetting is accelerated (Muhlert et al., 2011). In this study, it was reported that seven of the fourteen participants with TLE reported experiencing seizures between the 30-minute and 3-week testing interval (Muhlert et al., 2011), but that these participants did not differ in their performance relative to those who did not experience seizures. These results also highlight the difficulty in separating the degree to which this effect is a result of AA or RA.

10. Non-Human Animal Models of TLE and Fear Learning and Memory Deficits

The results of clinical research present some concerns in terms of replicability and generalisation. Even with the clinical methods attempting to examine specifically AA or RA effects of seizures on learning and memory, by nature of the disorder, participants with epilepsy already have neural networks that have been altered by seizures (Biton et al., 1990; Ohira et al., 2019), making it difficult to disentangle whether the effects can truly be concluded as AA and/or RA. Furthermore, it is impossible to have a truly homogenous sample of human participants with epilepsy (Scheffer & Berkovic, 2003) due to factors such as the varied age of onset, lifestyle, etc. (Biton et al., 1990; Ohira et al., 2019). Therefore, it is important to have non-human animal models of epilepsy that can allow us to examine the effects of seizures on fear learning and memory. Since information pertaining to fearful events is so critical to survival, it is important that these memories remain intact throughout the lifetime. The possibility that these memories can become lost following seizures is maladaptive to the organism. We need to understand the impact seizures can have on fear memories as to determine ways to prevent or minimize the impact of network alterations from inducing memory loss.

10.1. Recurrent Seizures Model of TLE

Kindling refers to the repeated intermittent administration of a chemical or electrical stimuli at a sub-convulsive threshold, whereby the seizures experienced are progressive across stimuli administrations (Dhir, 2012). Two models of chronic recurrent seizures are the chemoconvulsant pentylenetetrazol (PTZ) and electrical stimulation of the amygdala (Dhir, 2012; Hannesson & Corcoran, 2000). Both models of kindling can

induce synapse formation and reorganization, mossy fibre sprouting and hippocampal sclerosis (Cavazos et al., 1994; Samokhina & Samokhin, 2018), which occur within humans with TLE (Samokhina & Samokhin, 2018). PTZ kindling can occur over 7-20 or more treatments of intraperitoneal injections, typically every other day (Dhir, 2012; Ergül Erkeç, 2015; Samokhina & Samokhin, 2018). Rodents will experience a seizure for a few minutes before returning to baseline, without the need for intervention to stop the seizure (Dhir, 2012). Rodents treated with PTZ will experience seizures (ie. clonus, tonus, tonic-clonic seizures) in response to a challenge dose, weeks and/or months after the termination of kindling, as well as greater seizure score compared to control rats that were not kindled but receive a challenge dose (Becker et al., 1997; Corda et al., 1991; Mortazavi et al., 2005). Amygdala kindling can be induced in both a conventional (stimulation every day or every other day) or rapid method (multiple stimulations each day, across consecutive days) (Goddard et al., 1969; Lothman et al., 1985). Amygdala kindling also results in seizures that persist for a few minutes, and do not require intervention to stop them (Goddard et al., 1969; Lothman et al., 1985). Both PTZ kindling and amygdala kindling can result in spontaneous recurrent seizures SRS (Brandt et al., 2004; Hannesson & Corcoran, 2000; Samokhina & Samokhin, 2018).

Following PTZ kindling, increased neurogenesis within the DG, mossy fibre sprouting, synapse formation and reorganization as well as decreases in number of functional neurons within the CA1 and DG occurs (Mortazavi et al., 2005; Samokhina & Samokhin, 2018). Similar results have also been reported where there is a decrease in the number of neurons within the CA1 of rats that received either 11, 14 or 21 PTZ injections (Mortazavi et al., 2005; Pavlova et al., 2006), and decreased number of neurons within

the DG of rats that received 21 PTZ injections (Pavlova et al., 2006). Additionally, there have been reports of damaged neurons throughout the HPC (CA1, CA2, CA3, CA4, DG) following PTZ kindling (Franke & Kittner, 2001; Vasilev et al., 2018), and the resultant hippocampal damage showed a weak but statistically significant correlation with the number of clonic seizures experienced by the rats (Franke & Kittner, 2001). There is also evidence of damaged neurons in the basolateral amygdala (BLA) after PTZ, as well as in the basomedial nucleus, and some damaged neurons in the central amygdala (CeA), as well as the medial nucleus following PTZ kindling (Franke & Kittner, 2001). After amygdala kindling, there is apparent neurogenesis of DGCs (dentate granular cells) (Fournier et al., 2013; Parent et al., 1998; Scott et al., 1998) and mossy fibre sprouting that occurs (Cavazos et al., 1991; Ebert & Löscher, 1995; Osawa et al., 2001). Additionally, there is evidence of neuronal loss within different areas of HPC depending on the number of tonic-clonic seizures: CA1 and hilus after 3 seizures; CA3 after 30 seizures as well as the entorhinal cortex and the rostra1 endopyriform nucleus; CA2 after 150 seizures (Cavazos et al., 1994).

Following both PTZ and amygdala kindling, rodents have apparent learning and memory deficits in both fear and spatial memory tasks. After PTZ kindling, kindled rats have a decreased freezing response to foot shocks during CFC training and freeze less during a 24 hour retention test than controls (Szyndler et al., 2002). Also, kindled rats are able to learn an active avoidance task, but have impaired retention a week later as they no longer avoided the aversive compartment (Genkova-Papazova & Lazarova-Bakarova, 1995). Complementary findings show that PTZ-kindled mice require more trials to learn active avoidance than controls regardless of age (2 month, 6 months and 12 months of

age) (Mishra & Goel, 2012). Furthermore, after PTZ kindling, rats are able to locate a hidden platform comparably to controls across training trails during MWT, but spent less time in the target quadrant during the probe (Lamberty & Klitgaard, 2000). Additionally PTZ kindled rats have impairments in Radial Arm Water Maze (RAWM), as kindled rats make more errors than non-kindled rats (Mortazavi et al., 2005). Following amygdala kindling, freezing response to foot shock during training is equivalent to controls (Botterill et al., 2014; Fournier et al., 2013). However, decreased freezing response occurs in kindled rats during presence of the tone in a novel context and to the context alone (without the tone) compared to controls (Botterill et al., 2014; Fournier et al., 2013). Furthermore, amygdala kindled rats have no impairments in ability to learn platform location in MWT, despite kindled rats experiencing decreased motor activity during open field testing (Nieminen et al., 1992).

10.2. SE Model of TLE

Two widely used models of TLE via induction of SE in rodents are pilocarpine and KA (kainic acid) (Polli et al., 2014). They are often administered through single convulsive doses via intraperitoneal or intra-hippocampal injections (Polli et al., 2014). Both methods will result synapse formation and reorganization, hippocampal neuronal loss, synaptic reorganization and mesial temporal lobe sclerosis (Polli et al., 2014), all of which occur in humans with TLE. Pilocarpine induced SE persists typically for 30-120 minutes before intervention occurs to stop the seizures (Curia et al., 2008; Smolensky et al., 2019), and animals will develop spontaneous recurrent seizures (SRS) in the following weeks (Cavalheiro et al., 1991; Curia et al., 2008). Tissue analysis typically

reveals mossy fibre sprouting and sclerosis of the HPC (CA1, CA2, CA3, and Hilus) following pilocarpine induced SE (Curia et al., 2008; Polli et al., 2014). When SE is induced via KA, the seizures will persist for hours, and typically around 4 or 5 hours is when intervention will take place to stop the seizures (Cavalheiro et al., 1982; Zhang et al., 2002). Similarly to pilocarpine, SRS will occur in the following weeks after KA induced SE (Cavalheiro et al., 1982; Maia et al., 2014; Polli et al., 2014) – however, SRS occurs more frequently observed in the pilocarpine model than the KA model (Polli et al., 2014). Tissue analysis after KA induced SE will typically reveal mossy fibre sprouting and sclerosis of the HPC (CA1, CA3 and Hilus) (Polli et al., 2014; Zhang et al., 2002). Astrogliosis within the amygdala and HPC, as well as in the uncus, para- hippocampal gyrus and entorhinal cortex, following pilocarpine or KA treatment has also been observed to occur (Samokhina & Samokhin, 2018).

Alterations to these network are believed to cause learning and memory deficits (Fournier & Persinger, 2004). Following pilocarpine treatment, mice have been reported to have comparable learning (freezing response to foot shocks) and comparable freezing during retention tests 24 or 48 hours later relative to control mice, whereas during a 2 week retention test pilocarpine treated mice freeze less than controls (Zhang et al., 2010). Similar results have been reported where pilocarpine treated rats trained more than 7 weeks after treatment will rats will display decreased freezing relative to controls during a 24 hour retention test of tone-conditioned fear (tone co-terminating with shock during acquisition training) to both the context alone and the tone presented in a novel context (Smolensky et al., 2019). Following KA treatment, rats trained in tone-conditioned fear

conditioning 5 months after treatment will have decreased freezing during a 24 hour retention test relative to controls (Maia et al., 2014; Smolensky et al., 2019).

There have also been impairments in spatial learning and memory tasks following SE. After KA treatment, rats swam greater distances to locate the platform during MWT acquisition, and had a decrease in both the time spent in the quadrant and in the number of crossings in the former platform location during the probe trail (Maia et al., 2014). After pilocarpine treatment, rats also swim greater distances to locate the platform on the first day of training (Smolensky et al., 2019). Upon subsequent training sessions pilocarpine treated rats had greater swim distances during the first trial of training but had comparable swim distances relative to controls by the last trail (Smolensky et al., 2019). This pattern repeated, as when pilocarpine treated rats were returned to the pool for two more days of training, they swam greater distances during the first trials relative to controls and had comparable distances by the last trial – indicating ability to learn the platform location, but impairments in retention of the platform location (Smolensky et al., 2019).

10.3. Advantages and Disadvantages of Recurrent Seizures and SE Models

The first difference across the methods described above are those which require surgery (i.e. electrode or canula placement) and those which do not (i.e. intraperitoneal injection administration) (Dhir, 2012; Hannesson & Corcoran, 2000; Polli et al., 2014) versus. The benefit of using a method which consists of intraperitoneal injections is that there is no required post-operative time from surgery nor the risk of mortality during surgery (Dhir, 2012; Hannesson & Corcoran, 2000; Polli et al., 2014). The second

difference across methods is that SE can be achieved in a single treatment or in a single day (Polli et al., 2014), whereas recurrent seizures occur across days or weeks (Dhir, 2012; Ergül Erkeç, 2015; Samokhina & Samokhin, 2018). Although having treatment occur on a single day is both convenient and reduces the number of injections the rodents receive, one main issue with the SE model is that mortality rate is higher (Curia et al., 2008). For example, pilocarpine has been reported to have a mortality rate around 30% (Curia et al., 2008), whereas PTZ mortality rates usually fall around 10% (Mortazavi et al., 2005; Szyndler et al., 2002). Furthermore, researchers will suggest PTZ recurrent seizures once daily as this method frequently results in low mortality rate (Dhir, 2012; G. L. Holmes et al., 1999; Samokhina & Samokhin, 2018). Therefore, for the current experiments, recurrent intraperitoneal administration of PTZ will be used to induce produce kindled seizures in rats.

11. Objectives and Hypotheses

To date, the available evidence suggests that alterations made to memory networks can have varying effects on subsequent learning and retention of fear memories, as well as other spatial memories (Botterill et al., 2014; Genkova-Papazova & Lazarova-Bakarova, 1995; Maia et al., 2014; Mishra & Goel, 2012; Smolensky et al., 2019; Szyndler et al., 2002; Zhang et al., 2010). However, there has been minimal research examining effects of network alterations on retention when the alterations have occurred after the learning and memory stabilization has been completed. Some research suggests that inducing seizures immediately after fear learning will result in impaired memory retention (Palfai & Albala, 1976; Perlman et al., 1961), and impairments have also been

observed in spatial memory tasks as well (Naik et al., 2021). However, this approach does not allow for the stabilization of the memory trace to occur prior to alterations being induced.

Overall, my thesis will address the impact of repeated seizures on the ability to retain previously acquired context fear memories in rats. Therefore, I have proposed two main objectives that will be addressed:

11.1. Objective 1

To test the effects of recurrent seizures on the degradation of a robust fear memory across time, as well as assess the potential alterations which may promote differences in memory degradation. To carry out this aim, we examined freezing behaviour between PTZ-treated and Saline-treated (control) rats during retention testing of a contextual fear memory across various time points; during and after PTZ kindling termination. PTZ-induced seizures can have varying impact on fear learning and retention (Genkova-Papazova & Lazarova-Bakarova, 1995; Mao et al., 2009; Mishra & Goel, 2012; Palfai & Albala, 1976; Szyndler et al., 2002). For example, an avoidance response (via delivery of a shock) to bar pressing for food reward falls victim to RA up to 4 days after training (Perlman et al., 1961). Therefore, the first hypothesis is that the kindled rats will experience an accelerated decline in freezing behaviour across retention tests relative to controls, despite both groups having comparable acquisition and retention prior to PTZ-treatment.

From here, we also examine how repeated seizures impact subsequent learning. We hypothesize that the kindled rats will have deficits in subsequent CFC learning and

retention, however, will be able to learn a platform location in the MWT relative to controls. Any differences in retention and/or subsequent learning could be promoted by the seizure-induced alterations (Becker et al., 1997; Franke & Kittner, 2001; G. L. Holmes et al., 1999; Mortazavi et al., 2005; Park et al., 2006; Pitkänen & Lukasiuk, 2009; Samokhina & Samokhin, 2018). It is possible that these alterations could result in volumetric differences between PTZ-kindled rats and controls. Therefore, we hypothesize that the kindled rats will have a decreased volume in the CA1 and CA3, as well as increased volume in the hilus and DG, relative to controls.

11.2. Objective 2

To test the effects of recurrent seizures on the retention of a previously acquired fear memory, as well as assess the potential functional alterations which may explain differences in memory retention. To carry out this aim, we examined freezing behaviour between PTZ-treated and Saline-treated (controls) rats during retention testing of a contextual fear memory following termination of PTZ kindling. Consistent with the reasoning of the previous objective, the first hypothesis of this objective is that the PTZ-treated rats would freeze less than controls during a post-kindling retention test.

Then, to examine differences in retention-induced protein expression which may facilitate differences in freezing behaviour, timed perfusions will occur following the retention test to assess these differences within the HPC and amygdala. Research has found that behavioural differences between kindled rats and controls during retention testing are accompanied by a reduction in cFos expression between groups (Botterill et

al., 2014). Therefore, we hypothesize that the kindles rats will have lower cFos expression than controls within the CA1, BLA and CeA.

11.3. Relevance

Humans with TLE experience multiple facets of learning and memory impairments: such as AA and RA (Bell, 2012; Cassel et al., 2016; Helmstaedter et al., 1991; Lah et al., 2006; Muhlert et al., 2011; Narayanan et al., 2012; Viskontas et al., 2000; Yang et al., 2012). Humans can experience seizures and/or develop epilepsy at any age (Berg et al., 2010; Lah et al., 2006), and it is important to assess how seizures impact memories acquired across the lifespan. Non-human animal research tends to focus on answering questions which pertain to the possibility of seizures inducing AA of various types of memory, such as fear memories and spatial memories (Botterill et al., 2014; Genkova-Papazova & Lazarova-Bakarova, 1995; Maia et al., 2014; Mishra & Goel, 2012; Smolensky et al., 2019; Szyndler et al., 2002; Zhang et al., 2010). Research needs to begin examining the other facets of learning and memory impairments, as well how functionality of these networks is affected which may promote these deficits. This research will begin to shed light on the potential accelerated forgetting and RA of fear memories following seizures, as well as the alterations which occur to support them. As such, it will begin to expand on current learning and memory research, allowing for further understanding of these impairments.

CHAPTER II
Seizures Induce Accelerated Decay of Fear Memory

1. Introduction

Epilepsy is characterized by recurrent seizures, which are characterized by the synchronous firing of neurons, which cause structural and functional alterations (Stafstrom & Carmant, 2015). Seizures have been found to promote unique patterns of synaptic loss that is accompanied by reactive remodelling of synaptic connections of preserved neural circuits (Becker et al., 1997; Fournier & Persinger, 2004). This process eventually leads to aberrant changes in network function that in turn produce changes in behaviour (Becker et al., 1997; Fournier & Persinger, 2004).

Pentylenetetrazol (PTZ), a GABA-A receptor antagonist, is a chemoconvulsant drug used to model TLE in rodents (Dhir, 2012; Samokhina & Samokhin, 2018). PTZ kindling refers to repeated intermittent administration of PTZ at a sub-convulsive threshold, whereby the seizures experienced are progressive across treatment administrations (Dhir, 2012; Samokhina & Samokhin, 2018). Following PTZ treatment, there are alterations within the HPC, which includes loss of neurons within the CA1 and CA3 subfields as well as in the hilus (Pavlova et al., 2006; Samokhina & Samokhin, 2018). The pattern of cell loss within the CA1 and CA3 observed after PTZ kindling resembles what is observed in patients with TLE that show hippocampal sclerosis (Becker et al., 1997; Franke & Kittner, 2001; Mortazavi et al., 2005; Pitkänen & Lukasiuk, 2009). In addition to cell loss, several studies have also observed increased neurogenesis in the DG along with increased mossy fiber sprouting from dentate granule

cells into the hilus (G. L. Holmes et al., 1999; Mortazavi et al., 2005; Park et al., 2006; Samokhina & Samokhin, 2018).

In addition to seizure-induced hippocampal changes, rats also experience significant learning and memory impairments. Rats trained in a CFC task following a single tonic-clonic PTZ induced seizure express a reduction in freezing behaviour when the rats are returned to the context at a later time, suggesting AA effects (Mao et al., 2009; Szyndler et al., 2002). While the effects of seizures on AA-related deficits have been well described, the impact of these events on previously acquired memories remains unclear. Therefore, the aim of this experiment was to examine the impact of repeated seizure stimulation on the ability to successfully retain previously acquired context fear memories in rats.

The most obvious way to assess this would be to train rats in a task and examine for differences during a retention test after seizures have occurred. However, another way this could be addressed would be through the examination of accelerated forgetting (Cassel et al., 2016; Martinez & Rigter, 1983). This method would require re-exposure to a learning environment (Alberini & Ledoux, 2013; Lee, 2009), which allows us to determine differences in the rate of decay between rats that have and have not experienced seizures. Without any reinforcement of the memory during re-exposure, behavioural expression of the memory would gradually decrease in controls (Myers & Davis, 2002). If the rate of decline is greater in rats that have experienced seizures, then it would indicate that differences can be attributed to the experience of seizures rather than any natural decay that would be expected. This method of measuring RA is of interest because it allows researchers to examine rates of forgetting relative to the progressive

seizures that occur during kindling, versus examining only the cumulative effects of kindled seizures on memory retention. To address this first aim, we examined the retention of a contextual fear memory at various time points during and after completion of PTZ kindling. A PTZ-induced seizure occurring immediately after acquisition can impact fear memory retention (Palfai & Albala, 1976; Perlman et al., 1961), and RA of an avoidance response to bar pressing task (via shock administered) can occur when the seizure occurs 4 days after learning (Perlman et al., 1961). Therefore, we hypothesize that the kindled rats will experience an accelerated loss of memory across retention tests relative to controls.

Following the last retention test there is the opportunity to examine potential AA effects as well. There is evidence to suggest deficits in retention of fear memories following PTZ seizures (Mao et al., 2009; Szyndler et al., 2002), whereas there is inconsistent evidence in respect to fear learning following PTZ (Genkova-Papazova & Lazarova-Bakarova, 1995; Mishra & Goel, 2012; Szyndler et al., 2002). Additionally, there are inconsistencies in the evidence pertaining to the abilities of learning spatial information following PTZ seizures (Lamberty & Klitgaard, 2000; Mao et al., 2009). Therefore, it is hypothesized that kindled rats will not have impairments in the acquisition of fear memories, but will have impaired retention. Additionally, it is hypothesized that kindled rats will have consistent learning of a platform location in the MWT relative to controls.

If an accelerated decline in retention and subsequent learning and retention deficits are observed, they could be promoted by alterations induced by the recurrent seizures. And since there are apparent changes in structure of the HPC after seizures

(Becker et al., 1997; Franke & Kittner, 2001; G. L. Holmes et al., 1999; Mortazavi et al., 2005; Park et al., 2006; Pitkänen & Lukasiuk, 2009; Samokhina & Samokhin, 2018), it is possible that these structural changes could be observed through volumetric measurements. Therefore, the final hypothesis is that kindled rats will have a decreased volume in the CA1 and CA3, as well as increased volume in the hilus and DG, relative to controls.

2. Materials and Method

Twenty-five male Sprague Dawley rats, weighing approximately 150-220 g, were obtained from Charles Rivers Laboratories (St. Constant, Quebec). The rats were housed in standard laboratory cages with access to tubes as environmental enrichment, in a temperature-controlled room (21°C ± 2°C) with lights on at 07:00 h and lights off at 1900 h. Behavioural testing was conducted during light phases of the cycle. Food and water were available *ad libitum* throughout the duration of the study. All procedures were approved by the Trent University Animal Care Committee and complied with the Canadian Council on Animal Care.

2.1. Behavioural Tests

2.1.1. Context Fear Conditioning

Context fear conditioning was conducted in two standard conditioning chambers (30 x 26 x 26 cm), which were housed in sound attenuating cabinets. Each conditioning chamber consisted of clear (front wall/door, ceiling) and opaque plexiglass (side and rear walls). The floor of the chambers consisted of 21 steel rods (2 mm in diameter, spaced 1

cm apart) that was connected to an adjustable shock generator/scrambler (Ugo Basile, Verese, Italy) for the delivery of the foot shock. The chamber was illuminated by a 2.5 W white LED light. All conditioning sessions were video recorded using a webcam placed above the conditioning chambers and connected to a laptop computer. Freezing behaviour, defined as an absence of all movement except for those required for respirations, served as an index of conditioning fear. The conditioning chambers were cleaned between rats using Oxivar cleaner.

The contextual fear conditioning procedure consisted of 3 separate training sessions separated by 24 hrs. For each training session, the rats were transported to the testing room via a white transfer bucket and placed into one of the two preassigned conditioning chambers. The same conditioning chamber was used for all training sessions and retention tests. During the training sessions, the rats were placed into the conditioning chamber and allowed to explore the chamber freely for 3 min. After this period, 5 foot shocks (1.2 mA, 2 s duration) separated by a 60 s interval were delivered (total test time 8 minutes). Figure 1 illustrates the experimental design.

2.1.2. Retention Tests

For the retention tests (the first 1 days after training, the second mid-way through PTZ treatment, the third 2 days after completion of PTZ-treatment, the last 18 days after the third PTZ treatment), each rat was returned to their respective conditioning chamber for 5 minutes and the percentage of time spent freezing was recorded using an automated scoring system (AnyMaze, Stoelting CO USA). To minimize potential olfactory cues, the chambers were cleaned between rats using Oxivar cleaning solution.

2.1.3. Novel Context Test

One day after the last CFC Retention Test, the rats were placed into modified conditioning chambers and allowed to freely explore the environment for 3 minutes. The features of the chambers were modified from the original conditioning chambers to generate a unique context. This was accomplished through the use of checkered walls along the sides and rear facing walls of the chamber, the inclusion of constant background (white) noise (65 dB), lemon scent, and having the doors of the cabinets left opened during testing. During testing the chambers were wiped down with 70% (v/v) ethanol. Following the 3-minute acclimation period, a single foot shock was delivered (1.2 mA, 2 s) and the rats remained in the chamber for 60 s before being retrieved and returned to their home cages. During the session the percentage of time spent freezing during the 3-minute period before the onset of the foot shock (pre-shock) and 60 s interval immediately following the foot shock (post-shock) was recorded.

Twenty-four hours later, the rats were returned to the modified context for a 5-minute retention test. No foot shocks were delivered during this period.

2.1.4. Morris Water Task

Training and testing were performed using a circular pool (diameter: 140 cm; height 60 cm) filled with water (21 °C ± 2 °C) made opaque with nontoxic liquid paint. The pool was virtually divided into four equivalent quadrants: north-east (NE), north-west (NW), south-east (SE), and south-west (SW). In the water task, rats learn to locate a submerged, and hence hidden platform located 2 cm below the surface. The escape platform was made from clear Plexiglas and measured 11 cm in diameter. The location of

the escape platform was placed in the middle of one quadrant (NE) equidistant form the sidewall and the center of the pool. The pool was surrounded by several distal visual cues (e.g., wall posters, a cabinet, shelves, décor, etc.) that were present during all trials. The water was skimmed after each trial.

All rats were tested at approximately the same time of day, and each received 12 trials delivered in a single session. The location of the platform remained constant for each trial. Each trial started by gently placing the rat into the water with its head facing the wall of the pool in one of the quadrants not containing the platform. The release quadrant for each trial was pseudorandomized for each rat. Once released, the rat was allowed 60 s to find the submerged platform. If a rat did not mount the platform within this time, it was gently guided on to the platform by the experimenter. Rats were permitted to remain on the platform for 5 s (after locating or being guided) before being removed and returned to the white transfer bucket. The commencement of the next trial began 60 s later. Twenty-four hours later, the rats were returned to the pool to conduct a probe trial (30 s trial, platform removed) and an escape trail (60 s, platform available) 60s later.

All trials were tracked automatically by a digital tracking system (AnyMaze, Stoelting, IL) Barcelona) measuring latency to escape from the water (sec), the distance travelled to locate the platform (m), swimming speed (m/sec), and the percentage of time spent in each quadrant during all trials.

2.2. Pentylenetetrazol Kindling

To induce kindling, pentylenetetrazol (PTZ, Sigma-Aldrich, Cat# P6500, St Louis, MO USA) was administered at an initially subconvulsant dose (35 mg/kg, 1 ml per kg, dissolved in 0.9% w/v saline) on alternate days for 15 days (8 treatments). After each injection of PTZ or saline, rats were observed in a padded chamber (24 cm x 48 cm x 24 cm) for 30 minutes. The intensity of seizure behaviour was evaluated using established methodology (Dhir, 2012): where, 0 = no seizure; 1 = myoclonic jerks and/or facial automatisms (i.e. chewing); 3 = Straub's tail (dorsiflexion of the tail); 6 = forelimb clonus; and 8 = tonic extension. If a rat failed to progress beyond stage 3 events after two consecutive treatments, the dosage of PTZ was increased to 37.5 mg/kg, 40 mg/kg, 43 mg/kg and then 45 mg/kg (the maximum dose that could be administered). Any rats exhibiting severe seizures with tonic-clonic extension that persisted for 30 minutes received a single treatment of diazepam (5 mg/kg, i.p.). Saline-treated rats never displayed behavioural seizures.

2.3. Perfusions and Tissue Preparation

Twenty-four to forty-eight hours after MWT, rats were deeply anesthetized with sodium pentobarbital (240 mg/ml, Schering Inc., Montreal, Quebec OR Merck Animal Health, Canada) and perfused intracardially with 200-250 ml physiological saline followed by 200 ml of paraformaldehyde (4% paraformaldehyde, 0.1 M PBS, pH 7.4). The brains were extracted and postfixed in the same fixative for 24 hrs at 4°C before undergoing a series of sucrose infiltration at increasing concentration (10%, 20%, 30% (w/v) sucrose in PBS containing 0.01% (w/v) sodium azide). The brains were then

embedded in optimal cutting medium (OCT, Fisher Scientific Canada) before sectioning on a cryostat at a thickness of 40 μm. Sections were collected and stored in a PBS solution containing 0.01% sodium azide at 4°C until further processing. The tissue was collected using a 1/12 interval and stored in a PBS solution (0.1 M PBS, 0.01% sodium azide) at 4°C until further processing.

Immunostaining was performed on free-floating coronal sections (1 in 12 series) with all rinses and incubations carried out under gentle agitation. Briefly, sections were washed 3x in PBS (10 min each) followed by treatment with 3% (w/v) H2O2 at room temperature for 30 minutes to minimize endogenous peroxidase activity. Following this, sections were washed 3x PBS (10 min each) before being placed for 1 hr at room temperature in a blocking solution comprised of 1% bovine serum albumin, 5% normal horse serum, and 0.3% Triton X-100 dissolved in PBS. After blocking, the sections were then treated with a primary anti-mouse NeuN monoclonal antibody (1:1000, 24 hrs, 4°C, Chemicon) diluted in the previously described blocking solution. The next day, sections were rinsed several times in PBS (3x, 5 min each) followed by incubation with a secondary biotinylated antibody (horse anti-mouse, 1:500, 2 h, room temperature, Vector Laboratories). Immunolabeled cells were visualized using 2.5% (w/v) nickel ammonium sulphate, 0.02% (w/v) diaminobenzidine (DAB) and 0.000083% hydrogen peroxide, and sodium acetate (0.175 M, pH = 6.8) to yield a blue-black product. After sufficient colouration (~8 min), the reaction was halted by washing the sections several times in sodium acetate (3x, 5 min each) followed by PBS rinses (3x, 5 min each). The sections were mounted onto SuperFrost Plus glass slides and left to air dry overnight. Slides were

dehydrated through a series of alcohols, cleared in xylene, and coverslipped with DPX (Sigma Aldrich) mounting medium.

2.4. Hippocampal Volume Estimate/Quantification

Hippocampal subfield volumes were measured by means of point counting method using the Cavalieri's principle (Mouton, 2002). Section images were captured at a 2X magnification by a Nikon DS-Qi1Mc camera (Nikon Instruments, USA) on a Nikon C-TAQ microscope (Nikon Instruments, USA). Images were then processed in Fiji ImageJ (Version 2.0.0-rc-69/1.52p) and volume estimation was performed by placing a grid of points (0.05 mm^2) randomly onto the images. The points that intersected on each hippocampal subfield (CA1, CA3, dentate gyrus, and hilus), subfields defined using a rat brain atlas (Paxinos and Waston, 2007), were summed for the respective subfield. The volume of each subfield was determined by applying the following formula:

$$\Sigma P \times SSF \times t \times a/p$$

Where *ΣP* represents the sum of total number of points, *SSF* is the section sampling fraction (12), *t* is the section thickness (40 um), and *a/p* is the area per point (0.05 mm^2).

2.5. Statistical Analysis

All statistical analyses were conducted using the software SPSS Statistics (Version 26 and Version 27). Comparison of CFC acquisition and pre-kindling retention were completed using a 2 x 3 mixed model ANOVA and an independent t-Test respectively. A one-way repeated measures ANOVA was used to determine the progression of seizure scores across treatments, and a paired t-Test was used to compare

the frequency of scores of 6 or 8 during the first half and last half of seizure treatment. The subsequent CFC Retention tests were assessed using a 2 x 3 repeated measures ANOVA, as well as accessing relative change between tests using a series of one-way ANOVAs. An independent t-Test was conducted to compare difference scores between freezing during the last CFC retention test and the pre-shock period of the novel context acquisition test. Examining differences in freezing during the novel context retention test was examined with an independent t-Test, and then followed up by covarying for the effect of pre-shock freezing during the novel context acquisition test. A 2 x 12 mixed model ANOVA assessed learning during the MWT acquisition. A series of independent t-Tests examined differences between groups on swim distance for both crossing the platform location during the probe trials and escaping onto the platform during the escape trial of MWT retention test. Lastly, independent t-Tests compared volume between groups in the whole HPC, as well as in the CA1, CA3, DG, and hilus. The criterion for statistical significance was set at $P < .05$.

3. Results

During the process of kindling, 2 out of 13 rats (15%) died and another rat failed to develop at or beyond a level 6 seizure score, the data from these rats were not included in any statistical analyses. The sample size was reduced to: saline, n=12; and PTZ, n=10.

Furthermore, during the MWT Retention Probe trial, the video camera did not accurately track the swim path of one rat during retention testing, therefore this rat was removed from MWT Retention Probe analyses. The sample size for these analyses was: saline, n = 11; and PTZ, n = 10.

Lastly, only a subset of tissue from each group was stained with NeuN and analysed to compare volume. This subset selection was a result of time constraints, and therefore consisted of rats only from the first cohort; each cohort underwent the experiment offset by one day. The sample size across tissue analysis was: saline, n = 5 and PTZ, n = 5.

3.1. Effect of PTZ Kindling on the Retention of Prior Fear Conditioning

All rats underwent three days of trace fear learning followed by a retention test prior to being assigned into saline or PTZ kindled groups (see Figure 1 for experimental design). As shown in Figure 2a, all rats showed an expected increase in defensive freezing responses over the course of training [$F(2,40) = 276.99$, $P < .001$]. No significant main effect of group ($F(1,20) = 2.97$, $P = .100$) or interaction ($F(2,40) = 0.14$, $P = .867$) was found, suggesting that both groups showed a similar learning pattern. Importantly, there were no differences in freezing levels between the groups during the first pre-kindling retention test [$t(20) = 0.13$, $P = .895$] (see Figure 2b).

Two days after undergoing the pre-kindling retention test, rats began treatment with PTZ or saline. All PTZ treated rats showed the characteristic increase in both seizure frequency and intensity [$F(1,9) = 6.58$, $P = .03$, Figure 3a] over the course of treatment. As expected, the cumulative frequency of seizure severity displays that rats experienced more motor convulsive events (stages 6 and 8) over the last four treatment sessions (treatments 5 through 8) than during the first four treatment sessions (treatment 1 through 4) ($t(9) = 3.354$, $P = .008$; see Figure 3b).

To examine the effect of repeated seizure activity on the ability to retrieve previously acquired fear information, we conducted additional retention tests during and after PTZ kindling. The first retention test after seizures began (Mid-kindling) was completed during the midway point of kindling (Day 11) while two additional post-kindling retention tests (Post-Kindling1 and Post-Kindling2) were completed at 2- and 20-days post-kindling (Day 20 and Day 38) (see Figure 1 for the experimental design). A repeated measures ANOVA with group (PTZ vs. saline) as the between subject factor and retention tests (mid-kindling, post-kindling1 and post-kindling2) as the within-subject factor was used to examine for group differences in freezing levels across the test sessions. There was a significant interaction between group and retention test [$F(2,40) = 9.15$, $P < .001$] as well as a significant main effects for group [$F(1,20) = 15.65$, $P < .001$] and retention test [$F(2,40) = 56.67$, $P < .001$]. As shown in Figure 4, while both PTZ and control groups showed a progressive reduction in freezing over repeated testing, levels of freezing were significantly lower for the PTZ group compared to the saline controls for the Post-Kindling1 retention test ($P < .001$) and Post-Kindling2 retention test ($P < .001$), but not the Mid-Kindling retention test ($P = .158$).

Since the PTZ and saline control groups showed a general pattern of reduced freezing over repeated testing sessions, a phenomenon that could reflect extinction or retrograde amnesia, we computed the relative change in freezing across each retention test. The results of series of one-way ANOVAs showed that while the relative change in freezing from retention tests 1 and 2 was comparable between both groups ($F(1,20) = 2.18$, $P = .155$), the PTZ kindled rats exhibited a greater decrease in freezing from retention tests 2 and 3 than the saline controls [$F(1,21) = 12.14$, $P < .002$]. However, this

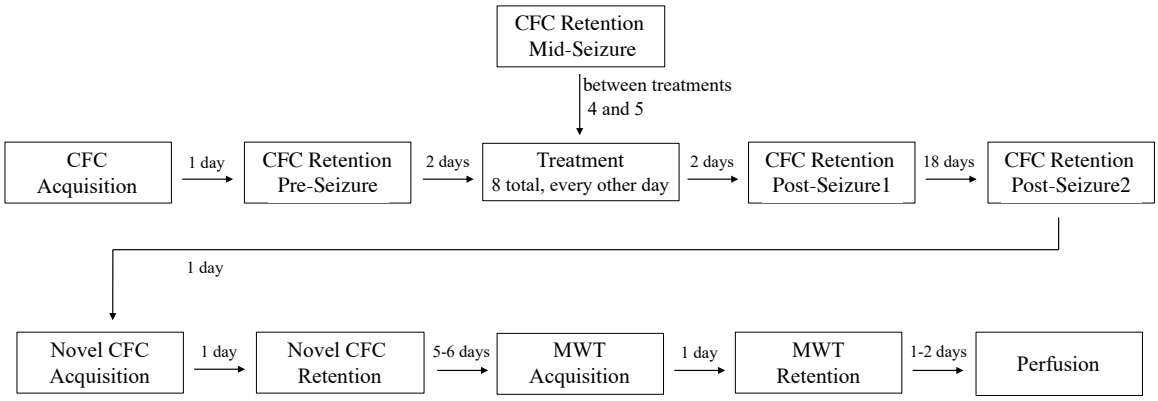


Figure 1. Illustration of the experimental design.

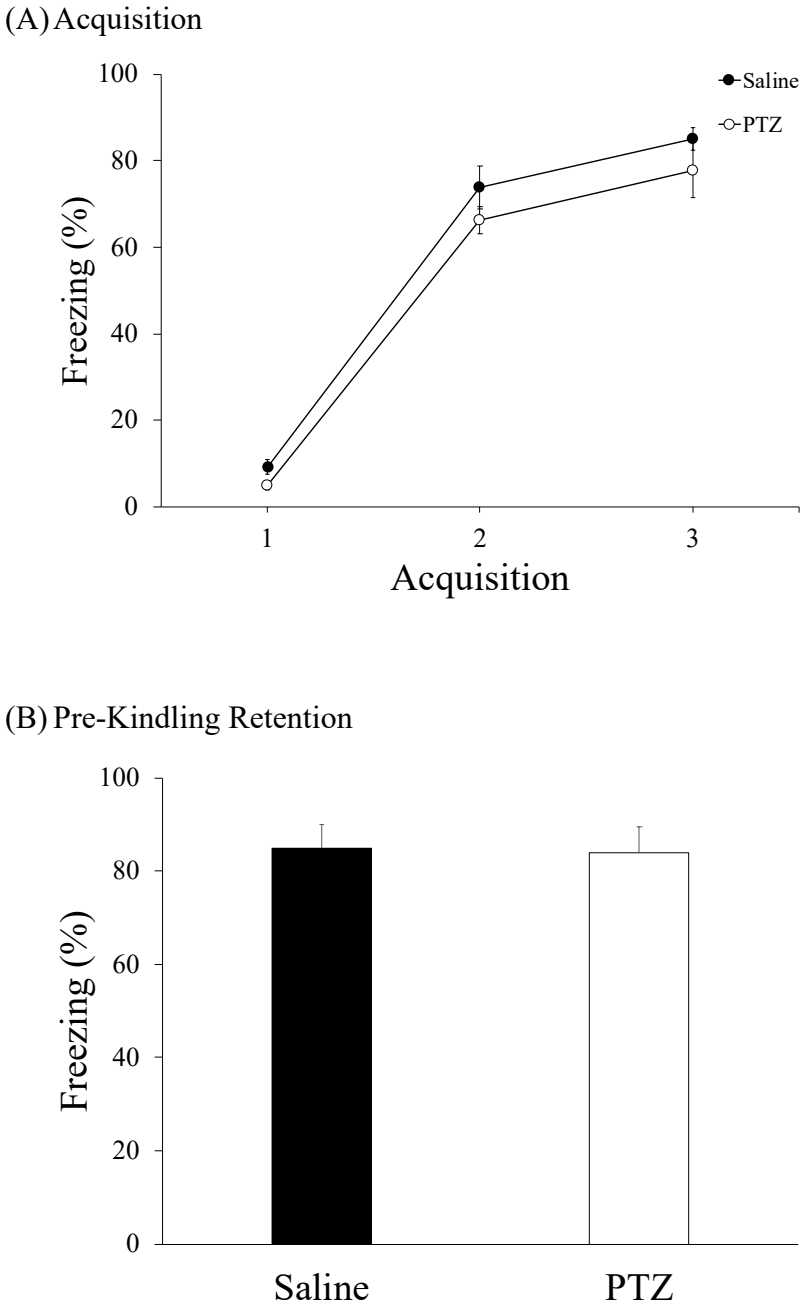


Figure 2. Illustration of the acquisition curve and pre-kindling retention test. (A) Acquisition curve illustrating the increase in freezing behaviour (mean and SEM) during the pre-shock period across training days ($P < .001$), which does not significantly differ across groups ($P = .867$). All rats in the experiment displayed similar learning experiences. (B) Freezing behaviour (mean and SEM) during the pre-kindling retention test. There were no significant differences in freezing between the control and PTZ groups ($P = .895$). All rats had comparable retention prior to seizures.

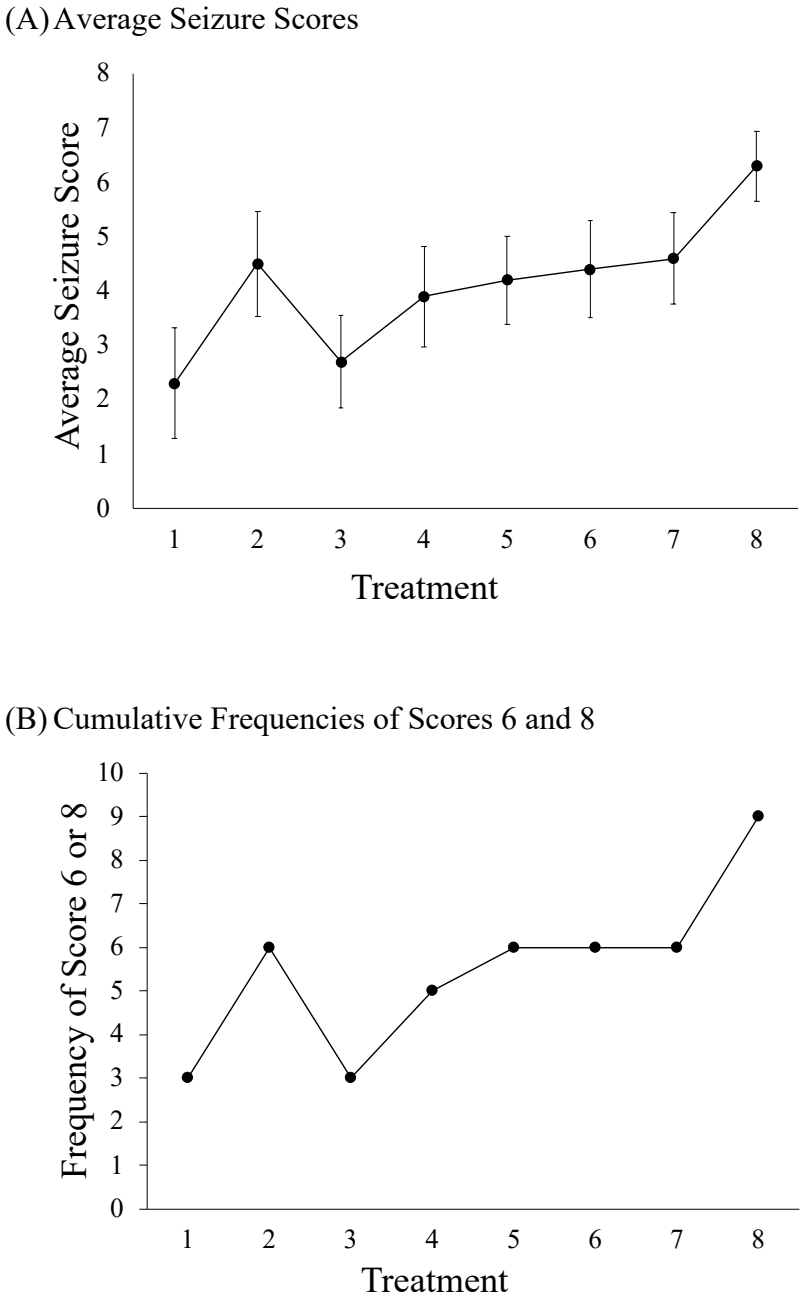


Figure 3. Kindling progression. (A) Kindling progression illustrating the linear increase in average seizure score and SEM across treatment days ($P = .03$). (B) Progression of the frequency of scores 6 and 8 across treatment days. There is a tendency for more scores of 6 and 8 on the last four treatment days compared to the first four ($P = .008$).

group difference was no longer significantly different over retention tests 3 and 4 ($F(1,20) = 0.53$, $P = .477$) indicating that the rate of amnesia or extinction appeared to be accelerated during the second week of kindling but then remained stable after kindling was terminated (see Figure 2b and Figure 4).

3.2. Effect of Prior PTZ Kindling on New Fear Learning

The results above indicate that chronic seizure stimulation produced a retrograde deficit associated with an impaired ability to recall a previously acquired trace fear memory. To determine if PTZ kindling could also disrupt the ability to acquire and form new memories, rats were tested on a novel contextual fear conditioning task. This procedure was performed twenty-fours after the last trace learning retention test (i.e. 21 days after the last PTZ treatment). For this study, rats were placed in a modified context from the one used for trace fear conditioning and after a 3-minute acclimatization period they received a single unpaired foot shock (1.2 mA, 2 s). Twenty-four hours later, the rats were returned to the conditioning chamber and freezing responses were recorded during a 5-minute retention test.

We compared freezing levels before the onset of the foot shock in the novel context (Figure 5a) with the levels of freezing exhibited during the last retention test (i.e. retention test 4; Figure 4). A series of paired t-tests revealed that both groups showed significantly lower freezing for the novel context compared to freezing exhibited during the last retention test (PTZ: $P < .001$; Control: $P < .001$). When freezing levels were examined twenty-four hours later after conditioning, the PTZ group exhibited significantly lower levels of freezing than the control group [$t(20) = 3.22$, $P = .004$,

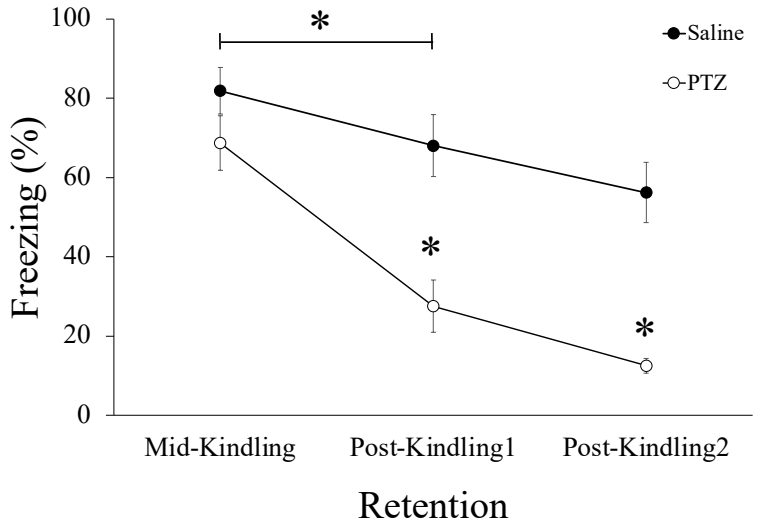


Figure 4. Illustration of the retention curve. Retention curve illustrating the decrease in freezing behaviour (mean and SEM) during retention tests ($P < .001$), which did differ by group ($P < .001$) and test ($P < .001$). Freezing did not differ between groups during the Mid-Kindling ($P = .158$) but did differ between groups during Post-Kindling1 ($P < .001$) and Post-Kindling2 ($P < .001$). Furthermore, the rate of decline between retention tests was only significantly different between groups from Mid-Kindling to Post-Kindling1 ($P = .002$); the rate of decline between groups was comparable between Pre-Kindling (see Figure 2b) and Mid-Kindling ($P = .155$), as well as Post-Kindling1 and Post-Kindling2 ($P = .477$). *$P = 0.05$

Figure 5b]. This group difference remained statistically significant even after covarying for the effect of pre-shock freezing [$F(1,19) = 6.35$, $P = .021$]. This indicates that kindled rats experienced a long-lasting anterograde impairment involving fear-related memory.

3.3. Prior PTZ Kindling Does Not Affect Water Task Learning

Given that PTZ kindling produced a disruption in the ability for the rats to successfully acquire new contextual fear learning, we then trained the rats in the MWT to examine if these impairments also extended to spatial memory. For this, we conducted MWT training 5-6 days after the novel context retention test [27-28 days after the last PTZ treatment]. A two-way repeated measures ANOVA with treatment (PTZ vs. saline) and trial (trials 1 through 12) was used to examine the average swim distance for each escape trial for both groups. The results revealed no significant interaction between treatment and trial [$F(11,220) = 1.72$, $P = .071$]. There was no significant main effect for treatment [$F(1,20) = 0.33$, $P = .573$]. However, there was a significant main effect for trial [$F(11,220) = 9.99$, $P < .001$] suggesting that both saline and PTZ groups showed a comparable decrease in swim distance across the training trials (see Figure 6).

An independent t-test revealed that during a probe trial conducted 24-hours later, there were no significant difference between groups on the distance swam to cross the platform location ($t(19) = 0.10$, $P = .920$; Figure 7a). Additionally, neither group showed a preference to the target quadrant during this probe trial (control: $t(10) = 1.36$, $P = .203$; PTZ: $t(9) = 0.56$, $P = .611$; Figure 7b). Furthermore, during the escape trial that followed, there was no significant difference in swim distance to escape ($t(20) = 0.59$ $P = .588$; Figure 7c). Together, this suggests that the PTZ treated rats do not have impaired spatial

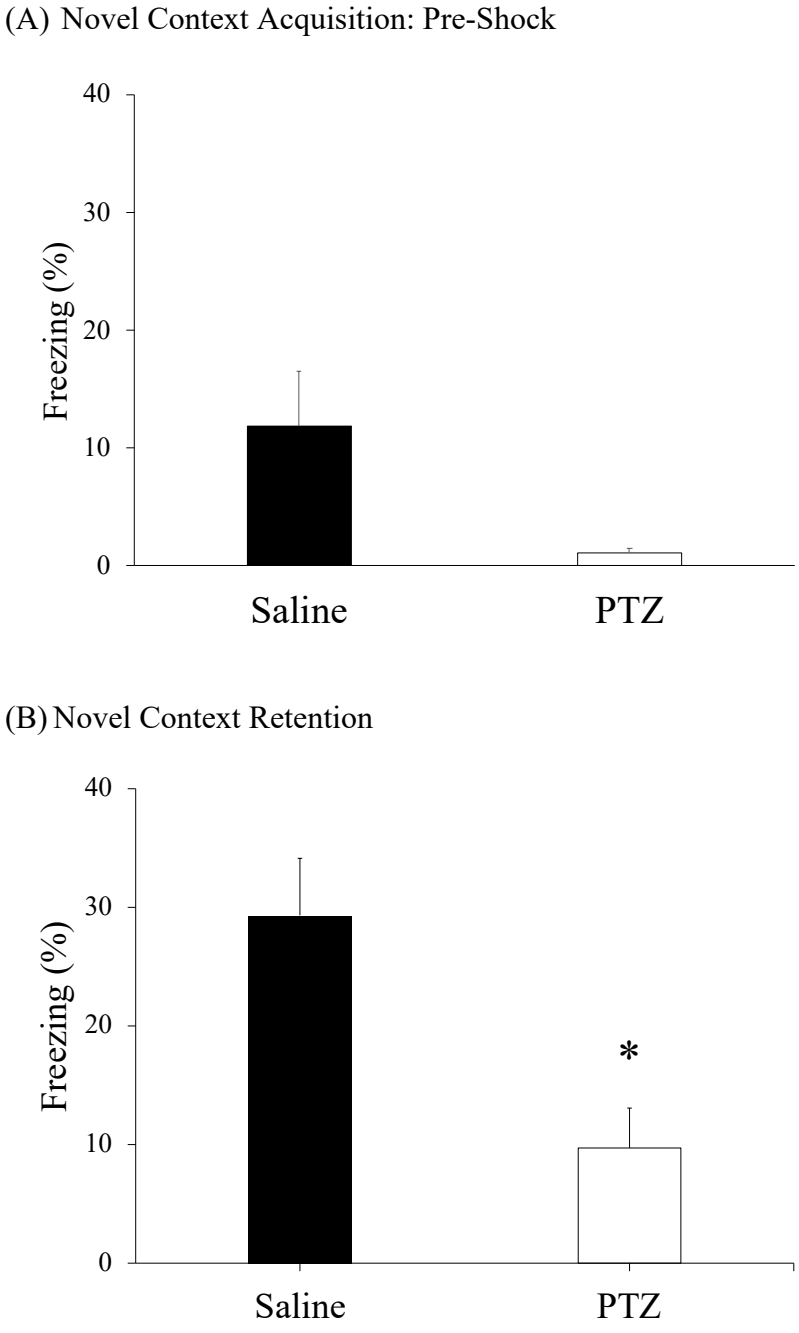


Figure 5. Freezing behaviour during the novel context acquisition and retention tests. (A) Freezing behaviour (mean and SEM) during the pre-shock period of Novel Context training day. Both the control and PTZ groups had significantly lower levels of freezing during this novel acquisition test than respective freezing levels during the last retention test (Control: $P < .001$; PTZ: $P < .001$). (B) Percent freezing during the Novel CFC Retention test. After covarying for the effect of pre-shock freezing, the PTZ-treated group froze significantly less than controls ($P = .021$). *$P = 0.05$

memory retention relative to controls.

3.4. Decreased Hippocampal CA3 Volume After PTZ Kindling

Following completion of the MWT, rats were euthanized (30 days after last seizure) and perfused to examine the impact of seizures on hippocampal volume. Hippocampal sections were stained with the neuron-specific antibody and volumetric analysis was conducted using the Caverali principle. A MANOVA revealed that there was no significant difference in the total volume of the HPC ($F(1,8) = 1.84$, $P = .212$; Figure 8a). However, upon further analysis, we found that kindled rats had significantly larger CA3 volumes than saline-treated controls [$F(1,8)= 7.57$, $P = .025$; Figure 8b]. There were no significant differences found in the volume of the CA1 ($F(1,8) = .01$, $P = .927$; Figure 8c) or dentate gyrus ($F(1,8) = .12$, $P = .735$; Figure 8d), as well as no significant differences in hilar volume ($F(1,8) = 1.48$, $P = .259$; Figure 8e). These results suggest that PTZ kindling produced a long-lasting increase in CA3 hippocampal volume.

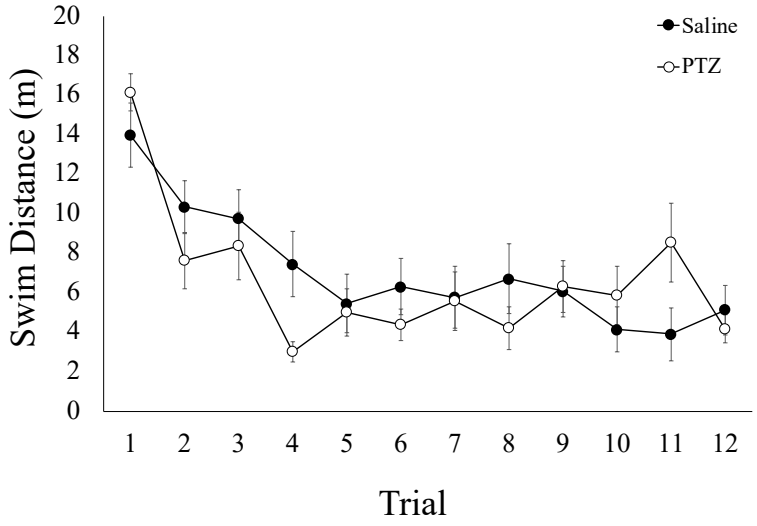
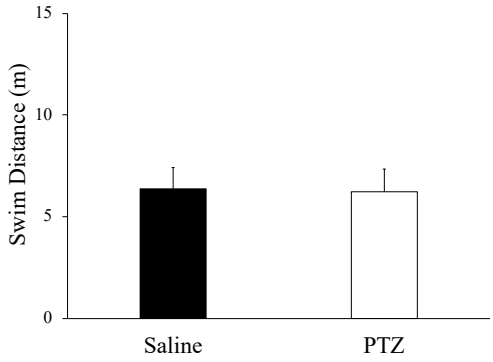
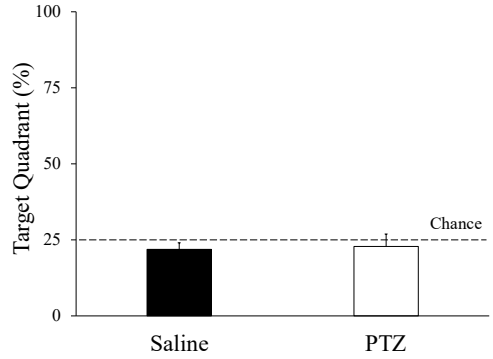


Figure 6. Illustration of acquisition curve during the MWT. Acquisition curve illustrating the decrease in distance travelled to the platform over trials during the MWT training day. There was a marginally significant interaction between trial and group ($P = 0.71$) and significant main effect of trial ($P = .001$), with no main effect of group ($P = .573$), indicating that both groups displayed similar learning experiences.

(A) Retention Probe Trial: Distance



(B) Retention Probe Trial: Quadrant Preference



(C) Retention Escape Trial: Distance

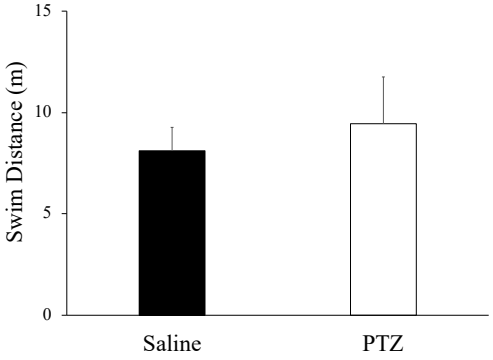


Figure 7. Behavioural data during the MWT retention test. (A) There are no significant differences between saline and PTZ groups during the MWT retention probe trails for distance to first crossing over the platform location (P = .920). (B) Neither group showed a preference for the target quadrant (control: P = .203; PTZ: P = .611) (25%; dotted line). (C) There were also no significant differences between groups during the MWT retention escape trial for distance to locating the hidden platform (P = .588).

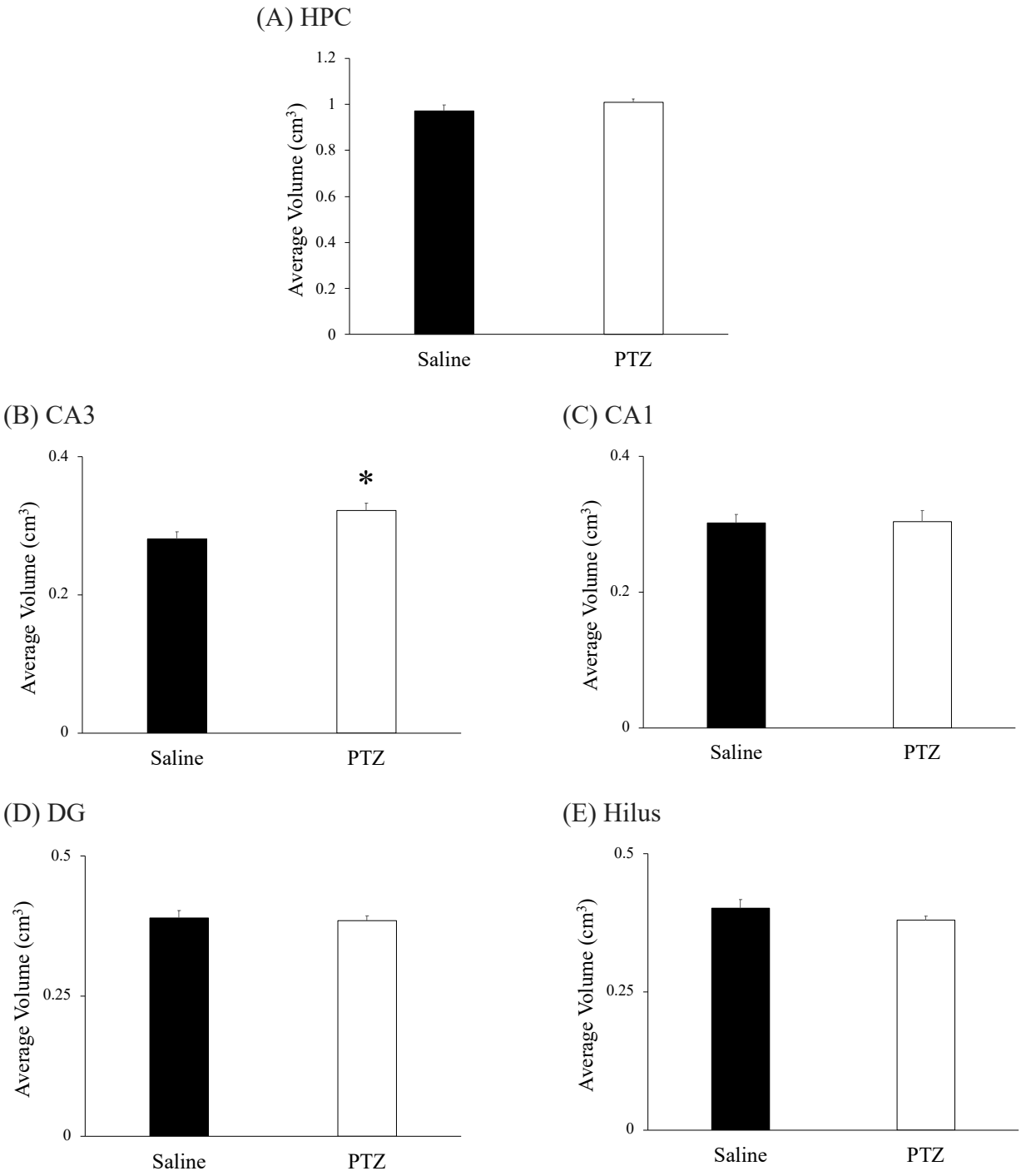


Figure 8. Volume throughout the HPC and Hilus. (A) Mean volume (SEM) of the total HPC, no significant differences between control and PTZ groups ($P = .212$). The PTZ group had increased volume (SEM) of the (B) CA3 compared to controls ($P = .025$), but no significant differences in volume (SEM) between groups was found in the (C) CA1 ($P = .927$), (D) DG ($P = .735$) or (E) Hilus ($P = .259$). *$P = 0.05$

4. Discussion

The current experiment found that rats which undergo repeated PTZ seizures show an accelerated rate of memory decay over time (indicating RA) compared to controls as inferred by the progressive reduction in trace fear memory across multiple test sessions. In addition, while we observed a clear anterograde amnesic effect of PTZ kindling, our data also revealed a sustained impact on context fear learning. For example, we found that PTZ treated rats continued to show impairments on novel fear learning. Interestingly, we did not observe an impact of prior PTZ kindling on the ability of rats to locate a hidden platform in the MWT relative to controls, suggesting that the impairments observed might relate to fear learning and retention. Lastly, we observed a significant change in hippocampal CA3 volume in rats that previously underwent PTZ kindling. This effect was not observed for other hippocampal subfields suggesting a highly specific impact of PTZ kindling on hippocampal circuitry.

4.1. Seizures and Accelerated Forgetting

Human patients with TLE and non-human animal models of recurrent seizures have been reported to experience accelerated forgetting in the context of AA (Aniol et al., 2013; Cassel et al., 2016; Narayanan et al., 2012). Humans with left TLE and right TLE can experience accelerated forgetting of verbal and non-verbal material, respectively, relative to controls when comparing the percentage of memory decay between a 30-minute and 4-week retention tests (Narayanan et al., 2012). Other researchers have found that humans with TLE have accelerated forgetting, relative to controls, of cued recall of visuospatial and verbal information during the 10-minute and 24-hour retention testing

respectively (Cassel et al., 2016). Cognitive decline observed in rats after a single tonic-clonic seizure has been reported where rats' differentiation between novel and familiar objects in NOR tasks is comparable to controls 10 days after seizures, but impaired after 70 days seizures (Aniol et al., 2013).

With the spontaneous nature of seizures in humans with TLE (Fisher et al., 2005; Stafstrom & Carmant, 2015), it is likely that these patients would have had seizures leading up to their participation in human research studies (Cassel et al., 2016; Narayanan et al., 2012). Which could explain why researchers will induce seizures in rodents prior to training to examine future forgetting across time (Aniol et al., 2013). This means that neural networks would already be in an altered state prior to learning. This is why the current findings of accelerated forgetting are intriguing, because the learning took place with a network which had yet to be altered by seizures. It is important to note that accelerated forgetting reported in human and non-human animal's is in the context of AA. However, accelerated forgetting is not a phenomenon exclusive to this experience, because the current experiment inducing post-training seizures also found this effect (RA) – i.e. indicating that the brain does not have to be damaged prior to learning for accelerated forgetting to occur.

In rodents, examining the effect of post-training seizures on fear memory retention is often done with the induction of seizures occurring immediately after training (within minutes of training completion). With reports indicating impairments in subsequent fear memory retention tasks (conditioned avoidance) after PTZ treatment (Palfai & Albala, 1976; Perlman et al., 1961). These methods examine the effect of seizures on a memory that is still completing the early stages of cellular consolidation, a

labile period during which memory is more susceptible to changes or loss (Kandel et al., 2014; Schafe et al., 2000; Schafe & LeDoux, 2000). Therefore, it is not entirely surprising that these memories were disrupted. Meanwhile, the current experiment had induction of seizures occur when the memory trace was considered to be outside of this labile time period (Alberini & Ledoux, 2013; Kandel et al., 2014; Lee, 2009; Schafe et al., 2000) yet still produce accelerated forgetting. However, it is important to note that cellular consolidation can take up to 100 hours to complete (Kandel et al., 2014; Schafe et al., 2000). With the context re-exposure prior to PTZ-treatment in the current experiment returning this memory to a labile state which must then undergo reconsolidation, it is possible that this memory was in this state during the early phases on kindling. Performing an additional experiment omitting any pre-seizure retention tests would allow for complementary results that the results of the current experiment are likely evidence of RA of a memory which was outside of the window of cellular consolidation.

4.2. Post-kindling Fear Memory Retention and Spatial Learning

Although the analyses revealed comparable decrease in swim distance across the training trials in the MWT, indicating comparable learning between groups, the retention test performed the next day indicates that neither group express accurate retention of the platform location. Interestingly, despite a drop in swim distance across the learning curve, there is no change in performance of either group after approximately trail 4 and/or 5. This is important to note because the swim distance during subsequent trails remains greater than that of the pool circumference (pool circumference = 1.4 m).

Therefore, one could argue that rats in the current experiment did not accurately learn the platform location during the training session. It might be necessary in future experiments to train rats with a stronger learning paradigm of MWT. One of which would be to increase the number of days/sessions that training occurs, such as 2 or 3 days of 12-trail sessions (Carr et al., 2016). Another method would be to train the rats to a criterion, i.e. less than 1.4 m swim distance on 3 consecutive trails (Lyeth et al., 1990). This would allow for researchers to ensure that rats are accurately locating the platform location prior to any retention tests occurring.

Since humans with TLE will inevitably have seizures before participating in research, it is not surprising that most rodent models of TLE adopt the method of inducing seizures prior to learning. This approach allows for researchers to examine for the effects of seizures causing AA. Researchers report AA for fear memories when training occurred both 24-hours (Genkova-Papazova & Lazarova-Bakarova, 1995) after completion of PTZ kindling and one week after the weekly challenging dose 6 weeks after PTZ kindling (Szyndler et al., 2002), as well as when a single convulsive seizure is induced within minutes before training (Mao et al., 2009). Interestingly, PTZ kindling also induced AA in the current experiment, but kindling had terminated 3 weeks before training had taken place. This provides evidence that alterations to neural networks resulting from PTZ-kindling have lasting effects on fear memories – however, future research could examine if this deficit is occurring during acquisition or at the time of retention. Examining cFos expression during post-kindling acquisition testing could examine if there are differences in network recruitment following PTZ-kindling that are driving this effect. Further exploration of cFos expression during post-kindling retention

testing could also examine differences in networks that may be involved in supporting the expression of fear memories.

In respect to spatial memory tasks, some researchers report that there are impairments in MWT acquisition of a hidden platform if a convulsive PTZ induced seizure occurs immediately before each of 2 training sessions (Mao et al., 2009), as well as during consecutive training on days 7-10 post-PTZ kindling (Szyndler et al., 2006). However, some researchers have found that there are no impairments of locating a hidden platform during MWT when 4-day training occurred 72-hours after kindling cessation (Lamberty & Klitgaard, 2000), as well as when training occurred two weeks after electrical amygdala kindling (Nieminen et al., 1992). Obviously, the effects of seizures on MWT acquisition are still debatable, and the results of the current experiment contribute to this debate in supporting that PTZ-kindling does not impair MWT acquisition.

Interestingly, in the current experiment, fear memory is subject to AA but spatial memory is comparable between groups during acquisition. One reason this might occur is the increased complexity of the MWT relative to CFC. In the MWT, increasing the task demand by removing extra pool cues has been shown to recruit additional networks (the anterior cingulate cortex) (Carr et al., 2016). In CFC, the animal is required to retain information about a single context, whereas the MWT requires additional faculties – such as locomotion and spatial navigation. Examining differences in the recruitment of these structures during post-kindling acquisition can provide insight into way spatial memory might be spared while fear memory falls victim to AA. If the increased task demand during MWT recruits more networks than CFC, it is possible that the damage incurred

via seizures does not have as much as an impact (i.e. having more networks involved can compensate for damage).

Another reason in which spatial memory is in tact whereas fear conditioning is impaired could be a result of damage incurred in specifically fear related memory networks. There is evidence of damaged neurons occurring in the basolateral amygdala (BLA) after PTZ, as well as in the basomedial nucleus, and some damaged neurons in the central amygdala (CeA), as well as the medial nucleus following PTZ kindling (Franke & Kittner, 2001). It is possible that damage occurred in the amygdala, thereby producing deficits in the ability to learn and/or express fear related information. Examining damage within the amygdala in future experiments would provide further insight into this possibility.

4.3. Volumetrics of the HPC

Literature often reports sclerosis of the CA3 region of the HPC after kindling (Franke & Kittner, 2001; Pitkänen & Lukasiuk, 2009). The current experiment finding that the CA3 volume is greater following PTZ-kindling is a novel finding. One way to further examine what might be producing this effect would be to examine the number of neurons within the structure. Unbiased stereology would be able to determine if there are differences in number of cells between kindled rats and controls. Researchers could then determine if persistent changes (i.e. tissue collection at 30 days or longer after kindling termination) are promoting this volume increase. Astrocyte swelling is proposed to contribute to seizure development (Murphy et al., 2017). If this type of alteration persists after seizures, it may be guiding this increase in volume. Further exploration into the

potential persistence of astrocyte swelling could give rise to further understanding of persistent alterations that promote learning and memory deficits.

Damaged neurons and cell death in the CA1, CA3 and DG (Franke & Kittner, 2001; Vasilev et al., 2018), and neurogenesis of DGCs (Fournier et al., 2013; Parent et al., 1998; Scott et al., 1998) with mossy fibre sprouting into the Hilus (Cavazos et al., 1991; Ebert & Löscher, 1995; Osawa et al., 2001) is commonly reported following seizures. The DG could be hypothesised to have an increased volume as a result of the increased neurogenesis from seizures (G. L. Holmes et al., 1999; Mortazavi et al., 2005; Park et al., 2006; Samokhina & Samokhin, 2018), however this was not observed in the current experiment. The brain is capable of some degree of repair and plasticity(Parent, 2002; Parent & Lowenstein, 2002). If repair to the network did occur in kindled rats of the current experiment, it could explain why there were no volumetric differences found in the DG. Using unbiased stereology not just for volume, but for cell estimation, one could quantify the number of neurons present in the DG of kindled and control rats to compare and determine if there are any signs of additional neurons, likely born through seizure-induced neurogenesis, integrating into the network (i.e. more cells in the kindled group than controls). The current experiment examined volume by staining the tissue with NeuN, which labels neurons throughout the brain (Vasilev et al., 2018), allowing for follow-up examination of cell quantification. However, it would still be unlikely to see a difference between kindled and control groups in this analysis because newborn cells do not remain within the network after prolonged periods of time (Aniol et al., 2013). Which provides confirmation as to why this specific analysis is not entirely necessary and would yield a similar conclusion that the morphological changes that occur at the time of seizure

are no longer detectable after 30 days. Additionally, potentially examining synaptic formation and/or connectivity (Curia et al., 2008; Polli et al., 2014; Samokhina & Samokhin, 2018) would provide further insight into alterations that promote RA.

4.4. Conclusions

As expected, recurrent seizures impaired the ability to retain fear information that was acquired prior to the seizure experiences (induced accelerated forgetting). It is important to recognize that 30-days after seizure termination the typical structural changes someone would expect to see following recurrent PTZ seizures are not present. Furthermore, these non-typical changes are capable of inducing AA of a fear memory, but the effects on spatial memory remain inconclusive. Future exploration into additional morphological and functional alterations that persist following seizures needs to be addressed.

CHAPTER III
Post-Kindling cFos Expression During Retention Testing

1. Introduction

In the previous chapter, repeated seizures resulted in fear memory retention deficits. Since seizures are known to result in changes to the morphology and functionality of various brain regions that are involved in learning and memory processes (Alberini & Ledoux, 2013; Fendt & Fanselow, 1999; Fournier & Persinger, 2004; LeDoux, 2014; Stafstrom & Carmant, 2015; Tovote et al., 2015), this was not a surprising result. For example, structures such as the HPC and the amygdala play a role not only in the initial input and consolidation of fear memories, but also in the retention of fear memory (Fanselow & Poulos, 2005; Poulos et al., 2009; Rudy et al., 2002; Tovote et al., 2015). As is evident in both patterns of activation and protein expression within these structures during fear memory acquisition and retention (Beck & Fibiger, 1995; Huff et al., 2006; Milanovic et al., 1998; Strekalova et al., 2003), as well as the impact of inactivation or lesions of these structures on fear memory acquisition and retention (Ji & Maren, 2008; Pitts et al., 2009; Poulos et al., 2009; Rogers et al., 2006; Sun et al., 2020).

In the previous chapter, the CA3 was the only subfield where morphological alterations were detected one month after the termination of kindling. This is particularity interesting as the rats had experienced a deficit in retention, but the CA3 is largely associated with acquisition of context fear rather than the support of fear memory expression (Daumas et al., 2005; Hunsaker et al., 2009; Ji & Maren, 2008; I. Lee & Kesner, 2004). One way this has been understood is by producing lesions to the CA3 (Daumas et al., 2005; Hunsaker et al., 2009; I. Lee & Kesner, 2004). Some researchers

have reported that following lesions to the CA3 rats display an initial acquisition deficit in freezing behaviour during context fear conditioning relative to controls (Lee & Kesner, 2004). This deficit does not persist as lesioned rats have comparable levels of freezing by the end of the conditioning session, and importantly have comparable freezing to that of controls when returned to the context 24-hours later (Lee & Kesner, 2004). This provides evidence that the CA3 does play a role in acquisition, as there were brief initial deficits, however the lack of impairment during the retention test indicates that the CA3 is not involved in expression. Other researchers have also induced lesions within the CA3 and concluded similarly stating that the CA3 in involved in acquisition of context fear (Daumas et al., 2005; Hunsaker et al., 2009).

Since the CA3 is associated with acquisition, and the focus of my thesis is retention, the structure examined in this chapter will be one that is associated with retention: the CA1 region of the HPC, as it is associated with acquisition and retention of associative memories, including fear association (Fanselow & Poulos, 2005; Hunsaker et al., 2009). Research has found that inactivation of the CA1 prior to trace fear learning results in decreased freezing relative to controls when returned to the aversive context during a retention test (Ji & Maren, 2008). Other research has found that dorsal or ventral CA1 lesions have comparable trace fear conditioned learning to that of controls but have impaired retention of the context (lower levels of freezing) relative to controls 24 hours later (Rogers et al., 2006). Interestingly, rats with ventral CA1 lesions have greater deficits in context retention relative to rats with dorsal CA1 lesions (Rogers et al., 2006).

The BLA is considered the input centre of the amygdala, and the CeA the output centre (Laurent & Westbrook, 2009; Sun et al., 2020). The BLA is also associated with

fear learning and retention (Gale et al., 2004; Poulos et al., 2009; Sun et al., 2020). Which can be seen when the BLA is inactivated or lesioned prior to CFC acquisition, there are deficits in freezing behaviour when returned to the context (Poulos et al., 2009; Sun et al., 2020). Projections between the BLA and CeA are believed to drive defensive behaviour expression (Sun et al., 2020), and the CeA is largely believed to be involved in simple reflexes and fear expression, such as startle or freezing responses (Fanselow & Poulos, 2005; Sun et al., 2020; Tovote et al., 2015), but it is also thought to play a role in fear learning (Tovote et al., 2015). Research has found that lesioning CeA 48 hours before CFC acquisition, or inactivating the CeA 15 minutes before CFC acquisition, both result in retention deficits when returned to the context an additional 48 hours later (Pitts et al., 2009). Interestingly, this deficit is not observed if the CeA inactivation only occurred 15 minutes before the retention test (Pitts et al., 2009).

Following learning and memory experiences, there is induction of the cFos protein, an immediate early gene that is expressed following neuronal activation (Botterill et al., 2014), within the brain (Milanovic et al., 1998) . When acquisition and retention pertain to fear related information, cFos expression increases within the HPC and amygdala (Milanovic et al., 1998). Therefore, cFos protein is often measured within these regions as an indicator of network recruitment during fear acquisition and retention testing (Milanovic et al., 1998). When fear learning (both trace and delayed fear conditioning) occurs after electrical kindling has terminated, rats express AA impairments in retention of these fear memories (Botterill et al., 2014). Interestingly, reduction of cFos expression in HPC and amygdala, as well as the parahippocampal regions, of kindled rats was associated with greater retention deficits (Botterill et al.,

2014). Similar tendencies for lower levels of cFos expression in the BLA to be related to decreased freezing behaviour have also been reported by others during retention testing (Santarelli et al., 2018). Research has examined differences in network alterations which could be promoting AA effects (Botterill et al., 2014), whereas the current experiment will examine potential network alterations which could be promoting RA effects. Since the focus of this experiment is not RA itself, rather changes to the network which might promote them, a less robust fear memory using a single CFC training session with fewer and lower intensity foot shocks is sufficient. We hypothesize that kindled rats will have lower levels of freezing during a post-kindling retention test, and that this will be accompanied by kindled rats also having lower levels of cFos expression within the CA1, BLA and CeA than controls.

2. Materials and Method

Twenty-four male Sprague Dawley rats, weighing approximately 150-220 g, were housed under the same conditions as the ones described in Chapter 2, pg 34.

2.1. Behavioural Tests

2.1.1 Context Fear Conditioning

The procedure used in this study is the same as previously outlined in Chapter 2, pg 34, with the following equipment modification: an adjustable shock generator/scrambler (Ugo Basile, Gemonio VA). As well, the procedure consisted of a single training session, with 3 foot shocks (1.0 mA, 2 s duration) separated by a 60 s interval were delivered (total test time 6 minutes).

2.1.2 Retention Tests

The retention tests were conducted in manner similar to that described in Chapter 2 with the modification that that the first retention test was conducted 24 hrs after training and the second retention test was conducted 48 hrs after completion of the last PTZ treatment. The duration of the retention test was 6 minutes.

2.2. BrdU Injections

Proliferation of cells in the DG in these rats was part of another project. The experimental procedure is reported, but as this was not the focus of the current experiment, it will not be reflected in the experimental design (Fig. 10), nor present in any statistical analyses. To label proliferating progenitor cells, rats were injected with 5-bromodeoxyuridine (BrdU, Sigma Aldrich Canada) 4 weeks before CFC training. Each rat received one intraperitoneal injection of BrdU (100 mg/kg, 20 mg/ml dissolved in saline with 0.007 N NaOH) for three consecutive days.

2.3. Pentylenetetrazol Kindling

To induce kindling, pentylenetetrazol (PTZ, Sigma-Aldrich, Cat# P6500, St Louis, MO USA) was administered at an initially subconvulsant dose (35 mg/kg, 1 ml per kg, dissolved in 0.9% w/v saline) on alternate days for 13 days (7 treatments). After each injection of PTZ or saline, rats were observed in a padded chamber (24 cm x 48 cm x 24 cm) for 30 minutes. The intensity of seizure behaviour was evaluated using established methodology (Dhir, 2012): where, 0 = no seizure; 1 = myoclonic jerks and/or facial automatisms (i.e. chewing); 3 = Straub's tail; 6 = forelimb clonus; and 8 = tonic

extension. If a rat failed to progress beyond stage 3 events after two consecutive treatments, the dosage of PTZ was increased to 37.5 mg/kg and then 40 mg/kg (the maximum dose that could be administered). No rats exhibited severe seizures with tonic-clonic extension that persisted for 30 minutes, therefore no rats required diazepam treatment. Saline-treated rats never displayed behavioural seizures.

2.4. Perfusions and Tissue Preparation

Animals were euthanized 90-120 minutes after completion of the last retention test. Tissue was processed as previously described (Kalinina et al., 2019). Immunostaining was performed on free-floating coronal sections (1 in 12 series for the CA1, 1 in 6 series for the BLA and CeA) with all rinses and incubations carried out under gentle agitation. Briefly, sections were washed 5x in PBS (10 min each) followed by treatment with 3% (w/v) H_2O_2 at room temperature for 30 minutes to minimize endogenous peroxidase activity. Following this, sections were washed 6x PBS (5 min each) before being placed for 1 hr at room temperature in a blocking solution comprised of 1% bovine serum albumin, 5% normal goat serum, and 0.3% Triton X-100 dissolved in PBS. After blocking, the sections were then treated with a primary anti-rabbit cFos polyclonal antibody (1:1000, 24 hrs, 4°C, Millipore Canada Ltd.) diluted in the previously described blocking solution. The next day, sections were rinsed several times in PBS (5x, 10 min each) followed by incubation with a secondary biotinylated antibody (goat anti-rabbit, 1:500, 2 h, room temperature, Vector Laboratories). Immunolabeled cells were visualized using 2.5% (w/v) nickel ammonium sulphate, 0.02% (w/v) diaminobenzidine (DAB) and 0.000083% hydrogen peroxide, and sodium acetate (0.175

M, pH = 6.8) to yield a blue-black product. After sufficient colouration (~20 min), the reaction was halted by washing the sections several times in sodium acetate (3x, 5 min each) followed by PBS rinses (3x, 5 min each). The sections were mounted onto SuperFrost Plus glass slides and left to air dry overnight. Slides were dehydrated through a series of alcohols, cleared in xylene, and coverslipped with DPX (Sigma Aldrich) mounting medium.

2.5. Unbiased Stereology

The estimated number of cFos positive cells were calculated in accordance with the optical disector method of unbiased stereology (Mouton, 2002). This method allows for unbiased estimation of the volume of an object of arbitrary shape and size. In conjunction with the program Stereologer, frame selection occurred at 10X magnification and cell quantification occurred at 100x in oil immersion by a Microfire CCD camera (Optronics, USA) on a Nikon Eclipse 80i microscope (Nikon Instruments, USA). The reference spaces (HPC subfield CA1 and amygdala subfields BLA and CeA) were defined using a rat brain atlas (Paxinos and Waston, 2007). Placing a grid randomly onto the tissue allowed for sampling of these subfields to occur. The number of cFos positive cells were counted at each grid point intersecting the respective subfield. Outlined in Table 1 are the specific parameters used to select grid points as well as the volume of tissue examined at each point.

Table 1.

Parameters used to complete unbiased stereology of cFos positive cells

Target Reference Space	Frame Spacing (μ)	Frame Area (μ^2)	Frame Height (μ)	Guard Height (μ)
CA1	150	4000	12	1.5
BLA	300	5000	12	1.5
CeA	150	5000	12	1.5

2.5.1. cFos Positive Count Adjustments

If tissue was damaged (e.g. tear in the targeted reference space in one hemisphere), it was corrected for by using the quantification score from the other hemisphere of the same section. To apply this correction, the output provided from the Stereologer Program was used to determine the number of cells counted on the intact half of the tissue. This value was then multiplied by two and used as the value for the whole section. From there, the total estimate was recalculated applying the following formula:

$$\Sigma Q^{-} \times \frac{1}{SSF} \times \frac{1}{ASF} \times \frac{1}{TSF}$$

Where: ΣQ^{-} represents the sum of the total number of objects/cells counted, SSF is the section sampling fraction (the number of sections sampled divided by the total number of possible sections), *ASF* is the area sampling fraction (the area of the optical disector divided by the area of the sampling grid), and *TSF* the thickness sampling fraction (interval at which the tissue was sampled divided by the average thickness of tissue).

This correction was only applied to samples which were missing half of the section. If a sample had more than one half of a structure missing (ie. two halves of different sections missing), but had only one half missing in another, then it was removed

from only the structure with multiple missing halves. Outlined in Table 2 are the number of brains in which correction methods were applied, as well as indication of which structures were impacted by excluding a brain that incurred too much damage for corrective methods to take place.

Table 2.

Breakdown of the number of brains in which the corrective method was applied and the number of brains removed for cFos quantification.

Target Reference Space	Corrected (n)	Removed (n)
CA1		
Saline	1	0
PTZ	1	0
BLA		
Saline	3	0
PTZ	1	0
CeA		
Saline	1	0
PTZ	0	1

2.6. Statistical Analysis

All statistical analyses were conducted using the software SPSS Statistics (Version 26). Comparisons of CFC learning and initial retention were completed using independent t-Tests. A one-way repeated measures ANOVA was used to determine the progression of seizure scores across treatments, and a paired t-Test was used to compare the frequency of scores of 6 or 8 during the first half and last half of seizure treatment. Comparison of the post-kindling retention test was completed using an independent t-

Test. A series of independent t-Tests was conducted to compare cFos expression were in the CA1, BLA and CeA. The criterion for statistical significance was set at $P < .05$.

3. Results

One of the PTZ rats died during the process of kindling and their data was subsequently removed from all analyses. Thus, the sample size for all behavioural experiments was saline n=12 and PTZ n= 11.

Unfortunately, some tissue was removed from the Fos analysis. The tissue from the PTZ-treated rat that died during kindling was not collected or stained. One brain from the saline-treated group was removed from cFos analyses across structures as an outlier in a conservative approach due to cFos counts being two standard deviations above the mean in the CA1. Additionally, 2 brains in the PTZ-treated group were removed across all structures because of improper sectioning. Due to complications in immunohistochemical processing, further sampling issues arose in the inability to sample a 1 in 6 interval to quantify the BLA and CeA of 2 saline-treated rats. Lastly, one PTZ-treated rat was removed from the CeA analysis due to missing tissue on more than one section (See Table 1 for breakdown of removal due to corrective method). The sample sizes for the CA1 were: saline, n = 11; and PTZ n = 9. The sample sizes for the BLA were: saline, n = 9; and PTZ n = 9. The sample sizes for the CeA were: saline n = 9; and PTZ n = 8. Outlined in Table 3 is a breakdown of final sample size in tissue analyses.

Table 3.

Breakdown of the number of brains removed because of being an outlier, improper sectioning, immunohistochemical errors, or missing tissue.

Target Reference Space	Initial (n)	Outlier (n)	Improper Sectioning (n)	Immunohistochemical Error (n)	Missing (n)	Final (n)
CA1						
Saline	12	1	-	-	-	11
PTZ	11	-	2	-	-	9
BLA						
Saline	12	1	-	2	-	9
PTZ	11	-	2	-	-	9
CeA						
Saline	12	1	-	2	-	9
PTZ	11	-	2	-	1	8

3.1. Effect of PTZ Kindling on the Retention of Prior Fear Learning

All rats underwent one day of CFC acquisition followed by a retention test prior to being assigned into saline- or PTZ-treated groups (see Figure 9 for experimental design). There were no significant differences in freezing behaviour during the post shock period of the acquisition task ($t(18.776) = 1.57$, $P = .134$, see Figure 10a), indicating that all rats had a comparable learning experience. Additionally, it is important to recognize that there were no differences in freezing levels between the groups when returned to the context 24-hours later ($t(21) = 0.04$, $P = .970$; see Figure 10b), suggesting comparable retention prior to kindling.

Two days after undergoing the pre-kindling retention test, rats began treatment with PTZ or saline. All PTZ treated rats showed the characteristic increase in both seizure

frequency and intensity [$F(1,10) = 16.08$, $P = 0.002$, Figure 11a] over the course of treatment. As expected, examination of the cumulative frequency of seizure severity

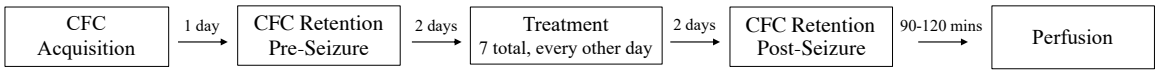


Figure 9. Illustration of the experimental design.

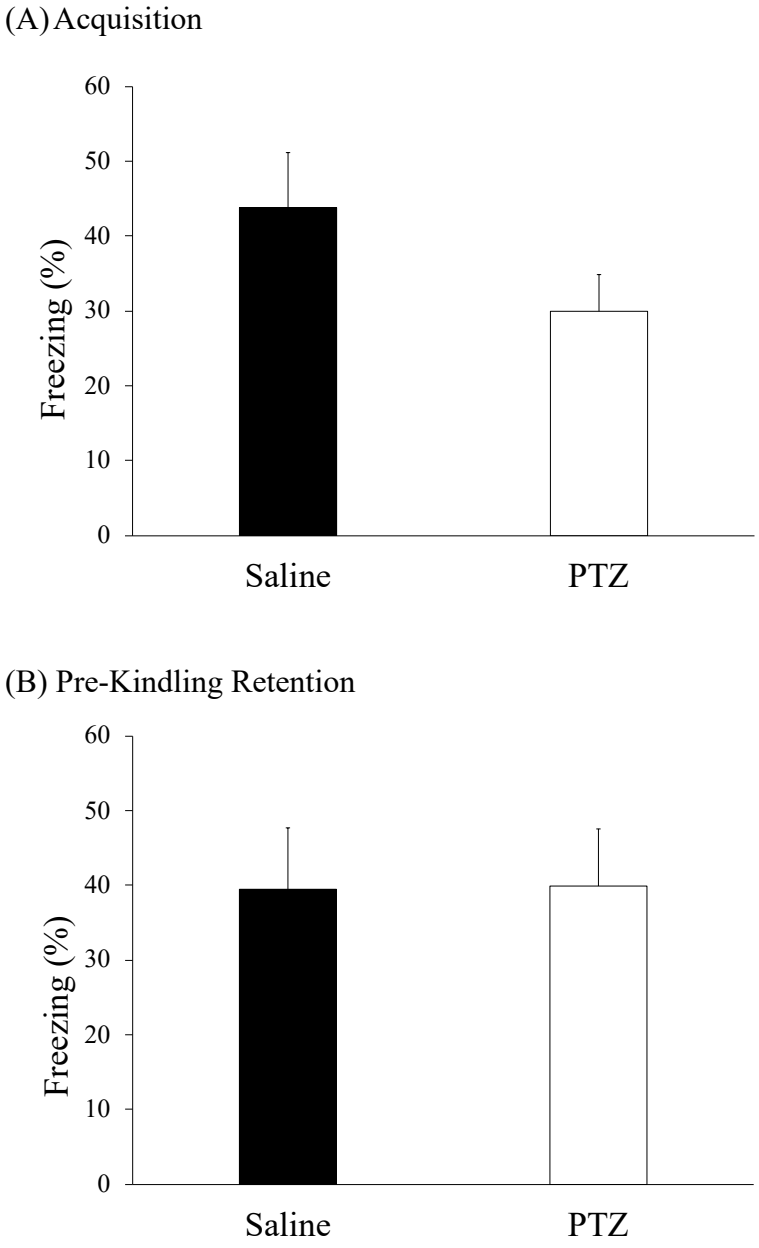


Figure 10. Freezing behaviour during acquisition and pre-kindling retention test. (A) Freezing behaviour (mean and SEM) during the acquisition test. There were no significant differences in freezing between the control and PTZ groups (P = .134). All rats had comparable learning. (B) Freezing behaviour (mean and SEM) during the pre-kindling retention test. There were no significant differences in freezing between the control and PTZ groups (P = .907). All rats had comparable retention prior to seizures.

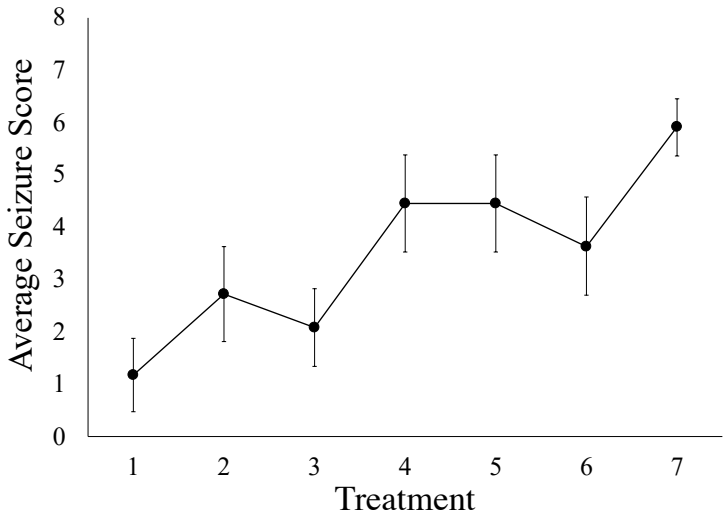
displays that rats experienced more motor convulsive events (stages 6 and 8) over the last three treatment sessions (treatments 5 through 7) than during the first four treatment sessions (treatment 1 through 4) ($t(10) = 3.63$, $P = .005$; see Figure 11b).

To examine the effect of repeated seizure activity on the ability to retrieve previously acquired fear information, we conducted a retention test 2 days after completion of kindling. As seen in Figure 12, an independent t-Test with unequal variance revealed that the PTZ-treated group froze significantly less than controls [$t(15.251) = 3.67$, $P = .002$]. This suggests that the PTZ-treated group exhibited RA following kindling.

3.2. PTZ Kindling Increased cFos Expression in the CA1

Rats were euthanized and perfused 90-120 minutes after the post-kindling retention test (2 days after last kindling session) to examine cFos expression between kindled rats and controls. An independent t-Tests revealed that PTZ-treated rats had significantly more cFos expression in the CA1 than saline-treated controls [$t(18) = 2.41$, $P = .027$*;* Figure 13a]. However, there were no significant differences found for cFos expression in the BLA ($t(16) = 0.55$, $P = .592$; Figure 13b) or CeA ($t(15) = 0.88$, $P = .395$; Figure 13c).

(A) Average Seizure Scores



(B) Cumulative Frequency of Scores 6 and 8

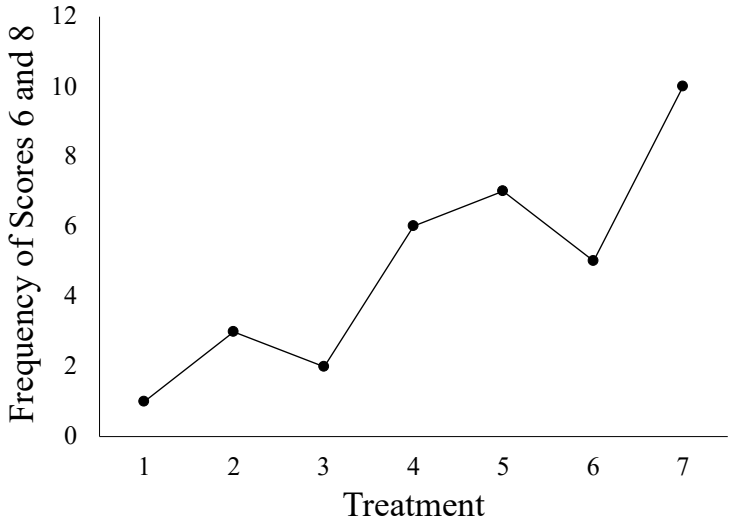


Figure 11. Kindling progression. (A) Kindling progression illustrating the linear increase in average seizure score and SEM across treatment days ($P = .002$). (B) Progression of the frequency of scores 6 and 8 across treatment days. There is a tendency for more scores of 6 and 8 on the last four treatment days compared to the first four.

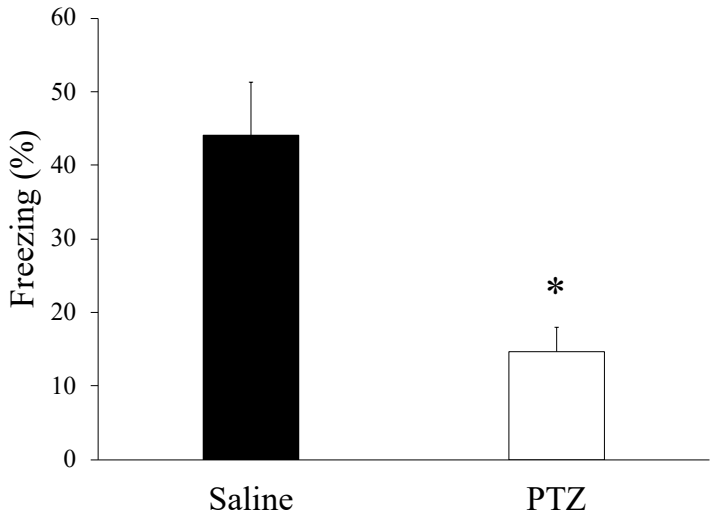


Figure 12. Freezing behaviour during post-kindling retention test. Freezing behaviour (mean and SEM) during the post-kindling retention test. The PTZ group froze significant less than controls ($P = .002$). Following seizures, the PTZ group experienced RA. *$P < 0.05$

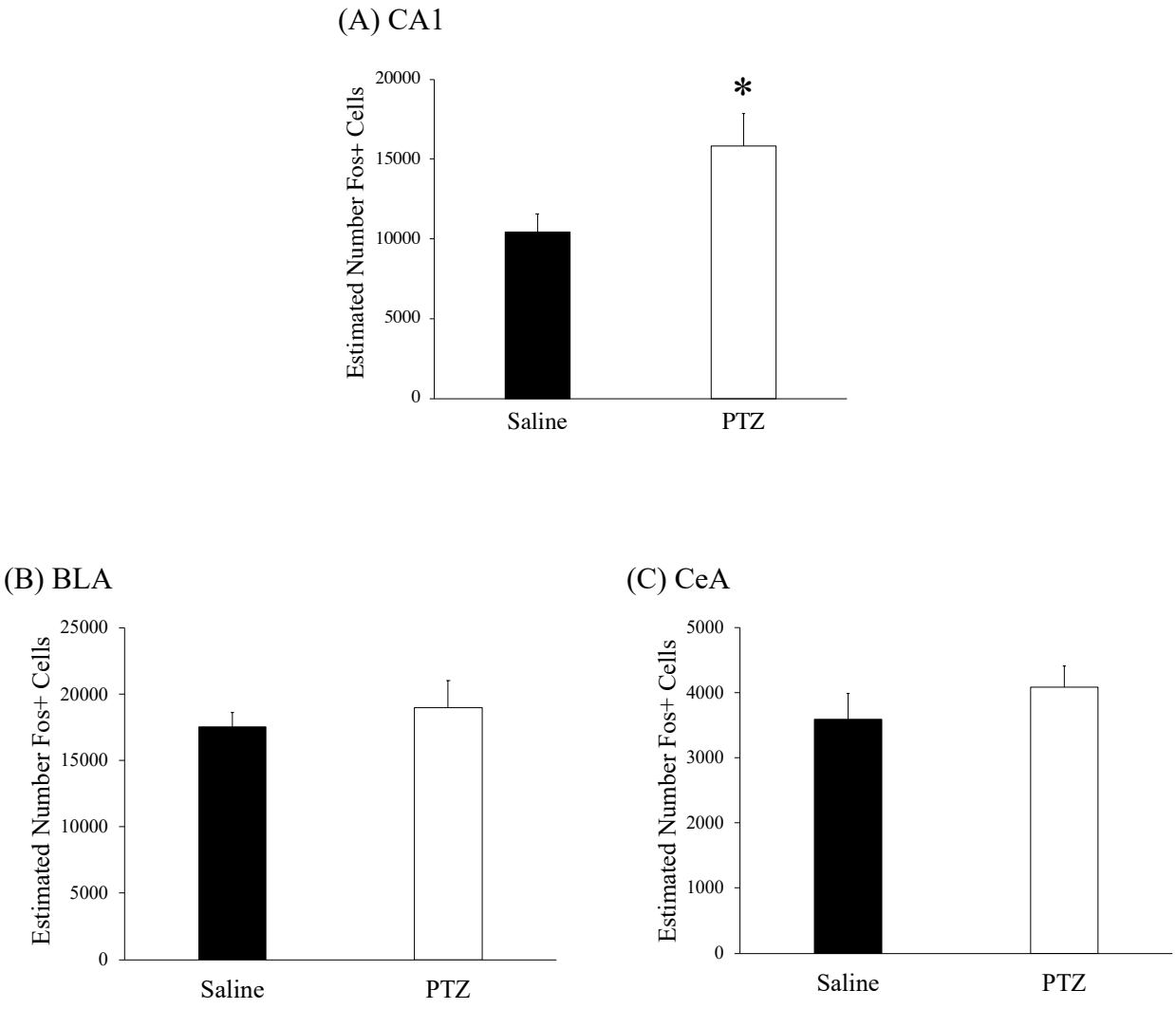


Figure 13. cFos expression within the CA1, BLA and CeA. (A) Mean (SEM) cFos estimate in the CA1, the PTZ-treated group had significantly more cFos expression than controls ($P = .027$). No significant differences in mean (SEM) cFos estimates between groups was found in the (B) BLA ($P = .592$) or (C) CeA ($P = .395$). *$P < 0.05$

4. Discussion

The current experiment found that rats which undergo repeated PTZ seizures express RA of a previously acquired fear memory, based on the decreased freezing observed during the post-kindling retention test. Furthermore, we observed significant increases of cFos expression within the CA1 of the PTZ-treated rats. This effect was not observed within the BLA or CeA.

4.1. Higher cFos Expression After Kindling

Rats were euthanized and perfused 90-120 minutes after the post-kindling retention test to examine cFos expression between kindled rats and controls. Researchers often report that cFos expression peaks between 60-90 minutes, however there is evidence that cFos can peak for as long as 120-180 minutes (Bertaina & Destrade, 1995; Farivar Reza, Zangenehpour Shahin, 2004; Fevurly & Spencer, 2004; Pace et al., 2005). Therefore, the range in perfusion times does not pose a concern in the current experiment.

The freezing behaviour of the PTZ-kindled rats during the post-kindling retention test is evidence of RA. Botterill et al. (2014) and Santarelli et al. (2012) have reported a tendency for higher levels of freezing behaviour to be related to higher levels of cFos expression in the HPC and amygdala (Botterill et al., 2014), or when examining the BLA (Santarelli et al., 2018), during both acquisition and retention tasks, respectively. Therefore, it was hypothesized that kindled rats would have lower cFos expression throughout the CA1, BLA and CeA than controls, however, this was not supported as the current experiment observed cFos expression to be greater in the kindled rats relative to controls within the CA1. It is possible that re-exposure to this context is comparable to

the experience of novel context exposure. If this was occurring, it would be expected that the PTZ-treated rats would have greater cFos expression in the CA1, BLA and CeA relative to controls as research suggests this tendency (Milanovic et al., 1998). This would support the reasoning that returning to the context is comparable to a novel environment for kindled rats, meaning that cFos expression would be greater in kindled rats than controls.

However, this tendency of greater cFos expression was not observed in the BLA or CeA. Freezing behaviour is greater during CFC retention when exposed to a context that looks similar to the training context, where a shock was delivered, than a context that looks dissimilar to the training context (Rajbhandari et al., 2017). This pattern is also observed in cFos expression during retention testing, where greater cFos is observed in mice exposed to a similar context than those exposed to a dissimilar context (Rajbhandari et al., 2017). This evidence would also provide reason to hypothesize that cFos expression in the BLA would be lower in the kindled group than controls, because the kindled group could be experiencing the context as novel (dissimilar) whereas the controls could be experiencing the context as a return to a familiar environment (similar and/or where they had once received foot shocks). It is possible that the results of the current experiment are just observed by chance. One way to confirm this would be to replicate and examine cFos in the BLA again. Alternatively, examining the entire BLA in entirety may not be the most effective way to examine for differences in cFos expression post-kindling. It is possible that further subdividing the BLA, as seen in reports by Hale et al. (2006) and discussed by Sun et al. (2020), to assess differences within subfields

may reveal changes in cFos expression that are undetectable when the structure is quantified as a whole.

In respect to the CeA, the lack of cFos expression differences between groups is not entirely surprising. Continuing with the idea that the return to the context is comparable to a novel context exposure in kindled rats, when examining cFos expression during an acquisition task with no foot shocks administered compared to cFos expression during a retention test where foot shocks were administered in the preceding acquisition task, the levels are cFos expression in the CeA appear to be relatively consistent between these situations (Milanovic et al., 1998). Additional groups would be needed to determine cFos induction between acquisition and retention to fully support this idea.

4.2. Future Directions

Future research could have additional groups, such as: home-cage controls (rats taken out of the home cage and sacrificed, naïve to the context and foot shock), saline-acquisition (rats that have never had seizures sacrificed 90-120 minutes after the acquisition test, exposed to the context and foot shock during acquisition), and PTZ-acquisition (rats that have had PTZ-induced seizures sacrificed 90-120 minutes after the acquisition test, exposed to the context and foot shock during acquisition). Having home cages controls would allow for confirmation of cFos induction beyond a baseline level of expression during both acquisition and retention tests. Although the baseline levels would likely be low (Huff et al., 2006), it is still useful to have this information to compare to as it would confirm cFos induction has occurred. This would also allow the researcher to better understand the expression of cFos during a retention test that occurs with so much

time between acquisition and retention (18 days) – since much of the research that has home cage controls have sacrificed animals following retention testing which occurred within week following acquisition (Beck & Fibiger, 1995; Milanovic et al., 1998; Strekalova et al., 2003). Also, having rats sacrificed following an acquisition test occurring after having experienced seizures or not would allow for the examination of potential differences in training-induced cFos expression after kindling. This could then be compared to cFos levels during no-seizure and post-kindling retention testing. Which could provide further insight into why there were no differences in cFos observed within the BLA and CeA of rats in the current experiment.

4.3. Conclusions

As expected, recurrent seizures impaired the ability to retain information that was acquired prior to the seizure experiences (induced RA). Although the cFos expression within fear learning and memory structures following seizures was not as expected, it highlights the need for future research to examine functionality of these networks following seizures. This will allow researcher to better understand how these networks are impacted by seizures, and therefore contribute to RA that occurs.

CHAPTER IV
General Discussion

4.1. Summary

Both preclinical and clinical studies have shown that repeated seizures can produce cognitive impairments (Fisher et al., 1998). Seizures induce alterations to neural networks, through both synaptic loss and synaptic remodelling (Becker et al., 1997; Fournier & Persinger, 2004). In people with TLE, these seizures and accompanied changes occur within the temporal lobe affecting structures such as the HPC and the amygdala (Fisher et al., 1998). Since both of these structures are associated with learning and memory (Fanselow & Poulos, 2005; Fendt & Fanselow, 1999; Tovote et al., 2015), it is not surprising that humans and non-human animals with temporal lobe seizures experience learning and memory deficits.

The deficits associated with seizure induced network alterations in non-human animals are largely examined in the context of AA (Botterill et al., 2014; Genkova-Papazova & Lazarova-Bakarova, 1995; Maia et al., 2014; Mishra & Goel, 2012; Smolensky et al., 2019; Szyndler et al., 2002; Y. Zhang et al., 2010). However, it is important to assess how these seizure-induced changes can impact a memory that was acquired prior to seizures occurring – a memory that is believed to have undergone the process of cellular consolidation, and as such, deemed to be stable (Kandel et al., 2014; Schafe et al., 2000). Exploration of seizure-induced network changes that may contribute to retention deficits, as well as activation of learning and memory networks that could support the conclusions of retention deficits followed. My thesis addressed the impact of repeated seizures on the ability to retain previously acquired context fear memories in rats. As expected, in Chapters 2 and 3, I found that PTZ-kindled seizures impair the

ability of rats to retain a previously acquired contextual fear memory, despite pre-kindling retention testing confirming that all rats had comparable fear memory prior to kindling. These results from my thesis contribute to the field of learning and memory research because it provides evidence that seizure-induces impairments are not solely a phenomena of post-kindling learning (AA); fear memories that are believed to be stable can also be susceptible to loss.

Furthermore, Chapter 2 revealed that 30 days after PTZ-kindling, the typical sclerosis of the CA3 following seizures (Samokhina & Samokhin, 2018) was not observed. Interestingly, I found that the CA3 of kindled rats was actually increased in volume. No differences in volume were found within the CA1, DG or hilus of kindled rats and controls. Although the differences I found are not consistent with both human and non-human research (Becker et al., 1997; Franke & Kittner, 2001; Mortazavi et al., 2005; Pitkänen & Lukasiuk, 2009), my experiment had more time between termination of kindling and tissue collection, which could be influencing these effects. Lastly, Chapter 3 revealed that cFos expression during post-kindling retention testing was only greater for kindled rats in the CA1, but comparable to controls within the BLA and CeA. Although I expected that kindled rats would have lower cFos expression than controls across these regions (Botterill et al., 2014; Santarelli et al., 2018), the potential of retention testing being novel environment exposure for kindled rats can support these results.

4.2. Kindling Impairs Retention of Fear Memories

Extinction occurs after completion of classical conditioning, and can be defined as a reduction in response to a conditioned stimulus (CS) after repeated exposures without

the presence of an unconditioned stimulus (US) (Herry et al., 2010; Maren & Quirk, 2004). For example, in context fear conditioning rats will associate a context (CS) as being aversive due to the presence of foot shocks (US) during exposure to the context. Extinction will occur when the rats are repeatedly returned to the context without receiving a shock – and will be evident in reduction of freezing behaviour with each re-exposure to the context. This occurs because every time a rat is returned to the context, the fear memory trace re-enters a labile state rendering it capable of adding new information – in this case, that the context no longer poses the threat of a shock – before reconsolidating to a stable memory trace (Alberini & Ledoux, 2013; Lee, 2009). The processes that take place to promote extinction are thought of as learning a new memory – essentially reintegrating the fear memory with a non-fear memory (Herry et al., 2010; Maren & Quirk, 2004). This belief is largely due to the phenomena of spontaneous recovery that can occur after successful extinction, and is defined as the spontaneous return of the associative fear behaviour after the passage of time (Herry et al., 2010; Maren & Quirk, 2004). An example of this could be the spontaneous recovery of freezing response when returned to a context days or weeks after having reduced freezing response to the context by the end of extinction taking place (Herry et al., 2010; Maren & Quirk, 2004).

Other markers of extinction include renewal and reinstatement (Maren & Quirk, 2004). During renewal testing of CFC, following training rodents undergo an extinction protocol in a novel environment. When returned to the original context, freezing behaviour often will be return to a level that indicates retention of the fear association to the original context (Maren & Quirk, 2004). During reinstatement testing of CFC,

following training rodents undergo an extinction protocol in the same context where training occurred. Upon completion of extinction, the rats are returned to the context and receive a single foot shock. Then, when returned to the context again the next day, levels of freezing will return to a level that indicates retention of the fear association to the original context (Maren & Quirk, 2004).

The method of measuring accelerated forgetting is similar to that of extinction, whereby the rats must be repeatedly returned to the context to measure reduction in freezing behaviour (Herry et al., 2010; Ishikawa et al., 2016; Maren & Quirk, 2004). Therefore, one might argue that the results of Chapter 2 can be consistent with that of extinction rather than accelerated forgetting. However, the results of Chapter 3 can be used to support that the results of Chapter 2 are more likely evidence of accelerated forgetting. In Chapter 3 there was no re-exposure to the context during the course of kindling, but there was a pre-kindling retention test which could have resulted in some new learning experience (as there was no foot shock present). Yet the results after kindling reveal that during the post-kindling retention test, controls still have levels of freezing that are comparable to the pre-kindling retention test performance, and only the kindled rats have a decrease in levels of freezing. This indicates that the decrease in freezing would not likely be a result of re-exposure to the context, but that of the kindling. Since the results of Chapter 3 would indicate the occurrence of RA (the inability to express the fear memory), the decreased freezing after kindling observed in Chapter 2 can be further supported as accelerated forgetting.

Another way one could further support the conclusions of amnesia would be to fully rule out the possibility of extinction by testing for spontaneous recovery, renewal

and reinstatement (Herry et al., 2010; Maren & Quirk, 2004). Testing for spontaneous recovery could be done by performing an experiment whereby multiple groups of rats undergo CFC training, followed by 2-weeks of kindling, and then each group undergoing post-kindling retention tests at varying time points. If there is a lack of spontaneous recovery at each time point (Herry et al., 2010; Maren & Quirk, 2004), it will allow researchers to confirm that the rats are experiencing amnesia. Additionally, testing for renewal could be done by performing an experiment whereby multiple groups of rats undergo CFC training, followed by 2-weeks of kindling, and an initial retention test in the original context. Further followed by each group going through an extinction protocol in a novel environment, terminating when the control group has reached a predetermined extinction criterion (for example, below 20% freezing). Then, by returning all rats to the original context, researchers could observe freezing behaviour to the original context (Maren & Quirk, 2004). If kindled rats still show a deficit relative to controls, it will allow researchers to confirm that the rats are experiencing amnesia. Lastly, testing for reinstatement could be done by conducting a similar experiment to that described during renewal testing, with the difference being that extinction occurs in the same context as initial training. Once the control performance reaches a predetermined extinction criterion, all rats would be returned to the context and receive a single shock. Being returned to the context the next day, researchers can examine for differences in fear behaviour between groups (Maren & Quirk, 2004). If the kindled rats still show a deficit relative to controls, it will allow researchers to confirm that the rats are experiencing amnesia.

4.3. Potential to Examine the Effects of Kindling on Other Types of Memory

Other fear-related behaviours that do not involve freezing as the sole measure of retention could also be examined in future studies to determine if these tasks are also susceptible to accelerated forgetting and/or RA. For example, the Shock Probe test would measure fear behaviour through avoidance and burying of the probe (Lehmann et al., 2005; Pinel & Treit, 1978). Additionally, shuttle box or passive avoidance tasks would measure fear behaviour through the avoidance of an aversive compartment of the box or the latency to step down off of the platform, respectively (Genkova-Papazova & Lazarova-Bakarova, 1995). Using these memory tasks in the future would not discount what was observed in the current experiment, they would complement them. They could explore the possibility that CFC memory is not the only type of fear memory that is susceptible to loss.

Not only could future research examine other types of fear memories, but they could also extend to examining the effects of seizures on other memory tasks, such as spatial memory. Both MWT and RAWM allow researchers to examine memory retention through examining distance travelled to locate a platform and the amount of time spent in the platform location when the platform is removed (Mortazavi et al., 2005; Szyndler et al., 2006). Assessing the potential for seizure-induced retrograde amnesia of spatial memory would help extend the current findings beyond fear associated learning and memory.

4.4. Kindling Induces Network Alterations

Since the HPC and amygdala are associated with fear memories (Fanselow & Poulos, 2005; Fendt & Fanselow, 1999; Tovote et al., 2015), and are largely impacted by seizures (Berkovic et al., 1990; Fisher et al., 1998; Stafstrom & Carmant, 2015), we examined for volumetric differences within the HPC in Chapter 2 and differences in cFos expression in both the HPC and amygdala in Chapter 3. The results of my thesis did not support what was expected. However, since the HPC and amygdala interact during learning and retention testing (A. P. R. Smith et al., 2006), potentially assessing how activation and connectivity between the HPC and amygdala are impacted by seizures could give rise to other explanations regarding how learning and memory processes are impacted after seizures. Post-kindling, researchers could selectively inactivate (Naik et al., 2021; Sacchetti et al., 1999) either of these structures in rats prior to acquisition and/or retention testing to examine how each structure contributes to learning and memory processes after seizure-induced network changes have occurred. Additionally, fMRI (Richter-Levin & Akirav, 2000) could be used in human research to examine differences in activation during acquisition and retention between humans with and without TLE. Especially since there are baseline differences in activation within the HPC of those with TLE (M. Holmes et al., 2012), which could impact recruitment of these structures during acquisition and retention testing.

Another structure that would be of interest in future research would be the CA3, since it is largely associated with learning (Hunsaker et al., 2009; Ji & Maren, 2008). Examining induction of cFos expression within the CA3 following the post-kindling retention test could provide evidence to support the idea of return to the context being

comparable to that of a novel environment for the kindled rats, versus a re-exposure to a familiar context for the controls. Some researchers have also examined the CA3in respect to dorsal CA3 versus ventral CA3 (Daumas et al., 2005). Examining cFos expression with this method could also provide further insight into the role of the CA3 in retention of fear memories.

4.5. Implications Beyond Seizures and Epilepsy

Kindling does not always result in lesions (Cavazos et al., 1994; Samokhina & Samokhin, 2018) whereas SE models of seizures often induce brain lesions (Curia et al., 2008; Polli et al., 2014), Both models induce cognitive impairments (Maia et al., 2014; Mortazavi et al., 2005; Smolensky et al., 2019; Szyndler et al., 2002; Y. Zhang et al., 2010), however, seizures are not the only method of inducing network alterations. Neurodegenerative disorders like Alzheimer's disease, which is characterized by memory loss (Larson et al. 2006; Scarmeas et al., 2009; Radak et al., 2010), induce lesions which promote this memory loss (Braak & Braak, 1995; Ito et al., 2010). TBI can also induce brain lesions and/or neuronal loss that are accompanied by cognitive deficits (Hicks et al., 1993). However, cognitive impairments after a TBI can also occur in the absence of lesions (Lyeth et al., 1990). Cognitive impairments following network alterations that do not induce lesions can also be observed through strokes (Batchelor et al., 2008; Karimian et al., 2018), whereby blood supply is cut off from an area of the brain which results in neuronal death (Johnson et al., 2016). Further research could compare cognitive impairments, specifically in the context of learning and memory, that are induced by various methods that do and that do not induce lesions. Additionally, examining the

impact of neuroprotective factors, such as diet and/or exercise, between these methods (those that do and those that do not result in lesions) could future provide insight into how to prevent learning and memory deficits.

4.6. Conclusions

My thesis examined the impact of recurrent seizures on the ability of rats to retain a previously acquired fear memory. We found that PTZ-induced seizures did impair the ability of rats to retain a fear memory and found some evidence of morphological and function changes which may promoted this deficit. This provides evidence that memories can be disrupted after the phase of consolidation is completed.

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