Assessing the Role of the Hippocampus in Amygdala Kindled Fear:

An Analysis of Environmental Habituation

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ABSTRACT

Amygdala kindling is commonly used to study the mechanisms involved in epileptogenesis, with long-term amygdala kindling providing a useful model of the behavioural disturbances— such as heightened anxiety— that can occur between epileptic seizures. The purpose of this thesis was to determine whether increased fear behaviours exhibited by long-term amygdala kindled rats are reflective of previously observed kindling-mediated alterations in the hippocampus. As the hippocampus plays an integral role in contextual learning, the ability of the animals to habituate to a novel environment was evaluated, in order to determine if the rats displayed impairments in this hippocampal-dependent function. In Experiment 1, long-term kindled rats demonstrated consistently elevated exploration and fear over repeated exposure to an initially novel open field, indicating impaired habituation. In Experiment 2, all kindled rats showed elevated exploration and an inability to form a home base in relation to static visual cues, again demonstrating an inability to habituate over repeated exposures to the initially novel environment. Rats that had received 30 or 60 stimulations demonstrated hyperexploratory behaviour and elevated fear, although this behaviour did dissipate to a certain degree by the final day of testing. Long-term kindled rats, having received 99 stimulations, demonstrated extremely heightened fear behaviours that interfered with normal exploration, home base formation and habituation. These fear behaviours included high levels of freezing, disorganized running, and purposive jumping from the open field. Taken together, these results indicate that long-term amygdala kindling does produce deficits in habituation to an initially novel environment. As habituation necessarily involves the hippocampal-dependent roles of contextual learning and memory, the current research suggests that long-term kindling does impair hippocampal function and that this

may contribute to kindling-induced fear behaviours. This research may help to understand the mechanisms involved in emotional disturbances experienced by human epileptics.

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DEDICATION

I dedicate this work to my family, a group of people who have taught me to be passionate about what I do. Your love and support have provided with the courage to take on new challenges.

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5-HT	Serotonin
5-HT_{1A}	Serotonin receptor subtype 1A
AD	Afterdischarge
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate
ANOVA	Analysis of Variance
BLA	Basolateral Amygdala Nucleus
BZ	Benzodiazepine
CA1, CA2, CA3	Ammon's Horn (Cornu Ammonis) regions 1,2,3 of Hippocampus
DG	Dentate Gyrus
DMTP	Delayed-Match-to-Place
GABA	γ - aminobutyric acid
$GABA_A$	γ - aminobutyric acid receptor subtype A
HPA	Hypothalamic-Pituitary-Adrenal Axis
IGF-1	Insulin-Like Growth Factor-1
M or *M*	Mean
MWM	Morris Water Maze
PB	Phosphate Buffer
PBS	Phosphate-Buffered Saline
RAM	Radial Arm Maze
RTC	Resistance to Capture
SC	Subcutaneous
S.D.	Standard Deviation
S.E.M.	Standard Error of the Mean
SGZ	Subgranular Zone
SPSS	Statistical Package for the Social Sciences
Stim	Stimulation
TLE	Temporal Lobe Epilepsy

CHAPTER 1:

General Introduction

Clinical Overview of Temporal Lobe Epilepsy

Epilepsy is a chronic neurological disorder characterized by recurrent, unprovoked seizures, which are instances of transient abnormal neuronal activity in the brain (Fisher et al., 2005; Seino, 2006). Due to the diverse presentations of this disorder, epilepsy is not seen as a single disease but rather as a group of syndromes that share the common characteristic of epileptic seizures. The prevalence of epilepsy ranges from 5 to 10 individuals for every 1000 individuals in developed countries (Tellez-Zenteno, Pondal-Sorda, Montijevic, & Wiebe, 2004). Temporal lobe epilepsy (TLE) is the most common form of epilepsy in adults, accounting for over half of all documented cases of epilepsy (Engel, 1998; Devinsky, 2004). Most patients with TLE experience complex partial seizures that often begin as a simple partial seizure or “aura" (a basic physical or mental sensation), followed by an impairment in consciousness (complex partial seizure), during which time the individual may display stereotyped movements called automatisms. In some patients, complex partial seizures may progress into secondary generalized tonic-clonic seizures. In most cases of TLE, the seizures originate within the mesial temporal lobe structures, which include the amygdala, hippocampus, and the parahippocampal gyrus. However, for a significant number of TLE cases the underlying

source of the epileptogenic activity cannot be directly localized, thus making treatment difficult if not impossible (Kohler, Carran, Bilker, O'Connor, & Sperling, 2001).

An additional feature of TLE is its high comorbidity with mood and anxiety disorders (Hermann, 1979; Devinsky & Najjar, 1999). Recently, it has been suggested that up to 50% of TLE patients may experience emotional disturbances between seizures (i.e., interictal) (Vazquez & Devinsky, 2003). Depression is the most common mood disorder reported by temporal lobe epileptics, whereas anxiety—the second most common disturbance— is perhaps more perplexing as TLE has been associated with several diverse anxiety disorders (including generalized anxiety disorder, panic disorders, and phobias) (Gilliam, 2003; Johnson, Jones, Seidenberg, & Hermann, 2004). The association of epilepsy with mood and anxiety disorders may be a result of the neuropathology associated with the seizures, psychosocial factors associated with the disorder, or even a result of the treatment itself, as several antiepileptic drugs (AEDs) have been reported to increase interictal behavioural disturbances in some epileptic patients (*see* Kohler et al., 2001).

There are numerous characteristics of epilepsy that make it difficult to study experimentally in clinical populations. Assessment of TLE patients is also difficult from both a neuropsychological and neurological perspective because functional testing may be adversely impacted by factors such as differences in baseline levels of functioning, side-effects associated with drug treatment, comorbid mood disturbances, and variability in seizure frequency and foci location. The heterogeneity in patient presentation of the disorder is a major obstacle impeding progress toward understanding the neuropathological basis of complex partial seizure disorders and their behavioural comorbidities. As a result, researchers have turned to animal models in order to gain

insight into the behavioural and neurostructural associations present in TLE.

Kindling is one animal model that has been particularly successful at reproducing the behavioural disturbances commonly seen in human temporal lobe epilepsy (Kalynchuk, 2000). By allowing precise control over the site of seizure origin, and seizure frequency and duration, kindling allows for the direct assessment of behavioural consequences that are uniquely impacted by the seizures themselves while minimizing confounding psychosocial factors that may be present in humans (Hannesson & Corcoran, 2000). Through the assessment of the neurobiological changes that occur in parallel with the behavioural consequences seen in TLE, kindling may provide insight into how recurrent temporal lobe seizures can mediate increases in anxiety. In the following document, I will assess how the increased anxiety-like behaviour seen in kindled rats may be a result of impaired hippocampal function, as the organization of this structure can be significantly altered by repeated seizures.

The Kindling Model

Kindling refers to the repeated administration of initially subconvulsive stimulations to a focal region of the brain that eventually leads to the development and increased intensification of convulsions (Goddard, McIntyre, & Leech, 1969). Initial stimulation produces an afterdischarge (AD) localized to the area of delivery, involving epileptiform activity at the site of stimulation that outlasts the duration of the stimulation (Goddard et al., 1969). With repeated stimulation, the AD lasts longer and spreads further from the site of stimulation, and is eventually accompanied by motor seizures (Racine, 1972a).

There are diverse methods of stimulation delivery, from electrical to chemical agents, with induction intervals varying from every few hours to every few days

(Hannesson & Corcoran, 2000; Mortazavi, Ericson, Story, Dulce, & Dunbar, 2005). In addition, several brain structures may serve as the site of stimulation with the vast majority of kindling studies focusing on mesial temporal lobe or subcortical regions. The typical kindling sites include the amygdala, hippocampus, perirhinal cortex, bed nucleus of the stria terminalus (BNST), piriform cortex, and caudate (Kalynchuk, Pinel, & Treit, 1998; Kalynchuk & Wintink, 2005; Hannesson & Corcoran, 2000). The number of stimulations delivered to the animal can also vary. "Partial kindling" models are those in which the rats receive few stimulations that evoke AD activity without any accompanying motor seizures (Racine, 1972a). "Short-term kindling" models are those in which rats receive between 15 and 20 amygdala stimulations, which is enough to produce a fully kindled state characterized by three to five consecutive fully generalized ("Class 5") motor seizures (McNamara, Kirkby, dePape, Skelton, & Corcoran, 1993; Racine, 1972b; Adamec, Shallow, & Burton, 2005). "Long-term kindling" models are those in which rats receive extended stimulations beyond those required for short-term kindling— generally from 60-100 stimulations. In some cases, more than 200 stimulations are given because this can produce a state of spontaneous seizures, which is the defining feature of epilepsy (Pinel & Rovner, 1978; Fisher et al., 2005). Although the majority of kindling research is carried out with "short-term kindled" rats (rats which have displayed between 3 and 30 generalized convulsions), studies focused specifically on the behavioral comorbidities associated with TLE often use the long-term kindling model because this is the version of kindling that produces the most robust and reliable changes in behavior (Kalynchuk, 2000).

Kindling as a Model of Temporal Lobe Epilepsy

There are numerous reasons why amygdala kindled rats are seen as not only valid

models, but preferable models, of human complex partial seizures with secondary generalization. The most direct proof of the functional capacity of this model is its ability to predict drug efficacy in humans with complex partial seizures (Adamec, 1990). With up to 70% of temporal lobe epileptics experiencing complex partial seizures that are pharmacoresistant, the ability to successfully predict the effectiveness of newly-developed anticonvulsants emphasizes the utility of this model (Coulter, McIntyre, & Löscher, 2002).

The primary criterion for the diagnosis of epilepsy in humans is the spontaneous occurrence of seizures. Although short-term kindling protocols do not result in the development of spontaneous convulsions, spontaneous seizure occurrence has been elicited in kindled animals using extended amygdaloid kindling protocols, as mentioned above (Pinel & Rovner, 1978). These procedures also produce the neuronal loss (Cavazos, Das, & Sutula, 1994) and mossy fibre sprouting (Sutula, 1991) that are often seen in resected tissue samples taken from human temporal lobe epileptics. Although these changes occur only after long-term kindling, two factors need to be considered: 1) that many temporal lobe epileptics have experienced a significant number of electrographic seizures before even the onset of motor disturbances and neuropathology become apparent, and 2) that both human epileptics and kindled animals show several aspects of cellular alterations that can proceed before the onset of visible histopathological changes (Kalynchuk, 2000; Devinsky & Najjar, 1999). Importantly, these early cellular changes can have significant consequences, as they facilitate the progression of the epileptic disorder and lay the foundation for the emergence of interictal behavioural psychopathology (Kalynchuk, 2000).

A very attractive feature of the kindling model is that it allows for the controlled

progression of seizures, facilitating the observation of related neuronal alterations and behavioural comorbidities at precise developmental time points (Hannesson & Corcoran, 2000). This is in contrast to chemoconvulsant models of epilepsy which, despite producing the gross histological damage similar to what is seen in human temporal lobe epilepsy, do not allow for the direct course of seizure development to be monitored.

Because of its clinical relevance, many researchers have used amygdala kindling to gain a better understanding of the interictal emotional disturbances that are commonly seen in patients with complex partial seizures (Kalynchuk, 2000). The most commonly studied emotional disturbances, repeatedly shown to occur after long-term amygdala kindling, are robust elevations in emotional behaviour.

Behavioural Consequences of Kindling

Hyperemotionality has long been investigated in kindling models. Adamec initiated this research by documenting fear behaviours after partial amygdala or hippocampal kindling in cats in response to threatening vocalizations (Adamec, 1975; Adamec, 1990). Pinel, Treit, and Rovner (1977) then identified an increased fear response in long-term amygdala and hippocampal kindled rats as measured by resistance to being captured from an unfamiliar environment. Since then, fear behaviour has consistently been seen in kindled rats using a variety of behavioral tests including increased defensiveness in social interaction and resident-intruder paradigms (Helfer, Deransart, Marescaux, & Depaulis, 1996; Depaulis, Helfer, Deransart, & Marescaux, 1997; Kalynchuk, Pinel, Treit, & Kippin, 1997), increased contextual fear conditioning (Barnes, Pinel, Francis, & Wig, 2001; Barnes, Pinel, Wee, Archambault, & Ailon, 2006), decreased exploration of the open arms in an elevated plus maze (Kalynchuk, Pinel, & Treit, 1998), increased fear-potentiated startle (Rosen, Hamerman, Sitscoke,

Glowa, & Schulkin, 1996), and decreased boldness on elevated bridges (Nieminen, Sirvio, Teittinen, Pitkanen, Airacksinen, & Reikkinen, 1992).

The most common measure of fear behaviour in kindled rats involves introducing the rat to a novel object or environment, particularly novel open fields (Hamovici, Wang, Cohen, & Mintz, 2001; Murphy & Burnham, 2003). Novelty has proven to be a particularly effective stimulus of fear behavior in long-term amygdala kindled rats. When introduced into an initially novel open field, kindled rats typically demonstrate significant increases in fearfulness through measures of increased freezing, hyperexploration, and increased resistance to capture (Kalynchuk et al., 1997). In addition, long-term amygdala kindled rats display sustained fear responses over repeated or enduring exposures to initially unfamiliar environments (Kalynchuk, Pinel & Treit, 1999; Wintink, Young, Davis, Gregus, & Kalynchuk, 2003), suggesting that an impairment in habituation to environments that should become increasingly familiar may occur after kindling.

Gray (1978) proposed an important role for the hippocampus and associated structures in mediating anxious behaviours in response to novel stimuli. Upon presentation of a novel situation, a conflict arises between approach behaviour (addressing a state of curiosity) and avoidance behaviour (addressing the fear of a potential threat). The conflict is mediated by the septo-hippocampal system (SHS), activating additional structures and ultimately determining the action to be taken (McNaughton & Gray, 2000). Although all animals exhibit a normal fear response that allows them to cope with expected or encountered dangers, Rosen and Schulkin (1998) suggested that one consequence of amygdala kindling is “fear sensitization”, in which overactivated fear circuits in the brain lead to pathological anxiety. Interestingly, excess

septo-hippocampal system activity, which would result after repetitive stimulation during amygdala kindling, leads to increased perception of environmental threats and excessively anxious responses to any type of novel circumstance (McNaughton & Corr, 2004). Thus, events deemed unfamiliar by the hippocampus are prone to instigate increased arousal and fear behaviours mediated by an already hyperexcitable fear system (Rosen & Schulkin, 1998).

Association of Long-Term Amygdala Kindling and Hippocampal Alterations

In a series of studies carried out by Kalynchuk and colleagues, interictal emotional behaviours seen in long-term amygdala kindled rats have been associated with hippocampal cellular alterations (Kalynchuk, 2000). In general, the identified alterations suggest an imbalance in the inhibitory (GABAergic) and excitatory (glutamatergic) systems within cellular subpopulations of the hippocampus. Ligand binding to inhibitory receptors (such as $GABA_A$ receptors and benzodiazepine receptors, BZ) is increased in the dentate gyrus of the hippocampus (Kalynchuk, Pearson, Pinel, & Meaney, 1999), whereas ligand binding to excitatory receptors (such as AMPA and NMDA) is decreased in kindled rats (McEachern, Kalynchuk, Fibiger, Pinel, & Shaw, 1996). Furthermore, kindling is associated with significant increases in serotonin 1A ($5\text{-}HT_{1A}$) receptor binding and mRNA expression in the granule cell layer of the dentate gyrus (Kalynchuk, Pinel, & Meaney, 2006). Activation of this receptor population hyperpolarizes cells by increasing intracellular levels of cAMP (Markstein, Matsumoto, Kohler, Togashi, Yoshioka, & Hoyer, 1999). This is significant because in mice, high levels of $5\text{-}HT_{1A}$ receptors have been associated with increased defensiveness and fearful coping responses that are similar to the behavioural changes seen after amygdala kindling (Korte et al., 1996).

These initial findings suggested that increased fear responses demonstrated by long-term amygdala kindled rats may be associated with impaired hippocampal synaptic plasticity and thus impaired hippocampal function. Importantly, it was recently shown that fear behavior in long-term kindled rats is accompanied by decreased FOS immunoreactivity in the CA1 and dentate gyrus regions of the hippocampus, as well as the piriform cortex, which is a projection area to the hippocampus (Kalynchuk et al, 2001). FOS is an immediate early gene that is thought to be indicative of cellular activity: When cells are active, FOS levels are high and when cells are quiet and FOS levels are low. Therefore, decreased FOS expression in hippocampal regions in kindled rats suggests that kindling could dampen hippocampal plasticity and impair behaviours that depend on normal hippocampal plasticity, such as behaviors related to cognition and emotion. One thing worth mentioning here is that kindling also decreases insulin-like growth factor-I (IGF-I) receptor binding sites in the CA1 region and dentate gyrus of the hippocampus providing further evidence for altered cellular growth and plasticity within the hippocampus (Kalynchuk, Meaney, & Kar, 2002).

Kindling may also affect normal stress responding. Kalynchuk and Meaney (2003) recently reported that kindling decreases glucocorticoid (GR) receptor mRNA expression exclusively in the dentate gyrus, suggesting that kindled rats may have some difficulty reacting normally under stressful conditions. Glucocorticoid receptors are instrumental in shutting off the physiological responses to stress. Therefore, the fear responses exhibited by kindled animals may be greater in magnitude and duration due to impairments in the stress circuit governed by GR receptors (McEwen & Sapolsky, 1995). As elevated glucocorticoid levels have been shown to impair explicit memory tasks in rats, such as spatial memory performance, it follows that— along with altered

hippocampal circuitry— long-term amygdala kindled rats should show deficits in tasks requiring spatial navigation.

Hippocampal-Mediated Behaviour

Because complex partial seizures most commonly originate within the amygdala or hippocampus, it should not be surprising that mesial temporal lobe epileptics often show deficits in memory. This is commonly seen as deficits in verbal memory, with deficits in visuospatial memory also common although to a lesser extent (Devinsky & Najjar, 1999). As the function perceived to be most indicative of a structurally and functionally sound hippocampus in animals is that of spatial cognition, spatial memory tasks are generally the focus of testing for hippocampal function in laboratory animals (Whishaw & Kolb, 2005).

Although the dorsal hippocampus plays a large role in spatial learning and memory, the ventral hippocampus has been associated with anxiety-related behaviours. These distinctions in intrahippocampal function are based on both behavioural studies and connectivity, with the dorsal hippocampus possessing visuospatial and sensory connections, whereas the ventral hippocampus possesses connections with the amygdala, hypothalamic-pituitary-adrenal (HPA) axis structures, and the prefrontal cortex. Bannerman and colleagues postulate that the connections between the amygdala and ventral hippocampus mediate anxiety behaviours, including the approach/avoidance conflict that determines whether an animal should approach a given stimulus to acquire more information (Bannerman, Grubb, Deacon, Feldon, & Rawlins, 2003). As amygdala kindling has been shown to cause a secondary disruption of the ventral hippocampus, there is growing evidence that this is the network mediating the fear-anxiety behaviours of amygdala stimulated animals (Howland, Hannesson, Barnes, & Phillips, 2007; Koch

& Ebert, 1998). Overall, this theory helps to explain the findings that an animal's decision to approach or avoid a novel stimulus may be impaired by a hippocampal lesion, thus predicting abnormal exploratory patterns (O'Keefe & Nadel, 1978).

Tests of Spatial Learning and Habituation

Spatial learning and memory are most often assessed through structured tests that require the animal to reach a goal. Two tasks that have been repeatedly utilized to assess spatial memory in rats are the radial arm maze (Becker, Walker, & Olton, 1980) and the Morris water maze (Moser, Krobert, Moser, & Morris, 1998). Both tasks require the rats to orient themselves according to previously learned static and external (allocentric) visual information in order to navigate through the apparatus and achieve their goal—whether it is a food reward or an escape platform.

Whishaw and colleagues argue that spontaneous open field exploration provides the most information regarding an animal's spatial learning and habituation, because the animal is less restricted than it would be in the imposed spatial and task-related circumstances of the radial arm maze or the Morris water maze (Wallace, Hines, & Whishaw, 2002; Lehmann, Clark, & Whishaw, 2007). Exploration in an open field is identified by the establishment of a "home base", a location where the rat spends the majority of its time (Eilam & Golani, 1989). An increased level of comfort, based on the learning of home base cues, is indicated as the rat demonstrates behaviours such as increased grooming, rearing, pivoting, exploration, stopping, and decreased speed of movement within the home base area (Golani, Benjamini, & Eilam, 1992). The spatial mapping theory proposed by O'Keefe and Nadel (1978) suggests that exploratory behaviour is completely reliant on hippocampal function and thus, that hippocampal damage should abolish exploratory abilities. Recent open field studies suggest that

hippocampal-lesioned rats display only minor impairments in exploration, whereas complete habituation to the environment remains impaired (Clark, Hines, Hamilton, & Whishaw, 2005). As an animal's ability to habituate is reliant not only on its spatial learning, but also on the perceived security of the environment, it follows that animals that are more fearful would experience greater impairments in habituation (Whishaw, Gharbawie, Clark, & Lehman, 2006).

The Impact of Kindling on Spatial Learning and Habituation

Assessment of spatial memory on both the radial arm maze and the Morris water maze reveals some disruptions in acquisition and retention in fully-kindled rats, but not partially-kindled rats (Leung et al., 1990; Sutherland & McDonald, 1990). Hannesson and colleagues (Hannesson, Wallace, Pollock, Corley, Mohapel & Corcoran, 2004) used a delayed-match-to-place task to distinguish reference memory in the standard Morris water maze task from measurements of spatial navigation and working memory (Steele & Morris, 1999). Their results indicated that short-term kindling of the dorsal hippocampus disrupted spatial learning as confirmed by impaired acquisition of the delayed-match-to-place task (Hannesson et al., 2004). However, short-term amygdala kindling generally produces no impairments in performance in the water maze (Nieminen et al., 1992). One study did reveal that rats subjected to extensive amygdala kindling (experiencing > 300 stimulations) showed small deficits in acquisition of this task (Cammisuli et al., 1997), but no other studies have assessed spatial memory in long-term kindled rats. The diversity in results with short-term kindling underlines the need for more research in this area, as it is currently difficult to determine which factors are critical: the site of stimulation, the number of kindling stimulations, or the fact that the task is acquired under novel circumstances that stimulate fear could all be important

determinants of performance.

Previously, Kalynchuk and Wintink (2005) used a delayed-match-to-place task in the water maze to try to rule out the potential confound of novelty, as it may impair performance in fearful rats that otherwise may have no deficits in cognition. In this version of water-maze testing, rats learn the task prior to the onset of kindling, before fearful behavior develops, and spatial learning is then re-examined at several time points during kindling. Amygdala kindled male rats showed impairments in spatial memory after 45 to 75 stimulations, confirming a deficit in hippocampal function (Kalynchuk & Wintink, 2005). Interestingly, Hines and Whishaw (2005) found impaired performance on the Morris water maze and complete deficits on the delayed-match-to-place task in hippocampal-lesioned rats. Furthermore, despite significant deficits in spatial cognition, the rats only showed impaired habituation when they were unable to integrate self-movement cues with the visual home base. These results support the conclusions of Niemenin and colleagues (1992) who, when working with models of fully amygdala kindled rats, concluded that the amygdala is not associated with spatial learning and memory unless emotional associations were involved, as would be the case in the formation of a home base for security.

In their discussion of the impaired habituation observed in hippocampal-lesioned animals, Whishaw and colleagues questioned the role of stress and anxiety in the activity observed in hippocampal-lesioned rats in an open field exploration (Clark, Hines, Hamilton, & Whishaw, 2005). The use of long-term amygdala kindled rats in the same paradigm offers the chance to clarify this observation through the assessment of a model involving not a complete structural hippocampal lesion, but rather a functional lesion specifically associated with increased anxiety observed in open field paradigms.

Specific Hypotheses and Purpose of the Study

The main purpose of this thesis is to determine whether the fear behaviour produced by long-term amygdala kindling is associated with a decreased ability to habituate to a novel environment. This decrease in habituation is hypothesized to be a behavioural demonstration of the increased arousal and anxiety exhibited by animals with hippocampal dysfunction.

In *Experiment 1*, fear and exploratory behavior was assessed in short-term and long-term amygdala kindled rats in order to identify the behavioural impact of a novel environment on the rats' ability to cope and habituate. It was hypothesized that the long-term amygdala kindled rats would show behaviours indicative of impaired habituation, including heightened fear and consistently elevated levels of exploration.

In *Experiment 2*, a larger open field was used to directly compare the behaviour of rats that had been kindled, potentially possessing a functional hippocampal lesion, on a task that was previously shown to be hippocampal-dependent. The addition of visual cues, including a home base cue, removal of walls, and a larger arena allowed for a more precise analysis of the exploratory, habituation, and fear behaviours displayed by kindled rats. The hypotheses were that long-term kindled rats would exhibit: 1) exploratory behaviours that are intact, although consistently elevated, 2) impaired ability to habituate to environmental cues and circumstances, and 3) heightened fear behaviours. Through the use of a model that creates a functional lesion, without complete destruction of the hippocampus and the complete abolishment of its function, the aforementioned experiments provide a promising way to establish the role of the hippocampus in the fear behaviour produced by long-term amygdala kindling.

Chapter 2: Experiment 1

Long-term Amygdala Kindled Rats Show Decreased Habituation in a Novel Open Field

The purpose of Experiment 1 was to determine whether the heightened fear behaviour of long-term kindled rats, previously observed in one-day novel open field tests, would remain elevated over five days of testing. Novelty is a stressor commonly used in testing paradigms to establish a rat's emotional responsiveness. As heightened fear behaviours are seen in kindled rats as a response to the novelty of the situation, a sustained elevation in fear behaviours should be indicative of impaired habituation to the environment. Kalynchuk et al. (1999) established that unfamiliarity played a significant role in the expression of kindled fear, as identified through elevated resistance to capture from a novel open field over 5 days of testing. A study performed using the same open field paradigm showed that, in contrast to controls, long-term kindled rats do not decrease their rate of exploration from the first to the final minute of testing (Wintink, Young, Davis, Gregus, & Kalynchuk, 2003). In Experiment 1, I set out to expand on the findings of Kalynchuk and colleagues through more elaborate examination of the kindled rats' exploratory behaviour and through a replication of the resistance-to-capture findings. In addition to the rats' distance traveled during the initial (30 second) exposure to the initially novel open field, exploratory behaviour over each (5 minute) session was

assessed. In order to assess the rats' adjustment to the environment, resistance to capture measures were also taken.

Methods

All handling, surgery, kindling, and testing were carried out by myself, to ensure that the methodology was consistent.

Subjects

The subjects were 33 adult male Long-Evans rats (approximately 300 g at the time of arrival) purchased from Charles River Canada (St. Constant, QC). Rats were individually housed in rectangular polypropylene cages in a colony room with the temperature maintained at 21°C and a 12:12 h light:dark cycle with lights on at 8:00 a.m. Purina rat chow and water were provided ad libitum. All experimental procedures were conducted in accordance with the University of Saskatchewan Animal Care Committee Guidelines, as outlined by the Canadian Council on Animal Care.

Surgery

Rats were given at least 7 days to habituate to the colony before undergoing stereotaxic surgery. At the time of surgery, all rats weighed between 275 and 325 g. Rats were anesthetized with isoflurane (4.5% for sedation, 3% maintenance dose; Abbott Laboratories, St. Laurent, QC) and given Anafen (ketoprofen, 10 mg/kg, 1 ml/kg, s.c., Merial, QC) as an analgesic. Rats were placed in a stereotaxic frame, and the skull was leveled with the incisor bar set at –3.3 mm (Paxinos & Watson, 1998). A midline scalp incision was made to expose the top of the skull and allow the overlying skin to be retracted. A single bipolar electrode (MS-303-2, Plastics One, Roanoke, VA) was implanted in the left basolateral amygdala, with the electrode tip aimed at -2.8 mm posterior, +5.0 mm to the left, and -8.5 mm ventral to the skull surface at bregma

(Paxinos & Watson, 1998). The electrode was secured to the skull with four stainless steel screws and dental acrylic. Finally, Hibitane (1% chlorohexidine acetate, Ayerst, Montreal, QC), an antibacterial and antifungal agent, was applied around the incision site in order to reduce the risk of infection and facilitate healing.

Kindling

After a postsurgical recovery period of at least 1 week, the rats were randomly assigned to one of three groups: one group received 69 sham stimulations followed by 30 kindling stimulations (30-stim group, $n = 11$), one group received 99 kindling stimulations (99-stim group, $n = 11$), and one group received 99 sham stimulations (sham-stim group, $n = 11$). Three stimulations were delivered each day (5 days per week) with a minimum of 3 h between consecutive stimulations. For each stimulation, the stimulation lead was attached, the rat was placed in a plastic box (60 cm x 20 cm x 15 cm), and a stimulation was delivered (1 s, 60 Hz, 400 µA square waves). After all convulsive activity had ceased, the lead was removed and the rat was returned to its home cage. Rats receiving sham stimulations were treated in exactly the same way except that no current was delivered.

In order to assess the progression of kindling, seizure severity was recorded in accordance with the convulsion classes described by Pinel and Rovner (1978) and Racine (1972b). The convulsion severity is as follows: Class 1: orofacial automatisms; Class 2: head nodding and orofacial automatisms; Class 3: head nodding, orofacial automatisms, and forelimb clonus; Class 4: head nodding, orofacial automatisms, forelimb clonus, and rearing; Class 5: head nodding, orofacial automatisms, forelimb clonus, rearing, and falling once; Class 6: head nodding, orofacial automatisms, forelimb clonus, rearing, and

multiple falls; Class 7: a Class 6 with running fits; Class 8: any of the preceding symptoms, along with periods of tonus.

Behavioural testing

Open Field. Behavioural testing began 1 day after the final stimulation. All behavioural testing took place during the light phase of the light:dark cycle. The open field was a square box (70x70x60 cm) with black wooden walls and a Plexiglass floor divided by tape into 36 identical (10x10 cm) squares. It was located in a brightly lit testing room that was separate from the colony room. Each rat was individually placed into a different corner of the unfamiliar open field for 5 min, while an experimenter who was unfamiliar to the rat sat quietly in the room, out of sight of the rat. All behavioral sessions were recorded on a video camera for off-line analysis. The distance traveled during the first 30 s and the total 5 min testing session was calculated for each rat. A rat was recorded as having traveled 10 cm when the centre of its back crossed from one 10 cm square into an adjacent square. This procedure was carried out for 5 consecutive days and every day the same equipment was used.

Resistance-to-capture. On the first and the last day of open field testing, each rat's resistance to being captured from the open field was assessed. This testing occurred at the end of the 5 minute open-field test, at which point an experimenter, who was unfamiliar to the rat, attempted to pick the rat up from above while wearing a large leather glove. The rat's resistance to being picked up was scored according to a 7-point resistance-to-capture scale (Kalynchuk et al., 1997): 0 = easy to pick up, 1 = vocalizes or shies away from hand, 2 = shies away from hand and vocalizes, 3 = runs away from hand, 4 = runs away and vocalizes, 5 = bites or attempts to bite, 6 = launches a jump attack. The

open field was cleaned thoroughly with Fantastik® solution in between each trial in order to minimize potential olfactory cues.

Histology

One day after the final day of testing, each rat was deeply anesthetized with sodium pentobarbital (Somnotol, MTC Pharmaceuticals) and perfused transcardially with 0.9% saline solution followed by 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS: 0.1 M, pH 7.4). All brains were kept in the 4% paraformaldehyde fixative for 48h and were then preserved in 0.5% sodium azide in 0.1 M PBS at 4°C until needed. Coronal sections were cut at a thickness of 50 µm on a vibratome and were collected in 0.1 phosphate buffer (PB). Every fourth section was mounted on a slide and stained with 0.1% cresyl violet. The position of each electrode tip was confirmed from the stained slides using a light microscope and a stereotaxic atlas (Paxinos & Watson, 1998).

Statistical analyses

The data were analyzed using Statistical Package for the Social Sciences v. 14.00 (SPSS, Chicago, IL, USA). Two separate two-way repeated measures analysis of variance (ANOVAs) were used to establish differences among the three groups (sham-stim, 30-stim, 99-stim) for either the total distance traveled during the first 30 s of testing, or for the total distance traveled on each of the 5 testing days. *Post hoc* analyses comprised one-way ANOVAs to identify between-group differences on Day 1 and Day 5, and paired t-tests to identify within-group differences in activity between Day 1 and Day 5. Group differences in resistance to capture were assessed in three ways. First, overall differences between different groups and between different days were measured three ways. A Kruskal-Wallis ANOVA was used to determine whether there were group

differences on resistance to capture between Day 1 and Day 5. *Post hoc* analyses were comprised of Mann-Whitney U tests to identify between-group differences on Day 1 and Day 5, and Wilcoxon-Signed Ranks tests were used to identify within-group differences in behaviour between Day 1 and Day 5.

Results

Histology

Figure 1.1 illustrates the electrode tip placement in each subject from Experiment 1. Rats were removed from the behavioural analyses if the electrode-skull apparatus became dislodged before the end of the experiment or if they were found to have electrodes terminating outside of the amygdala. Six rats were removed for these reasons, with the final analyses based on a total of 27 rats: 10 (sham-stim), 8 (30-stim), and 9 (99-stim).

Open Field Activity

Figures 1.2(A) and 1.2(B) illustrate the mean distance traveled by each group of rats during the first 30-s segment and the total 5-min segment, respectively, over the five days of open-field testing. The 99-stim rats displayed significantly lower activity levels in the first 30-s across all 5 days of testing, in comparison to both the 30-stim and the sham-stim rats. This was confirmed by a significant effect of group [F (2, 24) = 13.364, $p < .001$] with significant differences found between the 99-stim group and the 30-stim group ($p < .05$) and between the 99-stim group and the sham-stim group ($p < .001$). A treatment effect was also present for the total 5-min of testing [F (2, 24) = 5.40, $p < .05$]. In contrast to their behaviour in the initial 30-s segment, both the 99-stim and the 30-stim rats traveled a greater distance in the open field ($p < .05$) when compared to the sham-stim group. Finally, the sham-stim and 30-stim groups both showed a significant decrease in

activity over the 5 testing sessions [sham-stim: $t(9) = 2.760$, $p < .05$; 30-stim: $t(7) = 6.290$, $p < .001$], but the the 99-stim group did not [$t(8) = 2.056$, $p = .074$].

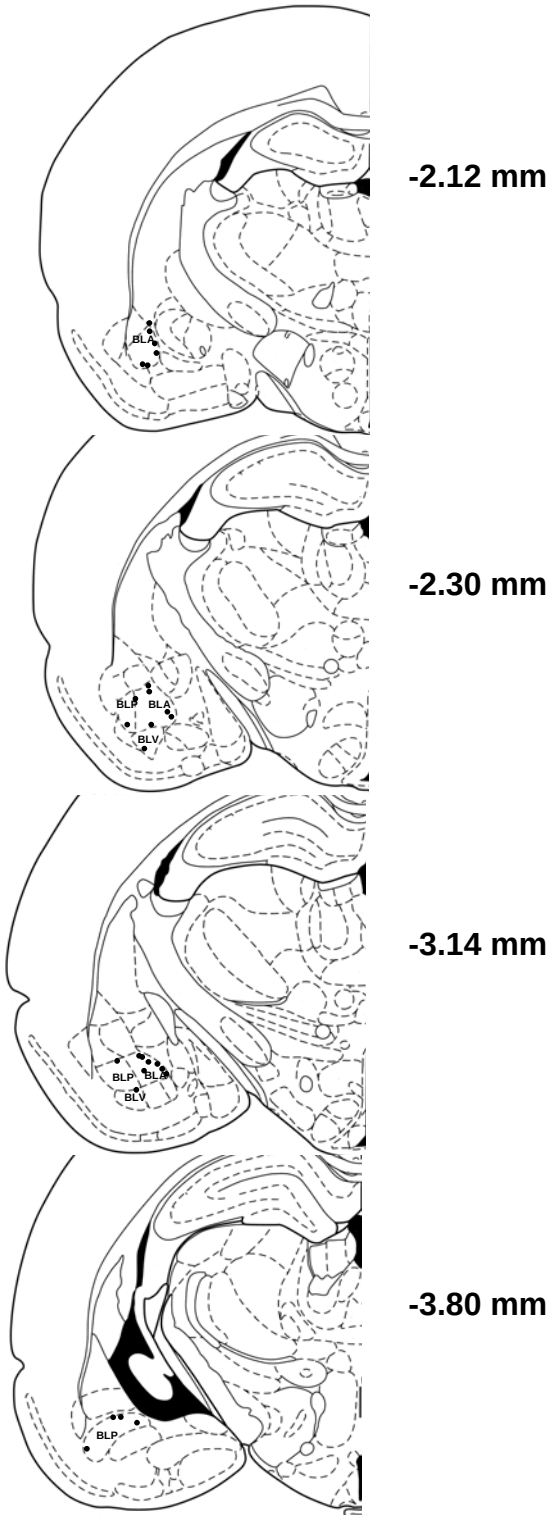
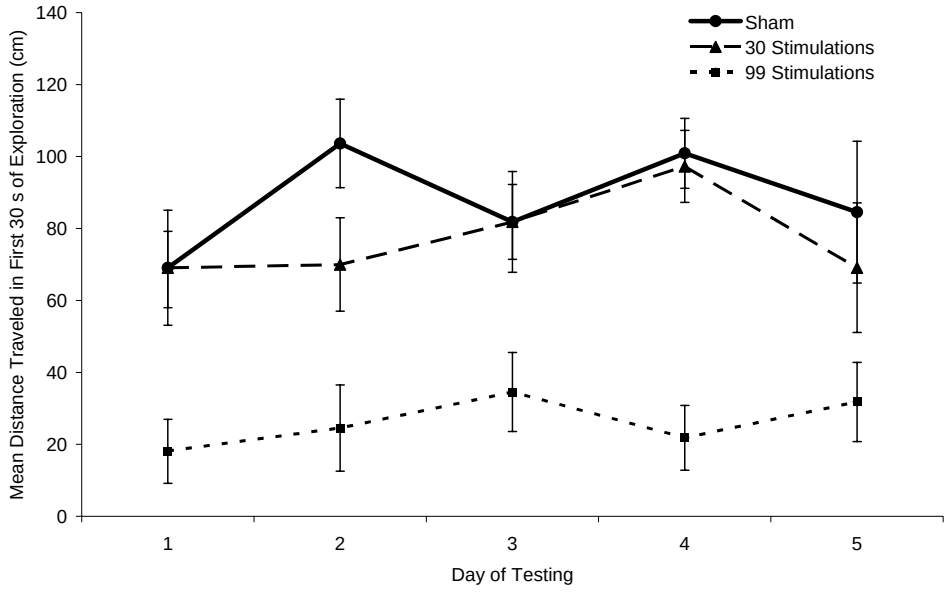


Figure 1.1. Histological results from the three groups of rats in Experiment 1. Only those rats for which data was used are shown, as their electrode terminated in the basolateral amygdalar nuclei (BLA, BLP, BLV).

A



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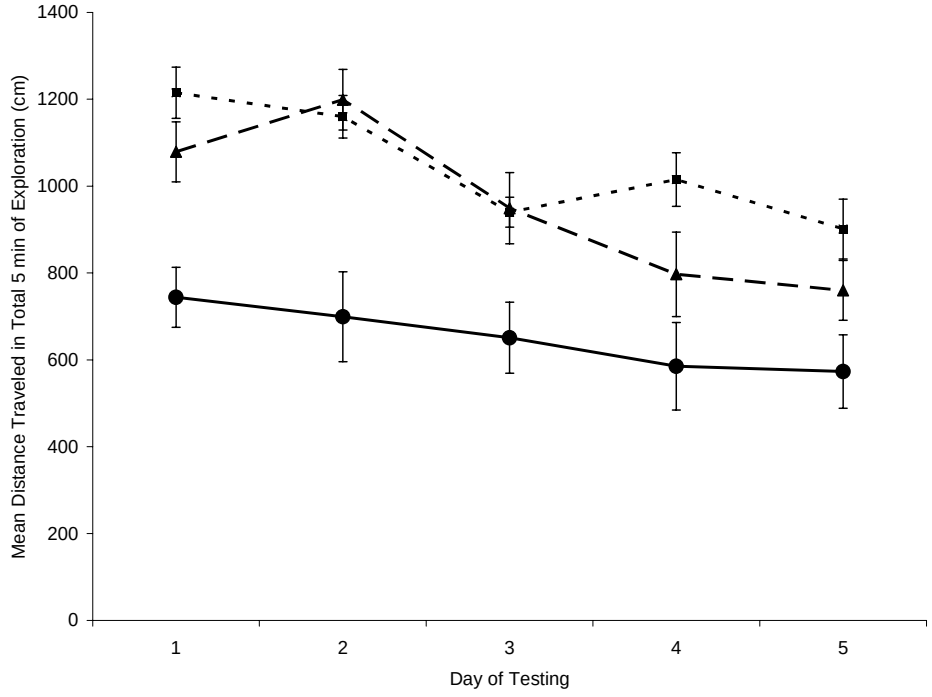


Figure 1.2. Mean (± SEM) distance traveled in first 30 sec (A) and total 5 min (B) of exploration by rats in each treatment group. See text for statistical analyses.

Resistance-to-Capture Test

Figure 1.3 illustrates the mean resistance to capture displayed by all groups on both Day 1 and Day 5. There was a significant effect of treatment group on resistance to capture on both Day 1 [$H(2) = 13.097$, $p < .05$] and Day 5 [$H(2) = 19.589$, $p < .001$]. On Day 1, kindled rats showed significantly higher resistance to capture scores compared to sham-stim rats [30-stim: $U = 12$, $p < .05$; 99-stim: $U = 2.5$, $p < .001$]; however by Day 5, the elevated resistance to capture levels displayed by kindled rats was reduced [30-stim: Wilcoxon $z = -2.232$, $p < .05$; 99-stim: $z = -2.263$, $p < .05$]. Importantly, although resistance to capture did decrease in kindled rats from Day 1 to Day 5 of testing, the Day 5 resistance-to-capture levels were still significantly higher in the kindled rats compared to the sham-stim rats [30-stim: $U = 3$, $p < .01$, 99-stim: $U = 0$, $p < .001$]. Finally, although high levels of resistance to capture appeared to decline with repeated exposure to the open field, it is of worth noting that the 99-stim group maintained a higher level of resistance ($M = 3.7$) relative to the 30-stim ($M = 2.3$) and sham-stim ($M = 0.67$) groups.

Discussion

The purpose of this experiment was to establish whether long-term amygdala kindled rats— previously shown to be highly fearful and hyperexploratory— would be able to habituate to a novel open field.

During the first 30 s of open-field testing, the long-term kindled rats were significantly less active than the rats in the other two groups across all days, indicating high levels of freezing and fear in the initially novel environment. Rats in the long-term kindled group showed increased freezing behaviour during the initial 30 seconds, whereas the rats in the short-term kindled and sham-stim groups began moving almost immediately.

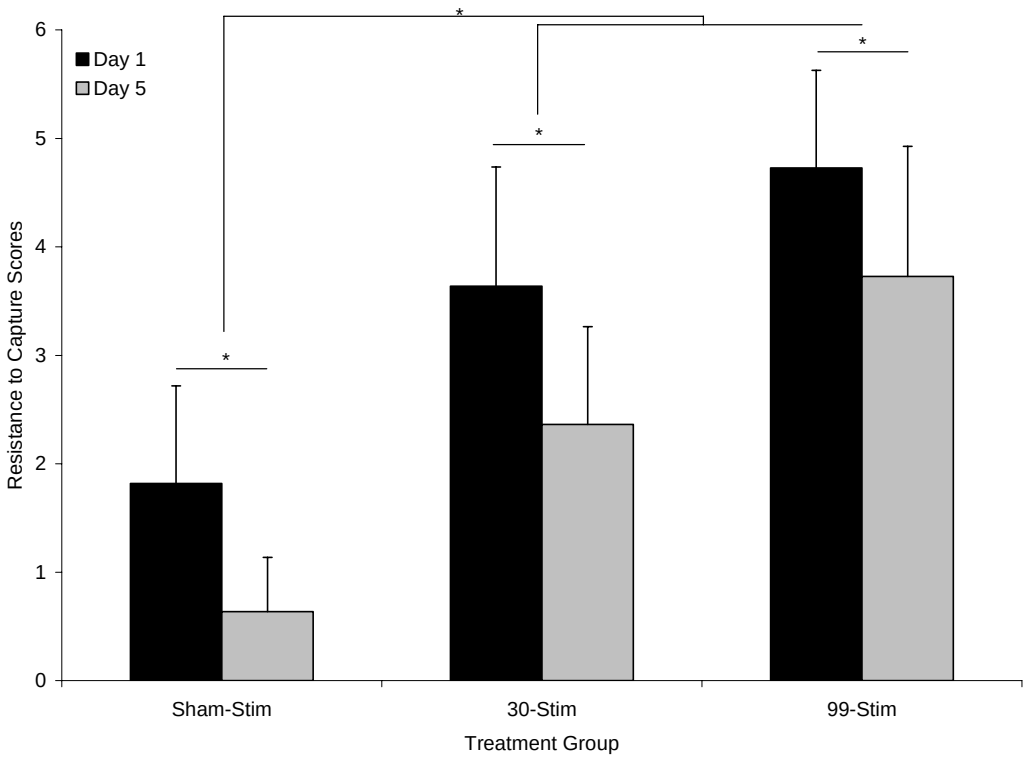


Figure 1.3. Mean (± SEM) open field resistance to capture scores for rats in each treatment group at the end of the first and last days of testing. * $p < .05$

The long-term kindled rats also displayed heightened levels of activity in comparison to the control group, with a minimal decrease in activity across the 5 days of testing. The short-term kindled rats also showed a heightened level of activity on Day 1; however, a significant decrease in distance traveled by Day 5 suggested that this group was able to habituate to the open field. The control group displayed low levels of activity that decreased minimally over the 5 days, indicating minimal fear and overall habituation.

Finally, the long-term kindled rats were significantly more resistant to capture from the open field at the end of the testing session. This fearful behaviour was extremely high on Day 1 and decreased only minimally by Day 5. The short-term kindled group displayed a moderate resistance to capture on Day 1 but had decreased to the level of controls by Day 5.

Taken together, the consistent levels of heightened exploration and fearfulness displayed by long-term amygdala kindled animals suggest a possible impairment in hippocampal function. In order to further assess this possibility, a more elaborate paradigm was used in Experiment 2 of this thesis. This open field paradigm has previously been used to assess the behaviour of hippocampal-lesioned rats, thus allowing a more direct comparison to the impairments in hippocampal function observed in the long-term kindling model.

Chapter 3:

Experiment 2

Long-term Amygdala Kindled Rats Show Decreased Habituation and Elevated Fear in a Novel Open Field

The purpose of Experiment 2 was to determine whether kindled rats show impaired habituation in an open field task that has been shown by Whishaw and colleagues to be sensitive to hippocampal lesions. Experiment 1 identified consistently elevated fear and impaired habituation in long-term kindled animals tested in an initially unfamiliar open field, but this open field was relatively small in size and there are no data about how rats with hippocampal lesions perform in this type of open field. Whishaw and colleagues (Clark et al., 2005) have used a much larger open field that allows for more complicated patterns of exploration in rats. Using this open field, they showed that hippocampal-lesioned animals are unable to habituate to specific aspects of an unfamiliar open field. In Experiment 2, there were two goals. The first goal was to gain increased support for the findings from Experiment 1 that long-term amygdala kindled rats show impaired habituation in an unfamiliar environment. The second goal was to compare the behaviour of long-term amygdala kindled rats to that of short-term kindled rats and control rats in a more elaborate environment in order to determine the specific hippocampal-dependent functional deficits, if any, that these treatment groups

had in common. In order to address these two goals, the current study used an open field that was very similar to the one used by Whishaw and colleagues in their studies of hippocampal-lesions rats.

Methods

All handling, surgery, kindling, and testing were carried out by myself, to ensure that the methodology was consistent.

Subjects

The subjects were 73 adult male Long-Evans rats (250-300 g at time of arrival) purchased from Charles River Canada (St. Constant, QC). Housing and care for the rats was identical to the methods outlined in Experiment 1.

Surgery

One week after arrival in the colony, the rats underwent stereotaxic surgery according to the methods outlined in Experiment 1.

Kindling

After a post-surgical recovery period of at least 1 week, the rats were randomly assigned to one of four stimulation groups: one group received 69 sham stimulations followed by 30 kindling stimulations (30-stim group, $n = 15$), one group received 39 sham stimulations followed by 60 kindling stimulations (60-stim group, $n = 12$), one group received 99 kindling stimulations (99-stim group, $n = 15$), one group received 99 sham stimulations (sham-stim group, $n = 15$). To ensure that no effects were caused by either the surgery or the 6 weeks of handling that the kindled rats received, two additional control groups were used: one group underwent surgery but received no additional handling (surgical control group, $n = 8$), and one group was anesthetized in the same manner as the surgical controls but underwent no additional surgical or

handling procedures (naïve control group, $n = 8$). Due to the large number of rats involved in the experiment, the animals were divided into 4 separate squads in order to allow the time necessary for kindling and behavioural testing. Kindling occurred in the same manner outlined in Experiment 1.

Behavioural testing

Open Field. All behavioural testing took place during the light phase, in an open field located in a brightly lit testing room that was separate from the colony room. The open field was a white, circular wooden table without walls with a diameter of 250 cm. As rats in the smaller open field tend to show thigmotaxic behaviour, the absence of walls had the purpose of potentially reducing this behaviour. The table was held 70 cm above the floor by a circular support (100 cm diameter) that allowed the table to be rotated. The testing room also contained numerous visual cues, including posters, lamps and carts. One primary visual cue, a large black box (40 cm x 60 cm x 80 cm) serving as a home base cue, was located 15 cm from the edge of the open field with its bottom level to the top of the table. This cue remained in one target quadrant for each of the testing sessions. At the start of each session, the rat was placed in the centre of the unfamiliar open field and allowed to explore for 30 min. The same 30 min exploration procedure was carried out for 5 consecutive days. The same open field, testing room, and experimenter were used throughout the 5 days of testing, and were unfamiliar to the rats on day 1 of testing.

Each 30-min testing session was recorded by an overhead digital camera and analysis of all behaviours was carried out using Ethovision 3.1 software (Noldus Information Technology, Leesburg, VA). For the purpose of analysis, the open field was divided into 4 quadrants (one of which was the home base cue quadrant) and 3 annuli

(outer, middle and inner), as seen in Figure 2.1. As habituation was the focus of the study, behavioural measures were compared for the first and fifth days of the study.

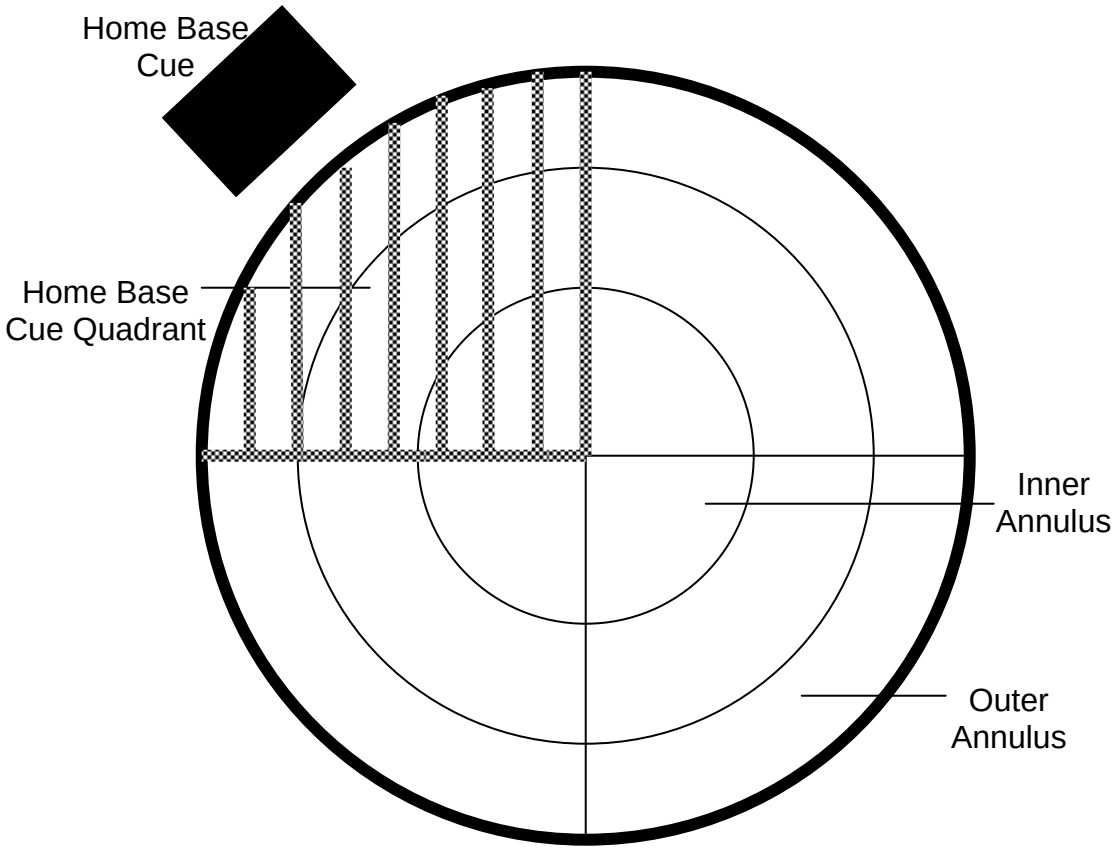


Figure 2.1. Schematic diagram of the open field used in Experiment 2.

Exploratory Behaviour. Exploratory behaviours assessed included total distance traveled, overall velocity of travel, annulus preference based on time and distance traveled, stopping frequency and duration, and rearing.

Home Base Behaviour. Of particular interest were behaviours that would indicate the establishment of a home base, analyzed as behaviours that occurred in the home base cue quadrant. These behaviours included time spent in the cue quadrant, relative distance traveled, relative velocity of travel, stopping frequency and duration, and rearing.

Fear Behaviour. Additional fear behaviours were scored manually and included initial freezing, stopping frequency and duration within the inner annulus, and escape behaviour. Escape behaviour was assessed as the number of purposive jumps that an animal made from the arena. These jumps were distinguished from falling as the animals would spend time looking off the edge of the arena and leap head first, whereas animals that fell were usually turning near the edge of the platform and fell rear first. If a rat jumped or fell from the table surface, it was promptly placed back on the table in the same location from which it had exited. It was anticipated that these jumps might occur as this behaviour had previously been observed in 99-stim rats tested on an elevated-plus maze (Kalynchuk et al., 1997).

Histology

Electrode placements were verified according to the methods outlined in Experiment 1.

Statistical analyses

The data were analyzed using SPSS 14.0. Separate repeated measures ANOVAs were used for each of the open field exploration and home base establishment measures. *Post hoc* analyses comprised one-way ANOVAs or paired t-tests in order to determine

between-group differences for these measures on Day 1 and Day 5 or within-group differences between Day 1 and Day 5. Between-group differences in the percentage of rats from each group that jumped off of the open field were analyzed using a Chi-square test. *Post hoc* non-parametric Mann-Whitney U tests were then used to identify specific between-group differences on Day 1 and Day 5.

Results

Histology

Figure 2.2 illustrates the electrode tip placement in each subject from Experiment 2. Rats were removed from the behavioural analyses if the electrode-skull apparatus became dislodged before the end of the experiment or if they were found to have electrodes terminating outside of the amygdala. Eight rats were removed for this reason, with the analysis based on a total of 63 rats: 14 sham-stim, 12 30-stim, 11 60-stim, 12 99-stim, 8 surgical control, and 8 naïve control.

Open Field Activity

As no significant differences were found between the three control groups (sham-stim, surgical control, naïve control) on any of the behavioural measures, these three groups were pooled in all analyses to form one control group. Thus, analysis of all behaviours was made between four groups: control, 30-stim, 60-stim and 99-stim.

Exploratory Behaviour. As can be seen in Figure 2.3, all kindled groups displayed similar levels of activity across both days, which were higher than the activity levels of the control group. Nevertheless, the distance traveled by the kindled groups decreased significantly by the final day. A significant difference in distance traveled was present for testing day [$F(3,59) = 50.15$, $p < .001$], group [$F(3,59) = 12.99$, $p < .001$], and a marginally significant interaction between group and day [$F(3,59) = 2.47$, $p = .070$].

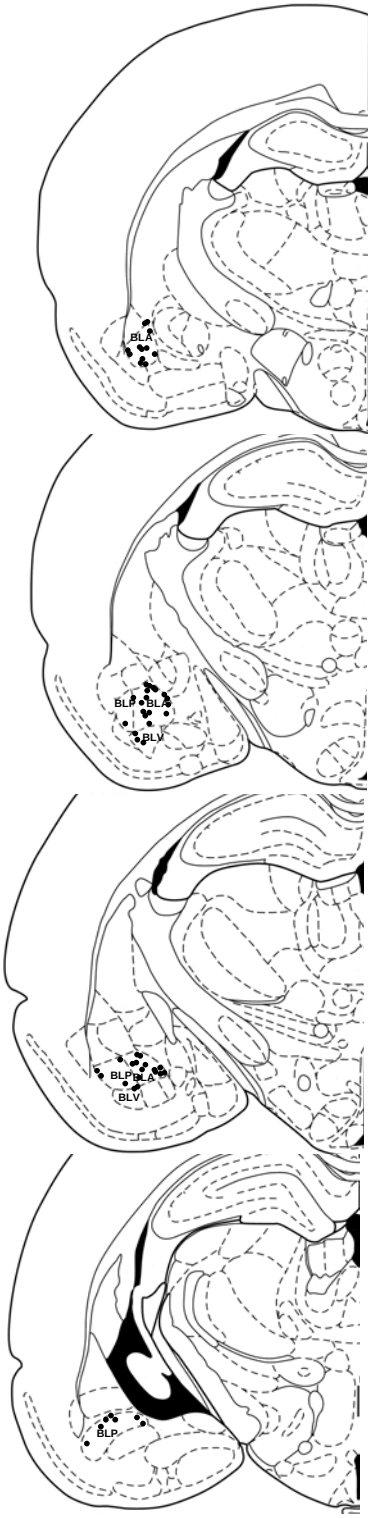


Figure 2.2. Histological results from the three groups of rats in Experiment 2. Only those rats for which data was used are shown, as their electrode terminated in the basolateral amygdalar nuclei (BLA, BLP, BLV).

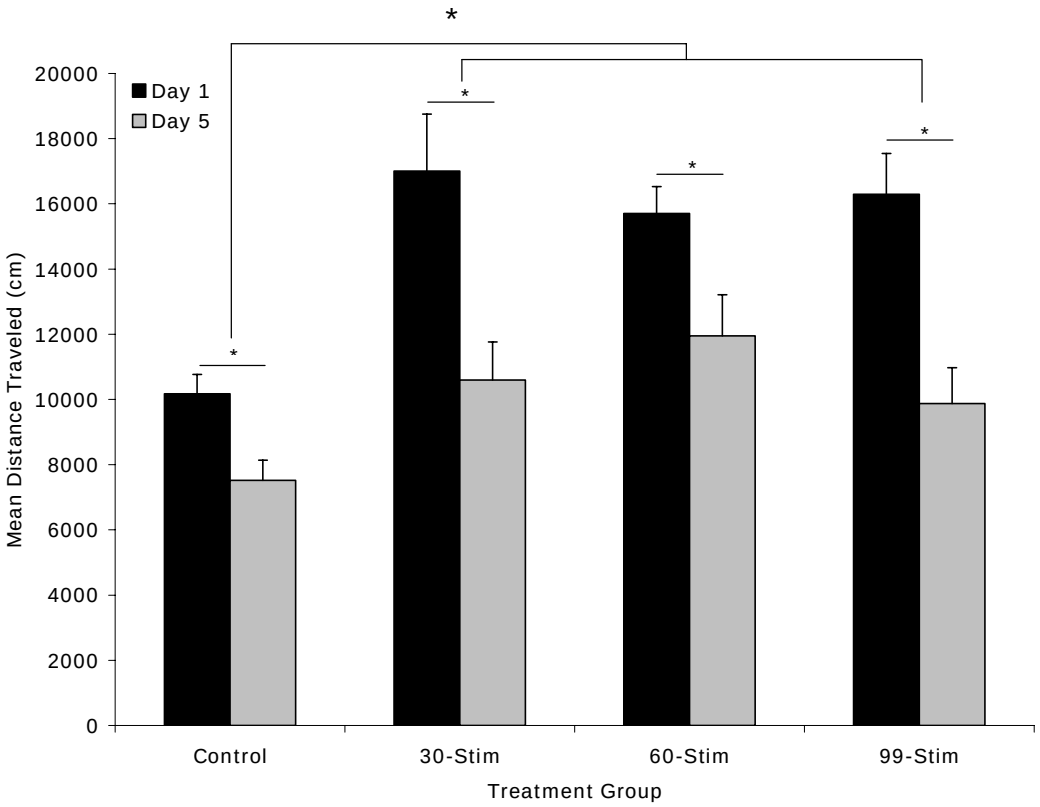


Figure 2.3. Mean (± SEM) total distance traveled by rats in each treatment group over the entire 30 minute testing session. * $p < .001$

Post hoc analyses revealed that all kindled rats showed a decrease in total distance traveled from Day 1 to Day 5 [30-stim: $t(10) = 4.34$, $p < .001$; 60-stim: $t(11) = 2.85$, $p < .01$; 99-stim: $t(12) = 4.18$, $p < .001$], with a smaller but still significant decrease in travel distance on the part of the control rats [$t(29) = 2.30$, $p < .05$]. The significant between-group differences for the total distance traveled on both Day 1 [$F(3,62) = 15.66$, $p < .001$] and Day 5 [$F(3,62) = 4.59$, $p < .01$] reflected differences between the kindled rats and the controls (all $p < .001$), with no significant differences between any of the kindled groups on either day of testing (all $p > .51$).

The decrease in mean velocity across all groups from Day 1 to Day 5 can be seen in Figure 2.4, which illustrates that the kindled animals started at much higher velocities than the controls. The statistical analyses revealed a significant difference in velocity for testing day [$F(3,59) = 60.71$, $p < .001$] and group [$F(3,59) = 9.49$, $p < .001$], and a significant interaction between day and group [$F(3,59) = 7.01$, $p < .001$]. There was a significant difference between groups on Day 1 [$F(3,62) = 19.78$, $p < .001$] as all kindled groups traveled at higher velocities than the controls ($p < .001$). This difference dissipated by Day 5 [$F(3, 62) = 2.65$, $p = .057$], as all groups approached the same velocity (all $p > .50$). Although the control group displayed no significant decline in velocity from Day 1 to Day 5 [$t(29) = .82$, $p > .41$], all kindled groups did display significant declines in velocity [30-stim: $t(10) = 4.56$, $p < .001$; 60-stim: $t(11) = 3.43$, $p < .05$; 99-stim: $t(12) = 4.21$, $p < .001$].

There were no significant differences in the time spent in the outer annulus, as all groups spent at least 1600 s in the outer annulus on both Day 1 and Day 5, illustrating that the rats maintained a preference for the outer annulus. Analysis of distance traveled revealed that there were significant effects of day [$F(1,59) = 78.96$, $p < .001$] and group

[$F(3, 59) = 9.29$, $p < .001$], and a significant interaction between day and group [$F(3, 59) = 7.76$, $p < .001$]. These results are visible in Figure 2.5 as all kindled groups decreased significantly in their activity level from Day 1 to Day 5 [30-stim: $t(10) = 5.41$, $p < .001$; 60-stim: $t(11) = 3.73$, $p < .01$; 99-stim: $t(12) = 5.48$, $p < .001$], whereas controls did not show the same decrease in activity [$t(29) = -2.74$, $p > .17$]. A significant effect of group was present on Day 1 [$F(3, 62) = 18.78$, $p < .001$], with all kindled groups traveling significantly greater distances than the sham-stim group (all $p < .001$). This effect of group was not present on Day 5 [$F(3, 62) = 1.61$, $p > .19$].

Figures 2.6(A) and 2.6(B) illustrate the mean frequency and duration of stops made on both Day 1 and Day 5. The total number of stops made by the rats was found to be significantly different by group [$F(3, 59) = 3.63$, $p < .05$], but not of day [$F(3, 59) = 2.38$, $p > .05$]. Additionally, the interaction was not significant [$F(3, 59) = 1.86$, $p > .05$]. The main effect of group was only present on Day 1 [$F(3, 62) = 2.925$, $p < .05$] with no specific between-group differences found to be significant. Figure 2.4(B) shows the different pattern that emerged in duration of stopping, as there was a significant effect of day [$F(3, 59) = 72.88$, $p < .001$] and group [$F(3, 59) = 7.417$, $p < .001$] but no interaction [$F(3, 59) = 2.008$, $p > .05$]. All groups spent a greater amount of time inactive on Day 5 than on Day 1 [control: $t(29) = -3.29$, $p < .05$, 30-stim: $t(10) = -5.10$, $p < .001$; 60-stim: $t(11) = -3.71$, $p < .05$; 99-stim: $t(12) = -6.88$, $p < .001$]. A significant difference was found between groups on Day 1 [$F(1,59) = 14.27$, $p < .001$], with all kindled groups differing from controls ($p < .05$) but not from each other. A significant between-group difference was also present on Day 5 [$F(1,59) = 2.96$, $p < .05$] but *post hoc* analyses revealed no specific differences between the treatment groups.

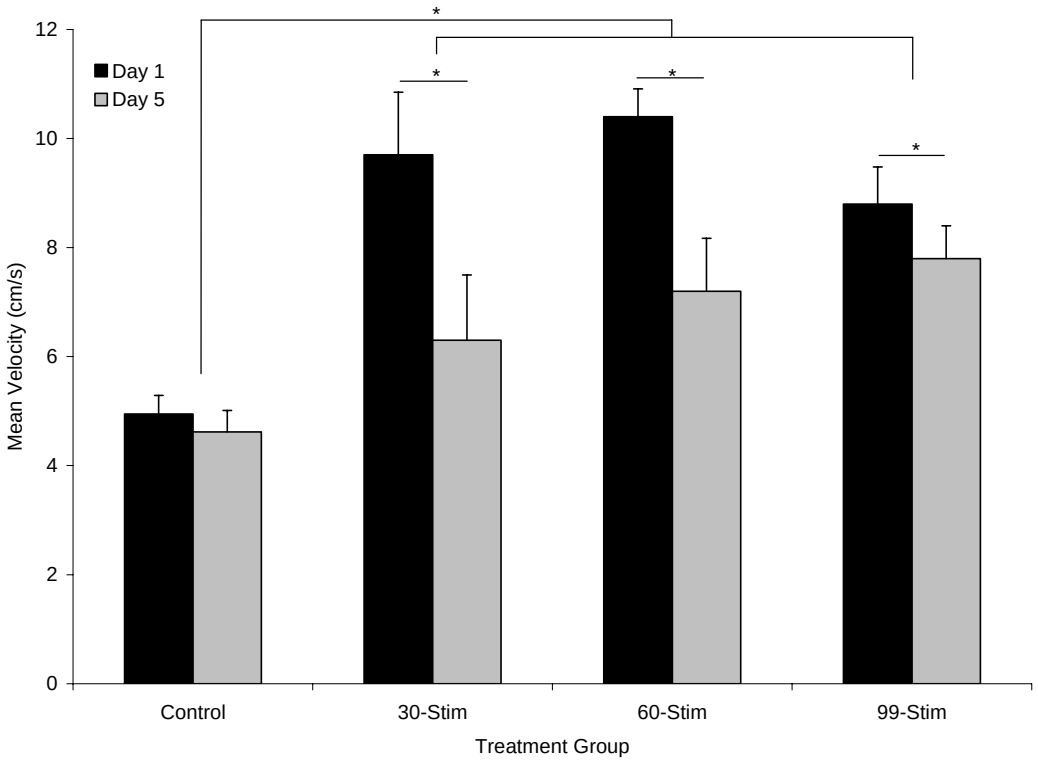


Figure 2.4. Mean (± SEM) total velocity of travel by rats in each treatment group over the entire 30 minute testing session. * $p < .05$

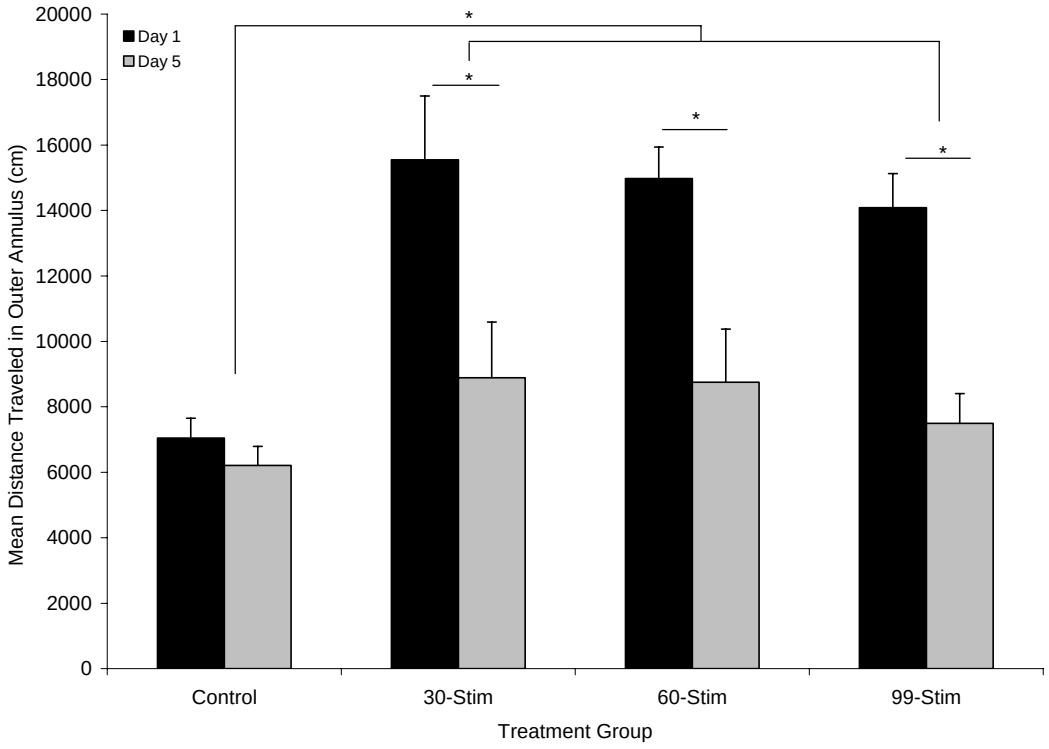
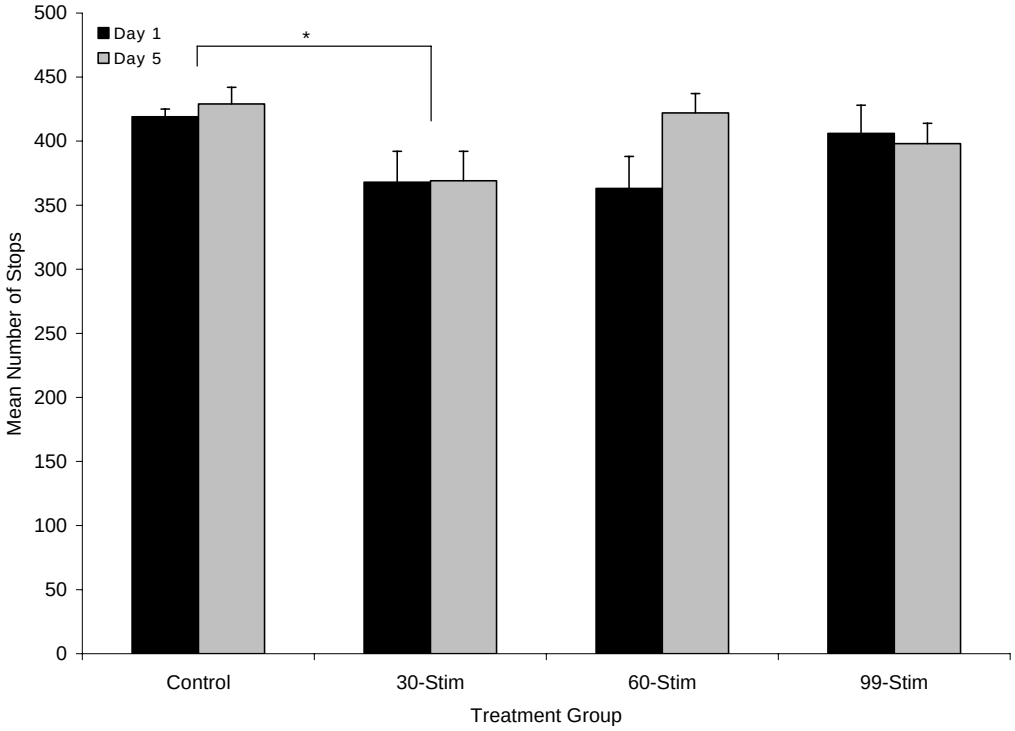


Figure 2.5. Mean (± SEM) total distance traveled in the outer annulus of the open field by rats in each treatment group over the entire 30 minute testing session. * $p < .05$

A



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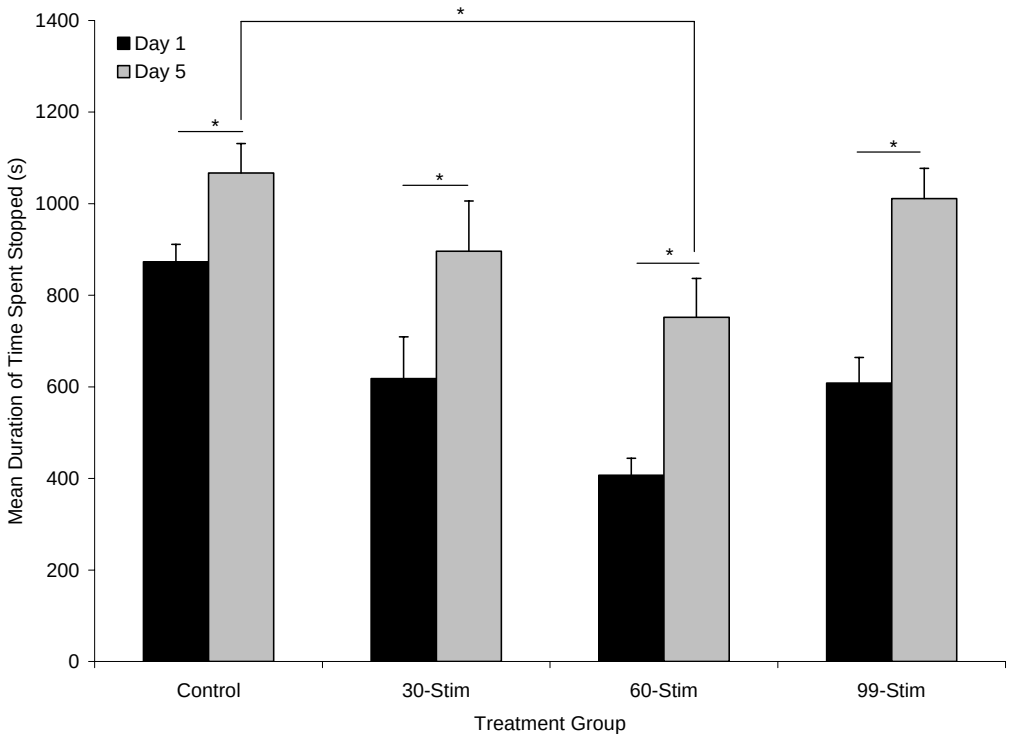


Figure 2.6. Mean (± SEM) number of stops made (A) and duration of time spent stopped (B) by rats in each treatment group over the entire 30 minute testing session. * $p < .05$

The groups also differed in terms of rearing behaviour. There was a significant main effect of day [$F(3, 59) = 4.671$, $p < .05$] and a significant interaction between day and group [$F(3, 59) = 3.542$, $p < .05$]. No significant effect of treatment was found [$F(3, 59) = 2.33$, $p > .05$]. As seen in Figure 2.7, all treatment groups showed decreases in the frequency of rearing whereas the control group showed an increase in this behaviour. These between-day effects were only significant in certain kindled groups [30-stim: $t(10) = 3.47$, $p < .05$; 99-stim: $t(12) = 2.87$, $p < .05$].

Home Base Behaviour. Figure 2.8 illustrates the mean time spent by each group in the home base cue quadrant. There was a significant main effect of group [$F(3, 59) = 3.817$, $p < .05$] but not of day [$F(1, 59) = .241$, $p > .05$], and there was not a significant interaction [$F(3, 59) = 2.082$, $p > .05$]. The time spent in the cue quadrant was only significantly different on Day 5 [$F(1,62) = 4.923$], and only the 99-stim group was different from controls ($p < .05$). The control group was the only group that showed a significant increase in the time spent in the cue quadrant [$t(25) = -2.76$, $p < .05$].

The distance traveled in the cue quadrant was also different among groups [$F(3, 59) = 13.47$, $p < .001$] as well as between days [$F(1, 59) = 47.83$, $p < .001$]. An interaction was also present [$F(3, 59) = 5.78$, $p < .01$]. As can be seen in Figure 2.9, the kindled animals traveled far greater distances in the cue quadrant than did the controls. This was reflected by the significant group differences on Day 1 [$F(3,59) = 24.93$, $p < .001$] and on Day 5 [$F(3,59) = 5.34$, $p < .05$], with all kindled groups different from controls ($p < .001$) but not from each other (all $p > .05$). There was a significant decrease in distance traveled by the rats in all kindled groups [30-stim: $t(10) = 3.187$, $p < .05$; 60-stim: $t(11) = 3.48$, $p < .05$, 99-stim: $t(12) = 4.84$, $p < .001$].

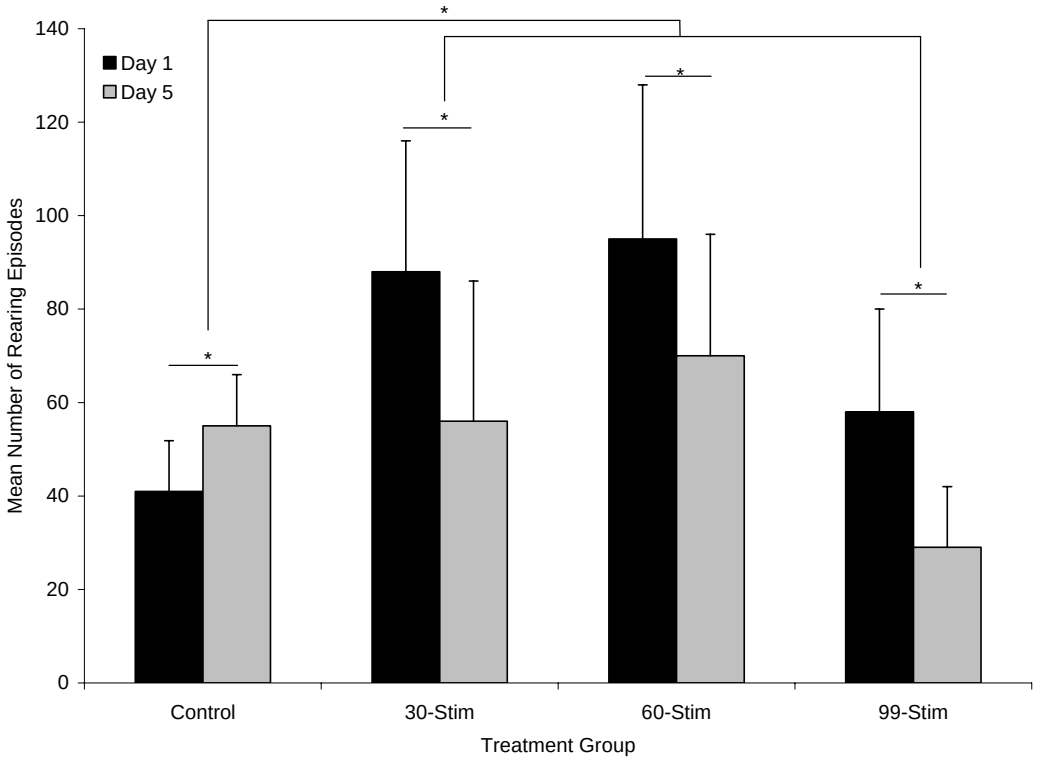


Figure 2.7. Mean (± SEM) number of rearing episodes by rats in each treatment group over the entire 30 minute testing session. * $p < .05$

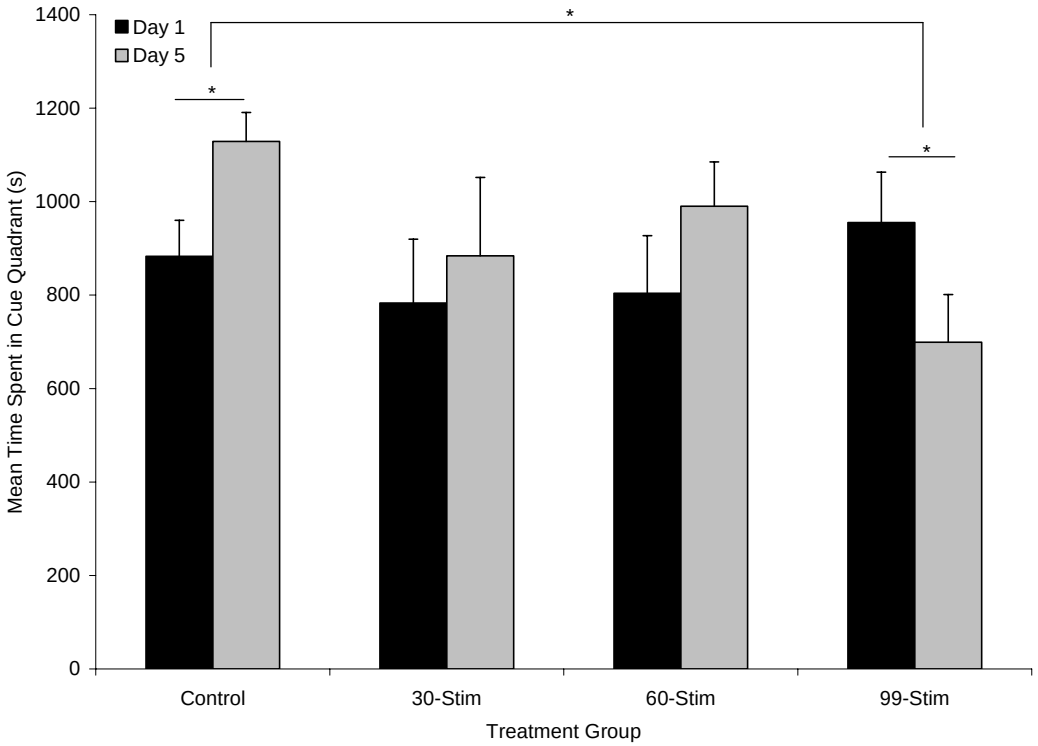


Figure 2.8. Mean (± SEM) time spent in the home base cue quadrant by rats in each treatment group over the entire 30 minute testing session. * $p < .05$

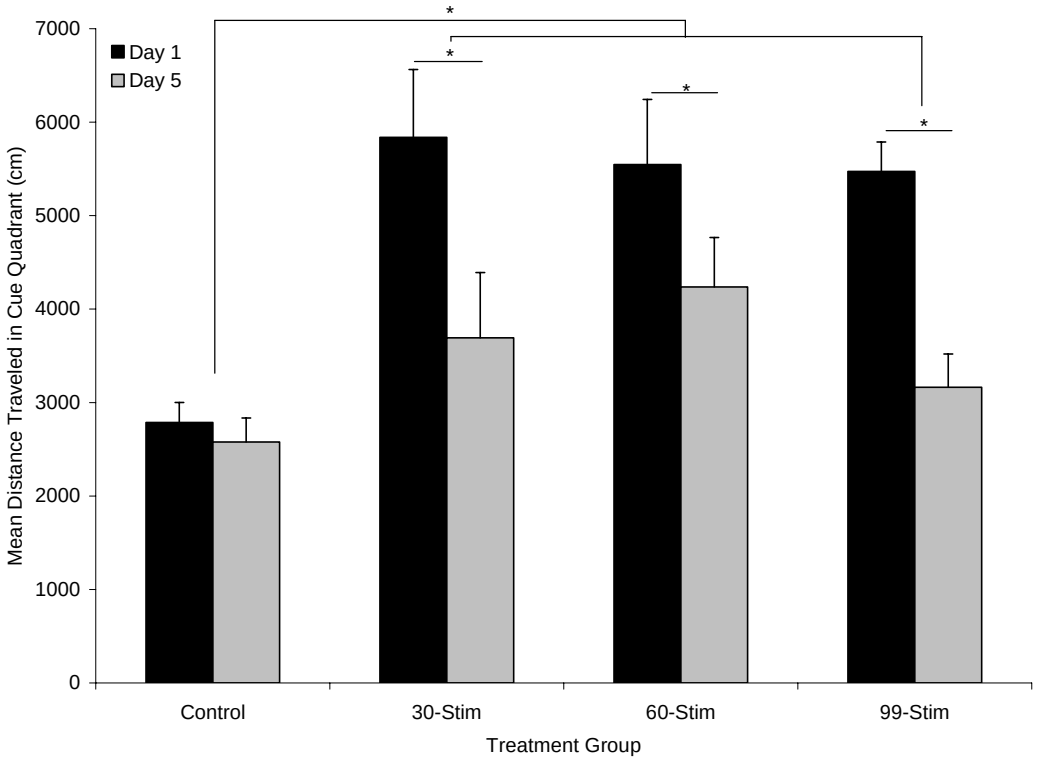


Figure 2.9. Mean (± SEM) distance traveled in the home base cue quadrant by rats in each treatment group over the entire 30 minute testing session. * $p < .05$

In contrast, the control rats showed lower levels of travel that decreased minimally, and non-significantly, from Day 1 to Day 5.

As can be seen in Figure 2.10, the mean velocity within the cue quadrant decreased for all groups from Day 1 to Day 5. There was a significant effect of day [$F(1, 59) = 9.20$, $p < .01$] and group [$F(3, 59) = 6.92$, $p < .001$]; however, there was no significant interaction between day and group [$F(3, 59) = 1.91$, $p > .05$]. Despite the far greater decreases in velocity exhibited by the kindled groups, they all remained significantly above the mean velocities demonstrated by the control group on both Day 1 and Day 5 (all $p < .05$).

The mean number of stops made within the cue quadrant identified no main effect of day [$F(1, 59) = 0.53$, $p > .05$], group [$F(3, 59) = 1.56$, $p > .05$], and no significant interaction [$F(3, 59) = 1.83$, $p > .05$]. Nevertheless, Figure 2.11(A) demonstrates quite clearly that the 99-stim group showed a decrease in the number of stops made, whereas all other groups showed increases in the number of stops. The time spent stopped in the cue quadrant showed a main effect of group [$F(3, 59) = 3.29$, $p < .05$] and day [$F(1, 59) = 9.637$, $p < .01$] however, there was not a significant interaction of group and day [$F(3, 59) = 1.29$, $p > .05$]. The 99-stim group was significantly different than controls ($p < .05$) and actually showed a minor decrease in stopping time in the cue quadrant from Day 1 ($M = 390.13$ s) to Day 5 ($M = 371.93$ s). This difference is illustrated in Figure 2.11(B).

Figure 2.12 displays the changes in rearing in the home base quadrant from Day 1 to Day 5. There was a marginally significant main effect of group [$F(3, 59) = 2.65$, $p = .057$], as well as a significant interaction [$F(3, 59) = 3.64$, $p < .05$]. There was no significant effect of day [$F(1, 59) = 1.18$, $p > .05$].

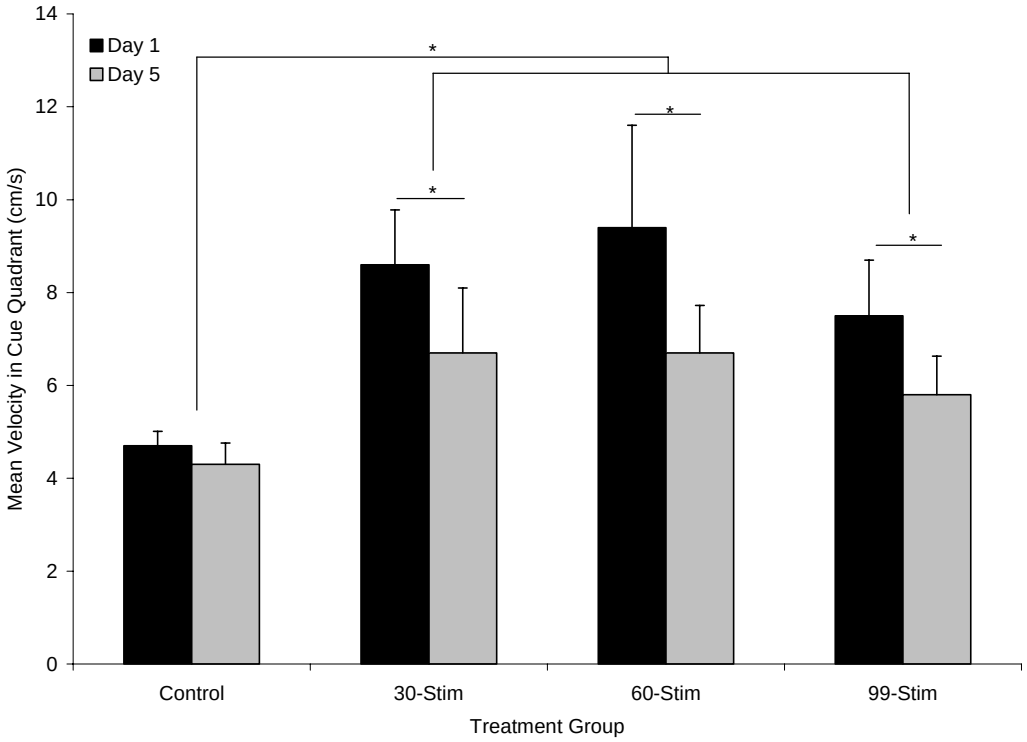
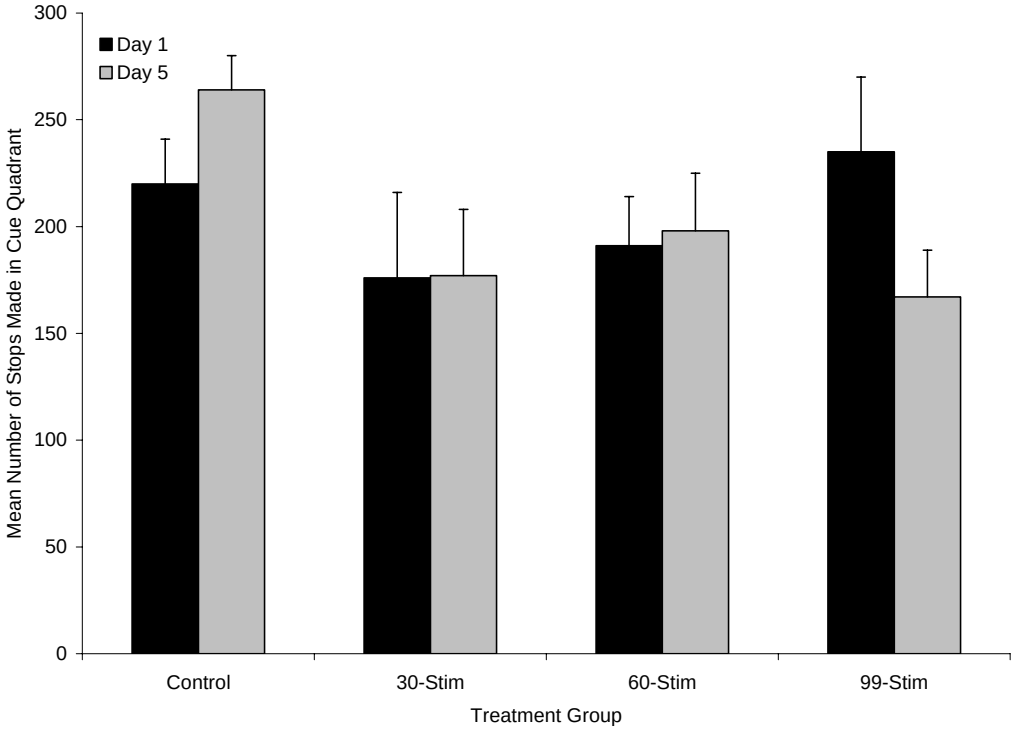


Figure 2.10. Mean (± SEM) velocity of travel in the home base cue quadrant by rats in each treatment group over the entire 30 minute testing session. * $p < .05$

A



B

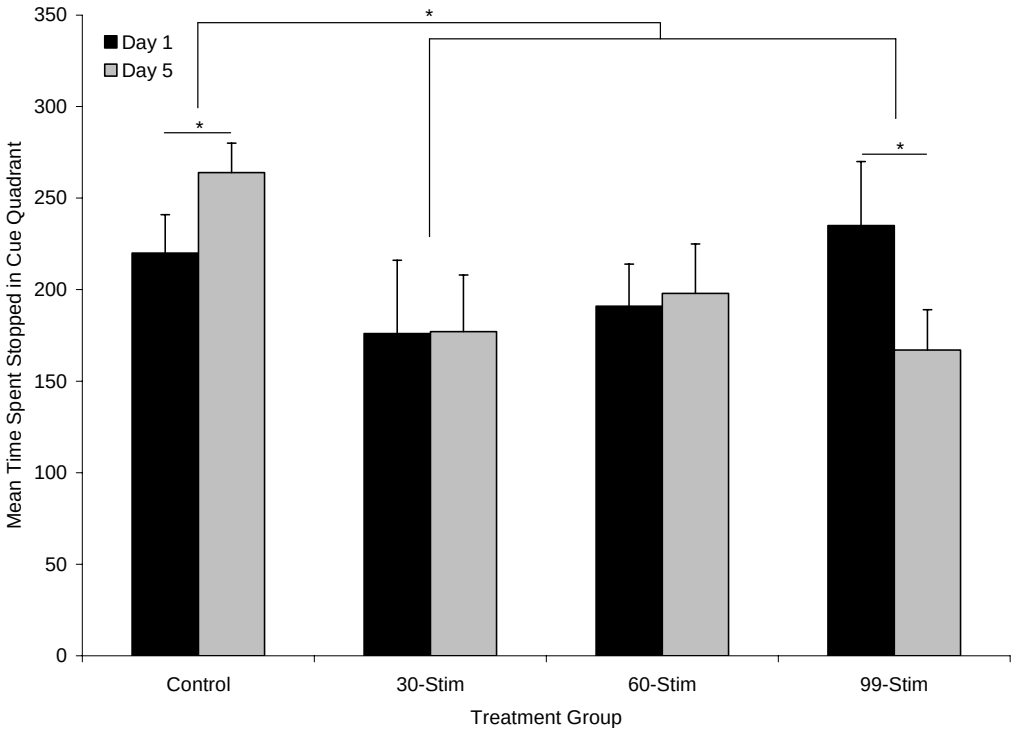


Figure 2.11. Mean (± SEM) number of stops made (A) and duration of time spent stopped (B) in the home base cue quadrant by rats in each treatment group over the entire 30 minute testing session. * $p < .05$

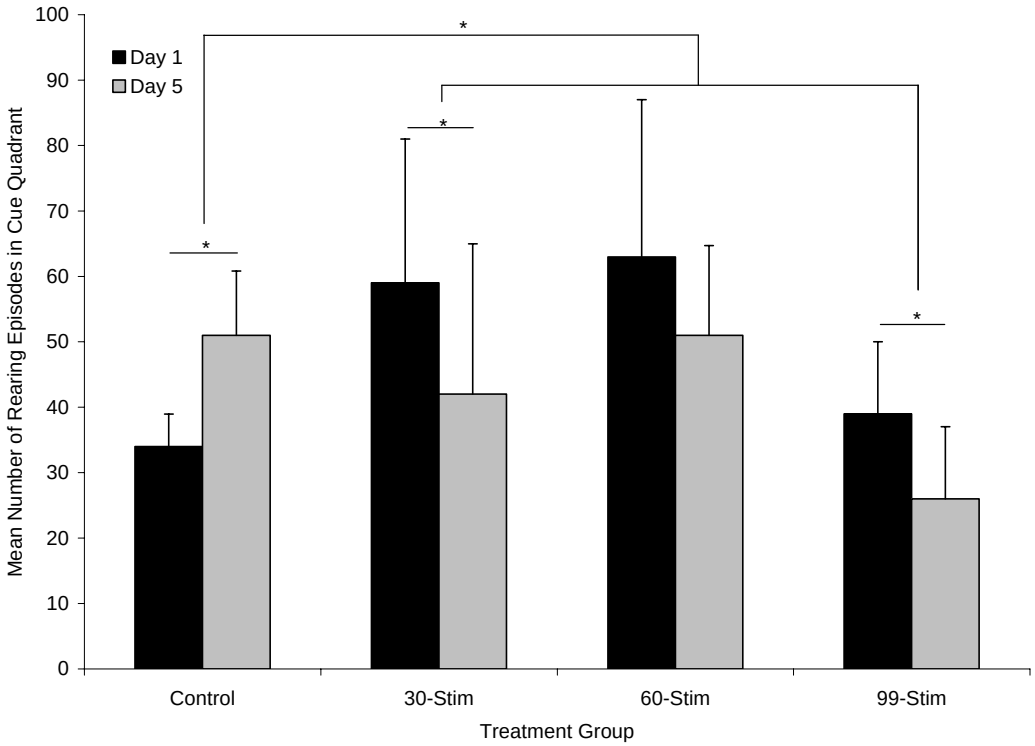


Figure 2.12. Mean (± SEM) number of rearing episodes in the home base cue quadrant by rats in each treatment group over the entire 30 minute testing session. * $p < .05$

A significant group difference was present on Day 1 [$F(3, 62) = 9.07$, $p < .001$], with the 60-stim group significantly different from all other groups ($p < .05$). As can be seen in Figure 2.10, all of the kindled animals showed decreases in rearing within the cue quadrant from Day 1 to Day 5, whereas only the control group showed an increase in this behaviour. Only two of the groups showed statistically significant changes in this behaviour from Day 1 to Day 5 [30-stim: $t(10) = 2.68$, $p < .05$; 99-stim: $t(12) = 4.08$, $p < .001$].

Fear Behaviour. There was no significant decrease in the initial time spent freezing from Day 1 to Day 5 [$F(1, 59) = .074$, $p > .05$], as illustrated in Figure 2.13. A significant effect of group was found for initial freezing [$F(3, 59) = 5.362$, $p < .01$], with a significant group difference on Day 1 [$F(3, 62) = 5.25$, $p < .01$] but not Day 5 [$F(3, 62) = 2.22$, $p > .09$]. The 99-stim group showed significantly more freezing than the control group on Day 1 and Day 5 ($p < .05$). Finally, there was no interaction of day and group [$F(3, 62) = 1.17$, $p > .05$].

There were no statistically significant differences between the groups in the frequency or duration of stops in the inner annulus. In regards to stopping frequency in the inner annulus, there was no significant effect of group [$F(3, 59) = 0.19$, $p > .05$] or day [$F(1, 59) = 0.03$, $p > .05$], nor was there a significant interaction [$F(3, 59) = 1.05$, $p > .05$]. Stopping duration in the inner annulus exhibited no significant effect of group [$F(3, 59) = 0.45$, $p > .05$] or day [$F(1, 59) = 0.35$, $p > .05$], nor was there a significant interaction [$F(3, 59) = 0.47$, $p > .05$]. Figures 2.14(A) and 2.14(B) illustrate the patterns observed, with the control group showing a decreased number of stops ($M_5 - M_1 = -16.52$) and decreased stop duration ($M_5 - M_1 = -19.30$) in the inner annulus. This pattern was similar in both the 30-stim and the 60-stim groups, who showed decreases to a

lesser extent. The opposite was found with the 99-stim group, who displayed an increase in the number of stops made in the inner annulus (M_5- M_1 = 30.08), indicating increased exploration of the area. The 99-stim group also showed an increase in stop duration in the inner annulus (M_5- M_1 = 74.31), demonstrating increased comfort in an area commonly avoided by fearful rats.

Figure 2.15 illustrates the percentage of rats in each group that jumped from the open field. Chi-square analysis identified a significant group difference in jumping behaviour for both Day 1 [χ^2 (3, N = 56) = 61.134, p < .001] and Day 5 [χ^2 (3, N = 56) = 117.462, p < .001]. An overall decrease in jumping was observed across all groups from Day 1 to Day 5 [χ^2 (3, N = 56) = 12.104, p < .05]. Post hoc analyses demonstrated that on Day 1, all of the kindled groups had a greater percentage of rats jumping in comparison to the control group (30-stim: U = 109, p < .05; 60-stim: U = 97, p < .05; 99-stim: U = 49, p < .001). By Day 5 however, only the 99-stim group still had a greater percentage of rats jumping in comparison to the control group, U = 98, p < .001.

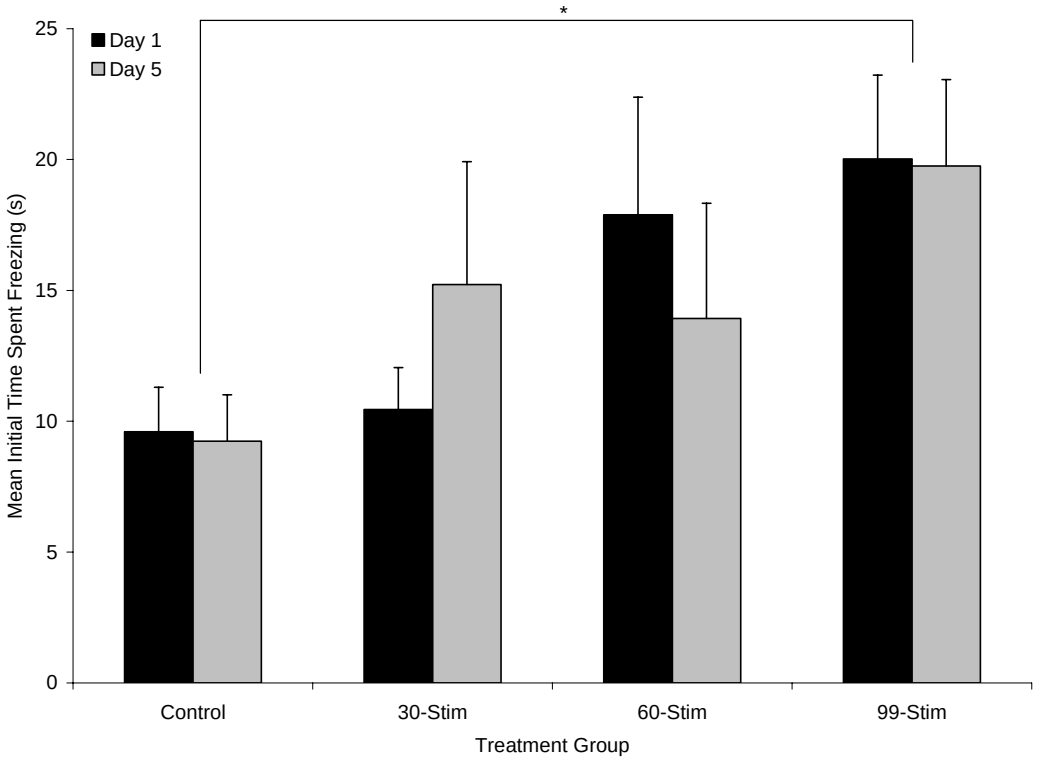
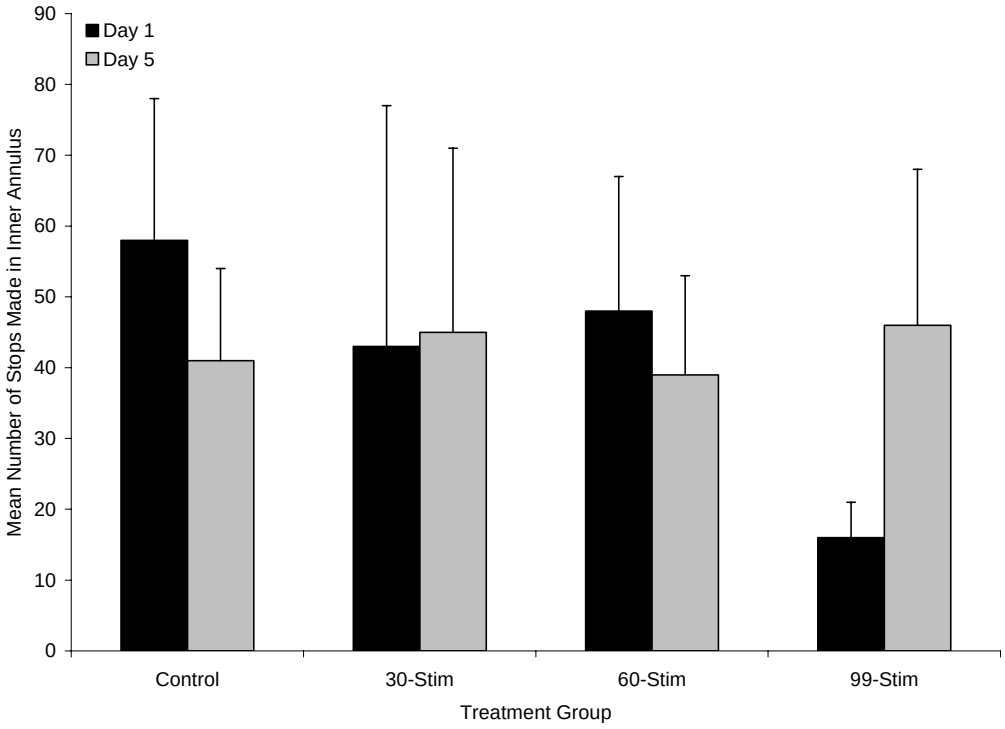


Figure 2.13. Mean (± SEM) initial time spent freezing by rats in each treatment group when first placed in the open field. * $p < .01$

A



B

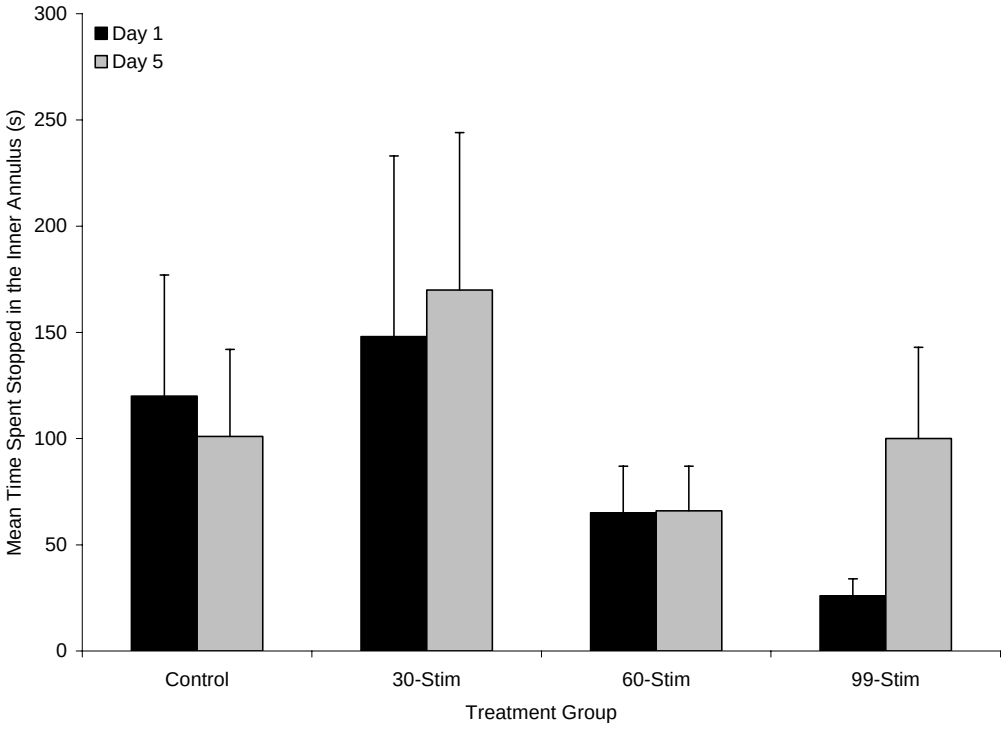


Figure 2.14. Mean (± SEM) number of stops made (A) and duration of time spent stopped (B) in the inner annulus by rats in each treatment group over the entire 30 minute testing session.

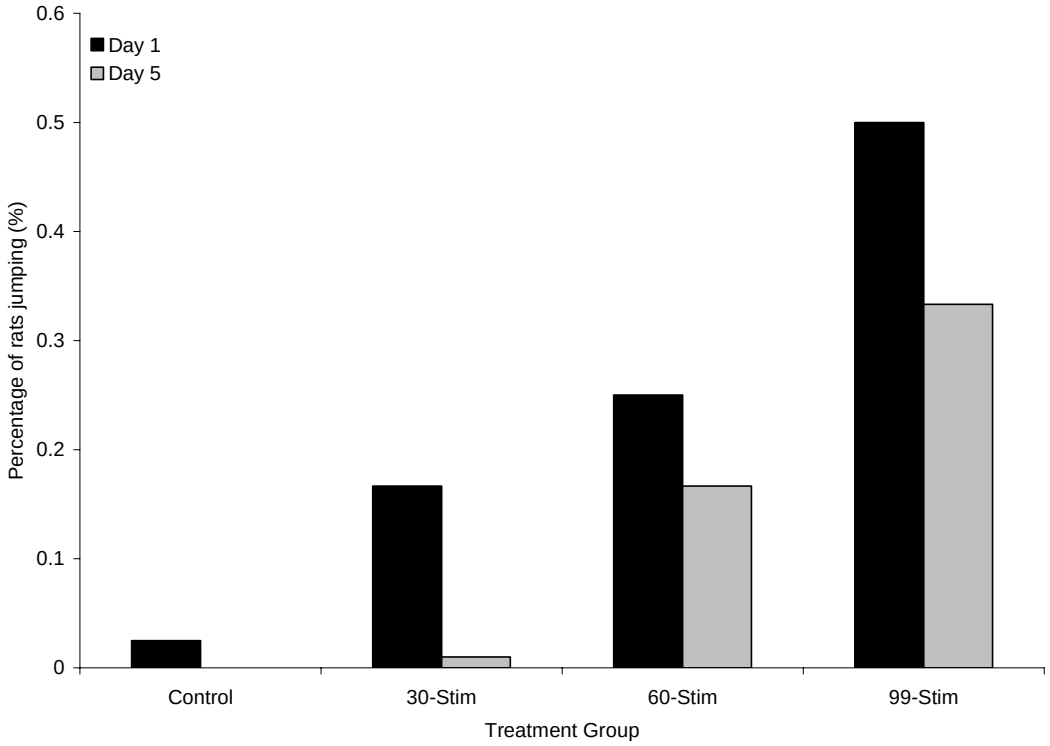


Figure 2.15. Percentage of rats in each treatment group that jumped from the open field.

Discussion

The purpose of this experiment was to determine whether kindled rats show impaired habituation in an open field task that has previously been shown to be sensitive to hippocampal lesions. The first goal of this experiment was to address the findings of Experiment 1 and to determine whether, when tested in a more elaborate environment and over a longer time course, kindled rats would still show impaired habituation and increased fear in a novel environment. The second goal was to identify any hippocampal-dependent functional deficits present in amygdala kindled rats when compared to control rats on measures of habituation, home base establishment, and fearfulness. The following discussion will address these goals more specifically, but a general summary can be made that in comparison to controls: 1) amygdala kindled rats were consistently hyperexploratory, despite decreased activity over the 5 days; 2) amygdala kindled rats showed a lesser ability to establish a home base; 3) long-term amygdala kindled rats showed heightened fear behaviour.

Exploratory Behaviour

All kindled groups traveled far greater distances than the control group on both days. Both kindled and control groups did, nonetheless, show decreases in the distance traveled across the 5 days, suggestive of some degree of habituation. This suggestion can also be supported by the measure of velocity. The kindled groups traveled at far higher speeds in comparison to controls on Day 1; however, this difference dissipated by Day 5 where all groups traveled at a similar speed. Although the change in speed was minimal for the controls, all rats exhibited a decline in their exploratory behaviour.

All groups traveled more in the outer annulus on Day 1 than on Day 5, indicating higher levels of exploration of the outer boundaries of the environment, as

well as observation of visual cues that were external to the open field. Despite the large decrease in distance traveled by the kindled rats from Day 1 to Day 5, all of the kindled groups consistently traveled greater distances in the outer annulus than did the control group. Although all groups always spent the majority of their 30 min sessions in the outer annulus, the difference in activity levels within this annulus emphasizes that there were different reasons for the time spent in this region. More specifically, the kindled rats continued to investigate the outer annulus and external cues, while the control rats remained stopped in one or two locations. Habituation to the environment was evident in the stopping behaviour of the control group, which displayed stops that were longer and less frequent on Day 5. Although the kindled groups did spend a longer time stopped by Day 5, the number of stops made did not change and remained significantly higher than the controls, indicating higher levels of exploration.

One result that was not predicted was that rearing behaviour, another indicator of exploration, decreased in all kindled groups but increased in the controls. As a majority of this behaviour occurred in the home base quadrant, the measure might actually indicate an increased level of comfort within the learned cue quadrant.

As exploratory behaviours did decline for both the kindled and control groups, it can be concluded that a certain degree of habituation did take place. The consistent hyperexploratory behaviour of all kindled groups in comparison to controls makes it difficult to conclude with certainty that the kindled rats did in fact habituate completely, as this behaviour is traditionally defined as a decline to almost complete inactivity (O'Keefe & Nadel, 1978).

Home Base Behaviour

Home base establishment is identified as the area within the field where an animal spends the majority of its time and where an increased comfort level is indicated through decreased speed of travel and increased exploration around the cue, including increased rearing (Eilam & Golani, 1989). In addition, the animal organizes its exploration around the home base as indicated by frequent stops of long duration at the home base (Eilam & Golani, 1989; Golani, Benjamini, & Eilam, 1992).

All groups showed at least a moderate increase in time spent within the cue quadrant by Day 5, with the exception of the long-term kindled group which actually showed a decrease in the time spent within this quadrant on Day 5. The organization of exploration also appeared to be altered in long-term kindled animals, as only this group demonstrated a decrease in the frequency and duration of stops made in the cue quadrant.

Some aspects of exploratory behaviour within the cue quadrant were altered for all kindled groups. Although kindled groups showed decreases in rearing and relative distance traveled within the cue quadrant, the controls demonstrated increased rearing and relative distance traveled around the cue. In addition, it was expected that rats would show a decreased velocity in the cue quadrant as an indication of their increased comfort level around a home base. The control group demonstrated a lowered velocity that decreased slightly from Day 1 to Day 5. In contrast, all kindled groups displayed a high velocity on Day 1 which decreased by Day 5 but still remained elevated relative to controls.

The measures related to home base establishment suggest identified impairments in all kindled groups, and furthermore, that long-term kindled rats appeared to show complete inability to form a home base.

Fear Behaviour

Compared to the control rats, only the long-term kindled rats showed significantly greater displays of fear. Long-term kindled rats had longer periods of initial freezing on both the first and the final days of testing. Figure 2.3 illustrates that the long-term kindled group spent a greater amount of time freezing, while rats in the control group tended to begin exploration almost immediately.

Although a few of the kindled rats in each group displayed purposive jumping on Day 1, the long-term kindled group had a significantly greater percentage of rats jumping on both the first and final days of testing. These results are particularly significant, as they replicate the purposive jumping behaviour previously observed in long-term kindled animals on an open-arm maze (Kalynchuk et al., 1997) and indicate that, in the open field paradigm, this behaviour does not dissipate over repeated exposure. Purposive jumping is worth investigating further as it is unclear how this behaviour may relate to other measures in the open field paradigm.

Fearful animals typically spend less time in the centre of an open field (Hannesson, Mohapel, & Corcoran, 2001), as was the case with the long-term kindled animals on the first day of testing in the open field. In contrast to all other groups, the long-term kindled group actually showed an increase in the time spent both exploring and stopping in the centre on the final day of testing. This result may reflect an increased level of comfort, as the group was more willing to explore an area previously perceived to be dangerous. Alternatively it may be that all other groups had habituated to the outer

annulus, while the long-term kindled group continued to explore areas of the arena with which it was unfamiliar.

Analysis of the fear behaviours indicate that only the long-term kindled animals are significantly more fearful, and maintain a significant level of fear, after 5 days of exposure to the unfamiliar open field.

Overall, the findings of Experiment 2 suggest that kindled rats show some ability to habituate to an unfamiliar open field. All kindled groups demonstrated some impairment in the ability to establish a home base, and the long-term kindled groups showed no establishment of, or habituation to, a cued home base. Finally, only the long-term kindled animals demonstrated consistently elevated fear behaviours. Taken together, these results suggest that amygdala kindled rats, particularly those having received long-term stimulation, show both impairments in habituation and increased fear behaviour in an initially novel environment. As habituation is a hippocampal-mediated behaviour, it can be concluded that long-term kindled rats show significant impairments in hippocampal function that may be mediating the previously observed hyperemotionality.

Chapter 4:

General Discussion

The purpose of the experiments in thesis was to determine whether increased fear behaviours shown by long-term amygdala kindled rats are reflective of kindling-mediated alterations in the hippocampal circuitry responsible for proper emotionality and contextual learning. More specifically, the two experiments evaluated the ability of the animals to habituate to a novel environment, as this may indicate how a hippocampal-dependent function could mediate the expression of increased fear in amygdala kindled rats. Experiment 1 demonstrated that long-term kindled rats do not show evidence of habituation, demonstrating consistently elevated exploration and fear in an initially novel open field that is relatively small in size. Experiment 2 demonstrated that kindled rats show elevated exploration and an inability to form a home base in relation to static visual cues, again demonstrating an inability to habituate to the initially novel environment. Significant elevations in fearfulness were also seen in the long-term kindled rats, ultimately providing an association between impaired habituation and increased fear in long-term amygdala kindled rats. The following discussion will more specifically address the behaviours observed in both experiments. The models and paradigms used will also be assessed in terms of their limitations and future lines of research that will strengthen and clarify the suggested association.

Long-Term Amygdala Kindling and Altered Hippocampal Function

The Interaction between Increased Fear Behaviour and Impaired Habituation

Traditional theories regarding rat behaviour in response to novel stimuli propose a progression in fear and defensive behaviours from freezing, fleeing and fighting depending on the level of threat that is perceived (Bolles, 1970; Gray, 1978; Rosen & Schulkin, 1998). Fear behaviours are determined by ongoing interaction between the hippocampus and the amygdala, which mediate dominant behaviours (initial freezing), increased defensive quiescence (cautious movement and stopping), and increased risk assessment (arousal and exploration) (McNaughton & Corr, 2004). Interestingly, the fear behaviours of the kindled rats in the current experiments provide support for the predictions of Rosen and Schulkin (1998), which suggest that increased stimulation of the amygdala creates a heightened perception of, and reaction to, novel stimuli that may be potentially threatening.

All rats in the experiments showed a brief freezing response as their initial fearful reaction the novel environment, as demonstrated by the short initial periods of freezing by both the control and 30-stim rats. In both experiments however, the 99-stim rats demonstrated excessively long periods of freezing, suggesting heightened fear reactions despite repeated exposure to the environment.

In both experiments, the 30-stim rats demonstrated initial caution as well as hyperarousal in the initial exposures to the open fields. The subsequent decrease in exploration and an increase in relaxed behaviours (i.e., grooming, sitting, and rearing) demonstrated by these rats indicate habituation. That is, the 30-stim rats no longer displayed the exploratory and fear responses used to address the novel environmental

stimuli. In Experiment 2, the 30-stim and 60-stim rats exhibited elevated fear, demonstrated by hyperexploratory behaviours and increased time peering over the edge, indicative of fleeing or attempted escape behaviour. A decrease in overall activity, mean velocity of travel and an increase in time spent stopped indicate habituation to the environment overall. In addition, a decrease in the time spent in the outer annulus does suggest a decrease in fear as the rats were spending less time searching for an escape. Nevertheless, a lack of home base establishment makes it tempting to assume that the animals demonstrated an impairment in habituation to specific contextual cues, as home base establishment is a key component to traditional definitions of habituation. An explanation for the lack of home base establishment could be that the rats did not perceive it as a source of safety because it did not provide them with the escape for which they were searching. This hypothesis is supported by the rats' continued exploration of the outer annulus after unsuccessful attempts to escape by jumping towards the home base.

While the 30-stim and 60-stim rats displayed similar behaviours, the 99-stim rats displayed behaviours indicative of fear that was even more heightened than that of the other rats. Upon initial exposure, the 99-stim rats appeared to display the same fear response as the other kindled animals, with an attempt to escape from the environment. However, the fear exhibited by these animals was even more elevated as over half of the animals jumped from the open field. This active avoidance, demonstrated as the most extreme possible attempt at escape, indicates a level of fear that extends beyond those demonstrated by the other kindled animals. This display of extreme fear is also evident in Experiment 1, where the resistance-to-capture measures for the 99-stim animals identify defensive attack behaviours that are the most extreme fear response.

While it is evident from the quantitative results of Experiment 2 that both the 30-stim and the 60-stim rats were significantly more active than the 99-stim rats, the reason behind this pattern of movement is not explained quantitatively. After a significant period of freezing and minimal movement, the 99-stim rats would typically dart to one edge of the table as though searching for an escape. At this point the rats either leapt from the table (over three quarters of the jumps that occurred took place in the first 10 min of testing) or froze for a large period of time before darting seemingly randomly to another edge. This behaviour explains why the 99-stim group was found to have lower average velocities, as half their time was spent frozen at the edge of the table.

The majority of the 99-stim rats did perceive some element of safety in the home base cue, as it was often the first location to which they would dart. Many of these animals perceived the home base cue as an escape, as they either jumped directly at the cue or attempted to climb closer to the cue by reaching towards it. In contrast to all other groups, the 99-stim group showed higher levels of activity in the cue quadrant on the Day 1 but their activity dropped to the same level as other groups by Day 5. This behaviour is likely indicative of the salience of the cue, as the rats clearly perceived the cue within minutes of their first exposure. Their inability to demonstrate home base establishment may indicate a deficit in normal exploratory patterns or in learning of spatial stimuli. This behaviour may also be a consequence of their inability to identify the cue as providing increased safety.

Ultimately, decreases in exploratory behaviour were observed in the 99-stim group, suggesting a certain amount of habituation. Nevertheless, these animals continued to show freezing, darting, and attempted escapes on the final day indicating elevated fear and unease in the open field. Although the control animals showed an appropriate fear

response, with decreases in fear behaviour and establishment of a home base by the final day, the kindled animals all showed a certain degree of impairment. The 30-stim and 60-stim animals showed moderate decreases in attempts to escape their environment but they did continue to demonstrate fear behaviour, and did not establish a home base location in which they demonstrated behaviours indicative of increased comfort levels (i.e., grooming, rearing). The 99-stim animals showed extensive impairments in moderating their behaviour, as they neither decreased their fearful escape behaviours to a large extent, nor did they establish a home base in which the demonstrated increased comfort.

Association of Long-Term Amygdala Kindling and Hippocampal Alterations: The Role of Neurogenesis and Cell Migration

As previously discussed, a significant pattern has emerged regarding the alterations in receptor and gene expression in the hippocampus that occurs in long-term amygdala kindled animals that display excessive fear (Kalynchuk, 2000). The majority of the changes in receptor subtype expression occur in the dentate gyrus, with a few additional changes in the CA1 region. These changes produce an overall increase in inhibition in the dentate gyrus, and surrounding structures by association, through an increase in expression of the inhibitory GABAergic system ($GABA_A$, BZ) and a decrease in expression of the excitatory glutamatergic system (AMPA, NMDA). These changes are further illustrated in decreased FOS immunoreactivity in the dentate gyrus, CA1 region and perirhinal cortex of long-term amydala kindled rats (Kalynchuk et al., 2001). Finally, decreased expression of growth factor binding sites (i.e., IGF-I) in the hilus and the granule cell layer of the dentate gyrus, as well as the CA1 region (Kalynchuk et al., 2002), suggest altered cellular growth and plasticity within the hippocampus.

Further investigations of the hippocampal changes associated with TLE have

begun to elucidate more structural alterations that may play a role in the observed behaviours. It has recently been established that aberrant neurogenesis and cell migration are observed in the dentate gyrus in both the rat pilocarpine model of TLE and in tissue resected from humans affected by retractable TLE (Parent, Elliot, Pleasure, Barbaro, & Lowenstein, 2006). Amygdala kindled rats also show this increase in dentate granule cell (DGC) neurogenesis after several generalized seizures (Parent, Janumpalli, McNamara, & Lowenstein, 1998; Scott, Wang, Burnham, De Boni, & Wojtowicz, 1998). In addition to the other cellular abnormalities in the dentate gyrus, such as mossy fiber sprouting and cell loss in the hilus, the ectopic migration of new dentate granule cells appears to be a prime culprit in the altered hippocampal function in temporal lobe epileptics (*see* Scharfman & Gray, 2007). Newly born DGCs appear to integrate abnormally into the existing hippocampal circuitry and are hyperexcitable, suggesting that they may disrupt appropriate function or cause an increase in the chance of spontaneous seizures (Scharfman, Goodman, & Sollas, 2000).

Research in our own laboratory, involving the rats from Experiments 1 and 2, has also identified DGC neurogenesis in long-term kindled rats (Fournier et al., 2006). In contrast to the sham-stim rats, the 99-stim rats showed ectopic migration of newly born cells into the hilus (Fournier, Andersen, Caruncho, & Kalynchuk, 2007). Both our results, as well as those recently published by Parent and colleagues, have also shown that kindled rats exhibit localized decreases in the expression of reelin, a signaling protein that is central to the healthy migration of DGCs (Fournier et al., 2007; Gong, Wang, Huang, & Parent, 2007). It is thought that reelin plays a key role in guiding new cells to the proper anatomical location and it also plays a role in synaptic plasticity, therefore, a lack of reelin could lead to ectopic cell migration (Ramos-Moreno, Galazo, Porrero, Martinez-Cerdeno,

& Clasca, 2006). Interestingly, reelin deficits in mice have been associated with impaired hippocampal plasticity as well as numerous cognitive deficits, including a specific deficit in contextual fear conditioning, a hippocampal-dependent form of learning (Qiu, Korwek, Pratt-Davis, Peters, Bergman, & Weeber, 2006).

Overall, the cellular alterations observed in long-term amygdala kindled rats provide further evidence for the role of hippocampal dysfunction in epilepsy-induced behavioural disturbances, providing molecular evidence for these behavioural abnormalities. The deficit in reelin, along with the behaviour that it appears to impair, provides intriguing insights into the mechanisms at work.

Further Exploration of the Environment:

The Impact of Long-Term Amygdala Kindling on Contextual Fear Conditioning

The two experiments discussed in this thesis have identified selected impairments in environmental, or contextual, habituation in long-term amygdala kindled rats. Although contextual learning and habituation rely heavily on hippocampal function, the contribution of the amygdala cannot be discounted due to its role in fear as it also relates to contextual learning (*see* Maren, 2005). Fear conditioning is a paradigm that can facilitate the distinction between the role of the hippocampus and the amygdala in a fearful environment. In fear conditioning, an unconditioned stimulus (US, typically a footshock) is paired with a conditioned stimulus (CS) to eventually elicit a cued response. In cued fear conditioning, the CS is a discrete cue, such as a light or a tone, whereas in contextual fear conditioning, the context in which the US was originally delivered serves as the CS.

It is commonly accepted that the amygdala is central to all fear behaviours, involving conditioning to either discrete cues or complex contextual cues (Phillips &

LeDoux, 1992; *see* Rosen, 2004). Temporary inactivation of the amygdala has identified a role for this structure in both learning and memory of discrete cues, such as a light or a tone, and complex cues, such as the environment in which the animal is present during an unpleasant experience (Donley, Schulkin, & Rosen, 2005; Maren, 1999). The hippocampus appears to mediate context-specific conditioned fear, as lesioning or temporary inactivation of the dorsal hippocampus at different time points in conditioning results in deficits in contextual fear acquisition, extinction or expression (Corcoran & Maren, 2001; Corcoran, Desmond, Frey, & Maren, 2005). Transient inactivation of the ventral hippocampus has also been shown to result in impaired context-specific learning (Bannerman, Rawlins, McHugh, Deacon, Yee, Bast, Zhang, Pothuizen, & Feldon, 2004; Hobin, Ji & Maren, 2006). The role of the hippocampus in conditioned contextual fear expression is suggested to be time-limited, with increased retrograde amnesia increasing with the amount of hippocampal damage (Lehmann, Lacanilao, & Sutherland, 2007).

Although few fear conditioning studies have been carried out with kindled animals, those that have been done revealed some interesting findings. Rosen and colleagues (1996) showed that partial amygdala kindled rats have an exaggerated fear-response when conditioned to a discrete cue. This could be predictive of the response seen in long-term kindling paradigms, which would likely produce heightened fear responses to both discrete and contextual cues. More recent research showed conditioned contextual fear responses in short-term amygdala kindled rats, using the context in which the animal was kindled as the conditioned stimulus (Barnes & Pinel, 2001; Barnes, Pinel, Francis & Wig, 2001). The rats showed increased fearfulness in the conditioned kindling context, as well as decreased seizure severity when rats were kindled in the unconditioned context. These studies provide a promising background for further exploration of contextual fear

conditioning as a tool for interpreting the impact of kindling on contextual memory.

The focus of the current studies has been to identify the impact of potential hippocampal dysfunction on fear behaviours in an initially unfamiliar environment. By further investigating the role of the hippocampus in environmental learning, we can strengthen a case for the role of unfamiliarity— resulting from a lack of contextual memory— in long-term amygdala kindled fear responses. Further exploration of context- versus cue-specific conditioning can help to identify the specific contribution of hippocampal-specific functional deficits to fear behaviours associated with long-term amygdala kindling.

Limitations of the Study and Future Directions

The two experiments carried out in this thesis were an attempt to gain an understanding of how the overly fearful behaviour of long-term amygdala kindled rats might be mediated by alterations in hippocampal cellular functions that moderate hippocampal-mediated behaviours, such as habituation. Habituation is generally seen as a change in responsiveness, as the initially unfamiliar circumstance changes from novel to familiar. Such responses necessarily involve a component of fear, as novelty receives a cautious and often fearful response when an animal does not know what threats it might encounter. As habituation requires both the amygdala and the hippocampus in order to mediate an awareness of contextual circumstances, as well as appropriate emotional responses, it is difficult to determine what structures are uniquely responsible for a set of actions. There are several lines of research that could help to further clarify the role that impairments in hippocampal function play in the excessive fear behaviours observed in long-term amygdala kindled rats.

Control Studies

As the present experiment examined the role of long-term amydala kindling on a hippocampal-dependent task, this behaviour would be best assessed by comparing amygdala kindled rats to rats with direct damage to the hippocampus. Rats with lesions or temporary inactivation, either to specific regions or to the complete hippocampus, consistently show deficits in spatial and contextual learning paradigms. Rats that have received short-term kindling of the dorsal hippocampus reliably exhibit deficits in spatial cognition, tested through Morris water maze tasks, including the delayed-match-to-place task (Hannesson et al., 2004; Hannesson et al., 2001). These findings have not been replicated with rats kindled in the ventral hippocampus, although context-specific fear behaviours have been identified in this population (Becker, Letzel, Letzel, & Grecksch, 1997). Ultimately, the use of long-term hippocampal-kindled rats would elucidate whether the results obtained are a consequence of site specificity, including a more direct clarification of the contextual versus emotional components of habituation.

Environmental Circumstances

The fact that the kindled animals in Experiment 2 showed extensive exploration and did not establish a home base is likely reflective of the increased fearfulness of these rats. Nevertheless, it could be that the rats did not set up a home base due to the environment in which they were tested. More homogeneous environments tend to lead to a faster decrease in activity levels and increase in home base establishment, with higher levels of exploratory activity found when the number of environmental features increases (Whishaw, Gharbawie, Clark, & Lehmann, 2006). This could explain the high activity levels of the rats, both in their initial exposure to the field as well as over the course of study; however, the control rats did not show the same heightened level of exploration. It is also possible that the animals did not set up a home base due to lack of salience of the

cue. This is highly unlikely, as similar cues used in paradigms with hippocampal-lesioned rats were dominant enough to prevent the rats from further environmental exploration (Clark et al., 2005; Whishaw et al., 2006; Wallace & Whishaw, 2003).

Exploratory Measures

Although the current experiments quantified exploratory and habituation behaviour based on measures of the total testing session, many studies examine the ability of an animal to establish and remember the home base cue by observing only a few excursions from the cue in order to determine whether the animal is exhibiting intact exploratory behaviour. Exploratory behaviour is seen as a direct consequence of the detection of novel stimuli with the goal of acquiring and updating information regarding learned spatial cues. However, in previous studies the specific behaviours involved in exploration were not originally identified (O'Keefe & Nadel, 1978). The more elaborate definition of exploration involves orientation around a home base cue, including travel oriented around the cue, as well as specific behaviours performed within the home base area (Eilam & Golani, 1989; Golani et al., 1992). As an extension, intact habituation is indicated by intact exploratory behaviour that has shown a marked decrease after the animal has learned the home base cue. An alternative method of identifying habituation in our animals, and one to be explored further, would be to identify and observe the first few exploratory excursions for each rat on each of the five testing days. Control rats would be expected to demonstrate increased home base behaviour with brief, circuitous exploratory trips that end with a direct return to the cue. Animals with impairments in home base formation would be expected to show less awareness of the home base, with decreased heading towards the cue quadrant, longer exploratory trips and indirect returns, if any, to the home base cue. Detailed observation of these excursions may provide

specific deficits and may ultimately clarify which, if any, of the observed behaviours can be solely attributed to deficits in spatial cognition as opposed to heightened fear.

Fear Behaviours

Whishaw and colleagues have carried out numerous studies using the open field paradigm used in Experiment 2; however, only once did they make reference to rats jumping from the platform (Lehmann, Clark, & Whishaw, 2007). In this instance, the authors state that the data from any animals that jumped from the apparatus was removed from the study, causing one to question how removing the data from these rats may have impacted the results. This circumstance also highlights a limitation of Experiment 2, as the behaviour of rats that performed purposive jumping may have been influenced by the handling necessary to return the rat to the open field.

Clinical Implications

The current studies served to assess chronic amygdala stimulation and its impact on the integrity of hippocampal structure and function. Two areas of behaviour were assessed: those involving contextual learning and memory, and those associated with pathological anxiety. These areas will be considered separately in terms of clinical implications of the current results.

Visuospatial Memory. Human mesial TLE, with seizures most often associated with dysfunction of the hippocampus or amygdala, is commonly associated with verbal memory deficits, with visuospatial memory deficits present to a lesser extent (Devinsky & Najjar, 1999). While spatial cognition is the behaviour most commonly assessed in animals in order to assess hippocampal function, verbal memory is more commonly used to assess human hippocampal function (Whishaw & Kolb, 2005). Research assessing

visuospatial memory in temporal lobe epileptics does occur, although these tests are quite basic, and involve small-scale spatial layouts [i.e., Wescheler Memory Scale-III (Visual Memory Indices), Brief Visuospatial Memory Test-Revised](Nunn, Graydon, Polkey, & Morris, 1999). Such testing has identified impaired performance in patients with right hemisphere damage, with impairments to a lesser extent identified in patients with left hemisphere damage (Nunn, et al., 1999). Visuospatial abilities assessed on a larger scale have identified deficits in topographical orientation and episodic memory in a virtual town (Maguire, Burke, Phillips, & Staunton, 1996; Spiers, et al., 2001). Results from these studies also associate the deficit in spatial orientation with those impacted by damage to the hippocampus of the non-dominant hemisphere (typically the right hemisphere). Interestingly, those with left hemisphere damage demonstrated deficits in context-specific memories, identified through an inability to associate events that occurred with the location in which they occurred (Spiers, et al., 2001). The present findings are encouraging, as they identify dissociation between the learning of contextual cues and the association of contextual cues with specific experiences. These results provide further reason to assess the performance of long-term kindled animals in contextual fear conditioning, as they may be able to habituate to an environment without being able to form cue- or context-specific associations. While long-term kindling does damage hippocampal function in both hemispheres there have, nonetheless, been distinctions found in behaviour after short-term kindling stimulation of the right versus the left hemisphere (Adamec, Shallow, & Burton, 2005). Such results suggest that there may be benefit to comparing contextual learning and memory performance in animals receiving long-term kindling from either the right or left amygdala.

Hyperemotionality. The potential for fear sensitization after repeated amygdala stimulation (or repeated mesial temporal lobe seizures) can easily be associated with TLE. Anxiety disorders are over-represented in the temporal lobe epileptic population, with a diversity of emotional disturbances experienced before, during, after, and most commonly between, seizures (Nees, Moriarty, Kitchen, & Trimble, 2001). While temporal lobe epileptics consistently exhibit higher mood and anxiety disorders than the average population, it is difficult to dissociate the neurobiological factors from the psychosocial factors contributing to these disorders (Gilliam, 2003). Researchers have attempted to separate these contributing factors through studies of patients pre- and post-temporal lobectomy. Individuals who stop experiencing seizures after surgery often show decreases in affective disorders, while those who experience less successful seizure outcome may exhibit minimal improvement in psychological state (Johnson, et al., 2004; Smith, Elliot, & Lach, 2004). This does present the possibility that a lack of seizures may be responsible for the neurophysiological mechanisms involved in improving one's psychological state. Nevertheless, it is equally possible to posit that a successful surgery outcome contributes to a healthier emotional and psychological state as the individual experiences a positive outcome and improved quality of life (Tellez-Zenteno, Patten, Jette, Williams, & Wiebe, 2007).

Kohler and colleagues (2001) argue that, in the case of complex partial seizures, there is an important role for the permanent alterations in neural circuits that may be mediating changes in mood and behaviour. Complex partial seizures are commonly preceded by physical or mental sensations identified as auras, with fear auras most commonly reported (Seino, 2006; Vazquez & Devinsky, 2003). Repetitive activation of the amygdala occurs as the seizures themselves lead to the conditioning of fear, cued by

the sensation of oncoming seizures or by the contexts which evoke seizure occurrence. Individuals may ultimately experience “higher-order” fear responses, in which novel contexts induce fearful responses despite the fact that have never been associated with the original fear auras or the seizures themselves (Kohler et al., 2001). This consequence is of particular interest as it not only demonstrates the persistence of panic disorders and generalized anxiety observed in this patient population, but also highlights which patient populations could most benefit from medical interventions (Fisher et al., 2005). Patients experiencing fear auras, and thus seizures thought to originate from the amygdala, have demonstrated equal success in seizure control through both surgical and anti-epileptic treatments (Johnson et al., 2004). Nevertheless, this same population displays a persistence of behavioural disturbances in both treatment circumstances (Kohler et al., 2001). The common hypothesis is that the forced normalization of limbic pathways, necessary to increase seizure control, actually exacerbates behavioural abnormalities through an imbalance caused in the neurological inhibitory functions developed by the epileptic brain in its own attempt to decrease seizure occurrence (Griffith, Bandler, & Engel, 1987; Kohler et al., 2001). Clearly, this hypothesis cannot be adequately assessed in a clinical population, although it does help to identify specific mechanisms that are in accordance with current areas being assessed in animal research. Ultimately, the long-term kindling model will continue to serve as a necessary tool in understanding the neurobiological basis of hyperemotionality without the limits of factors involved in clinical research.

Conclusions

The purpose of this thesis was to determine whether increased fear behaviours seen in long-term amygdala kindled (99-stim) rats are a result of hippocampal-dependent

functional deficits. The hippocampal-dependent task used was that of habituation to a novel environment, as novelty is known to exacerbate the high levels of fearfulness observed in long-term kindled rats. In contrast to controls, none of the kindled rats demonstrated the ability to establish a home base in the second experiment. The 30-stim and 60-stim rats were hyperexploratory and showed elevated anxiety, although certain decreases in fearful behaviour indicate that these animals did show moderate habituation. Consistent across both experiments was the finding that the long-term amygdala kindled rats exhibited persistent elevations in arousal, anxiety, and escape behaviours, indicative of decreased habituation. The 99-stim rats showed a complete inability to cope with the fear-provoking circumstances. Taken together, these results suggest that although certain hippocampal-related behavioural abnormalities are already present by 30 and 60 stimulations, it is the long-term (99-stimulation) kindled rat that exhibits extreme deficits in the regulation of its emotionality and spatial learning.

Long-term amygdala kindling has repeatedly been used to elucidate the mechanisms that may be responsible for the interictal behavioural disturbances associated with TLE. In addition, this model has been considered for its role in fear sensitization, as it may provide insight into the pathology underlying the development and expression of anxiety disorders. The mechanism has been elegantly identified by Rosen and Schulkin (1998), as a system in which normal fear behaviour has developed into pathological anxiety due to over-stimulation, and thus hypersensitivity of the fear circuit. By developing a better understanding of all physiological mechanisms involved in kindling-related behavioural abnormalities, with a particular focus on the altered hippocampal circuitry, we may be able to gain insight into the role played by the hippocampus in diverse neurological and behavioural disorders.

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