THE ROLE OF THE HIPPOCAMPUS IN AMYGDALA-KINDLED FEAR IN MALE AND FEMALE RATS

by

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Dedication

To my Smarty Pants colleagues:

Lisa M. Fiorentino, Marsha R. Penner, Sandra A. Wiebe

Melanie McFadyen, Sara Burke, Nicole A. Young, & Sarah A. Johnson

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Abstract

The amygdala-kindling model is often used as an animal model of epileptogenesis; however, it has also proven useful for studying fear sensitization, as a model of interictal (i.e., between seizure) anxiety. The expression of fearful behaviour has been well characterized in kindled male rats but not in females. Furthermore, the neural mechanisms underlying kindled fear is not fully understood. Consequently, sex differences and the neural mechanisms of kindled fear are addressed in this thesis. In Experiment 1, kindled males and females are compared on several measures of fearfulness and found to exhibit similar levels of kindled fear. In Experiment 2, the hypothesis that kindled fear is associated with hippocampal-dependent dysfunction was tested. This was found to be true, particularly for kindled males. The results of Experiment 2 also indicated that the kindled females that were exposed to the hippocampal-dependent test showed reduced fear compared to those that were not exposed to the task. In Experiment 3, the role of hippocampal cell proliferation was examined in relation to kindled fear. The results showed that cell proliferation was elevated prior to the manifestation of kindled fear, suggesting that it could contribute the development of kindled fear. In Experiment 4, the hypothesis that exposing kindled rats to hippocampal-dependent environmental enrichment would reduce the magnitude of fearfulness, as was shown in Experiment 2. The results supported the hypothesis, particularly for the kindled males. Also in Experiment 4, the survival and dispersal of proliferating hippocampal cells was examined as a function of kindling and enrichment; however, neither manipulation altered cell proliferation. The results of these experiments suggest that behavioural treatments that target the hippocampus are beneficial in reducing the magnitude of kindled fear. The results offer new insights into non-pharmaceutical alternative treatments of anxiety.

List of Abbreviations

5HT	serotonin
$5HT1_A$	serotonin receptor subtype 1_A
AD	afterdischarge
ADT	afterdischarge threshold
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate
ANOVA	analysis of variance
Bz	Benzodiazepine
BrdU	bromodeoxyuride
CA3, CA2, CA1	Ammon's Horn (cornu ammonis) regions 1, 2, 3
DAB	diaminobenzidene
DG	dentate gyrus
DMTP	delayed-match-to-place
EEG	electroencephalograph
EPSP	exctitatory postsynaptic potential
GABA	gamma-amino buteric acid
$GABA_A$	GABAergic receptor subtype $_A$
GCL	granule cell layer
HPA	Hypothalamic-Pituitary-Adrenal Axis
M	mean
MAM	methyloxymethanol acetate
MWM	Morris water maze
NHS	normal horse serum
NMDA	N-methyl-D-aspartate
OF	open field
PB	phosphate buffer
PBS	phosphate-buffered saline
RAM	radial-arm maze
RTC	resistance to capture
SD	standard deviation
S.E.M.	standard error of the mean
SPSS	statistical packages for the social sciences
SSC	saline sodium citrate
Stim	stimulations
TBS(t)	tris buffered saline (triton)
WM	water maze

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Chapter 1: General Introduction

Anxiety is a common human phenomenon often produced by high stress levels, perceived and physiological. Clinical anxiety can take the form of generalized anxiety, panic disorder, and agoraphobia, to list only a few. In its pathological form, anxiety generally makes individuals incapable of daily functioning and is consequently a major mental concern in western society with an estimated lifetime prevalence of 15% to 25% (Reiger, Boyd, Burk et al., 1988; Kessler, McGonagle, Zhao, et al., 1994). One reason that the prevalence rates of anxiety are so high might be because anxiety stems from the basic emotion of fear. In fact, anxiety has been described as maladaptive or inappropriate fear (Kalin, 2003; Kandel, 1983). The mechanism by which fear becomes maladaptive is not known; however, one possibility is that anxiety develops by a sensitization of fear circuits in the brain and/or a sensitization to fear-inducing stimuli. Accordingly, repeated exposure to a fearful experience (real or perceived) could induce a sensitivity that primes the system to experience fear. In this way, fear could intensify in a maladaptive or inappropriate manner to a degree that eventually produces clinical anxiety.

Post (1992) has hypothesized that some forms of mental illness (i.e., depression, anxiety, panic) arise as a result of a sensitization process similar to the one outlined above. For example, with depression, repeated exposure to stressful life events is believed to result in gradually worsened bouts of depression, that eventually lead to depression in the absence of triggering stressors. This is modelled through animal behaviour in which symptoms of depression (i.e., learned helplessness, anhedonia) can be induced through repeated exposure to inescapable shock (Wagner,Hall, & Cote, 1977), forced swimming (Porsolt, 1977), chronic mild stress (Willner, Muscat, & Papp, 1992) and early life exposure to chronic variable stress (Zurita, Martijena, Cuadra et al., 2000). This idea that precipitating events can lead to changes in mental health is interesting and intriguing especially given that the brain is wired in a manner that allows for adaptive neural changes (i.e., neuroplasticity). Therefore, animal models of brain sensitization may be an important way to study the neuroplasticity and mechanisms associated with the development of certain mental illnesses, including anxiety.

Anxiety is a curious disorder that often entails the potentiation of normal fear reactions that would constitute maladaptive or inappropriate fear responses described above. Interestingly, some of the information processed through the hippocampus is also fear provoking. For example, consider one's response to the unfamiliarity of an environment. This novelty is often associated with common feelings of anxiety. This is true in humans and forms the basis of many anxiety tests in laboratory animals (e.g. File, Mabbutt, & Walker, 1988; Pellow, Chopin, File, & Briley, 1985; Walsh & Cummins, 1976). Fear of unfamiliarity conveys the difference between anxiety associated with the potential for danger (i.e., unknown surroundings) rather than the fear of immediate danger (known and visible predator). A similar conceptualization between present and anticipated fears has been given elsewhere (Blanchard, Yudko, Rodgers, & Blanchard, 1993).

As mentioned above, anxiety is very prevalent in society and understanding the mechanisms underlying its development would benefit many individuals. One way to examine the development of anxiety is through animal models that sensitize the neural circuits of fear (Bear, 1979; Rosen & Schulkin, 1998). One particularly novel model of anxiety is the kindling model of fear sensitization (Kalynchuk et al., 1997). The kindling technique involves repeatedly delivering electrical stimulations to specific brain areas, conventionally as a model of epileptogenesis. The stimulations initially evoke no behavioural or electroencephalographic (EEG) responses but after a few stimulations seizure activity is observable and, with time, full generalized convulsions develop (Goddard, McIntyre, Leech, 1969). Interestingly, this process of brain sensitization (or 'kindling') in animals also produces highly fearful behaviour that intensifies with an increase in the number of stimulations (see Kalynchuk, 2000). For example, rats subjected to 20 amygdala kindling stimulations show few increases in fearful behaviour, whereas rats subjected to 100 amygdala kindling stimulations show dramatic increases in fearful behaviour, such as increased resistance to capture, increased fleeing time, and increased freezing in a novel open field (Kalynchuk, 1997). Accordingly, the kindling model provides a unique opportunity to study the mechanisms by which appropriate and adaptive behaviour (i.e., fear) becomes inappropriate and maladaptive (i.e., anxiety or panic). There are a host of neural changes involved in the development of kindling, although very few have specifically been considered in the context of kindling-induced fear. An overview of these neural changes will help expose the dynamics of kindling and could help our understanding of now anxiety develops in kindled rats. Therefore, in the following sections, the kindling model and the neural changes associated with kindling are described in detail.

The Kindling Model

Kindling (Goddard, McIntyre, & Leech, 1969) refers to a process by which repeated administration of initially subconvulsive electrical stimulations to a particular region of the brain eventually results in generalized convulsions. The stimulations are normally short (1 ms), low amplitude, high frequency (60 Hz) bipolar square wave pulses generally delivered to temporal lobe brain regions including limbic and parahippocampal structures (i.e., amygdala, hippocamfpus, perirhinal cortex, piriform cortex). The stimulations are normally delivered once daily until generalized convulsions are elicited. Kindling has been extensively studied as a model of epileptogenesis and, more generally, as an example of neural plasticity and the behavioural changes associated with this plasticity.

Once initiated, kindling appears to be permanent (Goddard et al., 1969; Cavozos, Golaria, & Sutula, 1991; Wada & Sato, 1974), and it results in few signs of neural damage (Goddard et al., 1969; Racine, 1972b; Sutula, He, Cavazos, & Scott, 1988). Kindling is a good example of sensitization because responses to the initially subconvulsive electrical stimulations progress into full motor convulsions. Racine (1972a) classified these motor convulsions with a scale that remains the most widely used measure of convulsion severity. Briefly, rats initially respond to each kindling stimulation with a brief behavioural arrest (class 0); however, within a few stimulations they begin to show motor responses that include eye blinking and ear twitching (class 1) followed by head nodding and jaw clonus (class 2). Forelimb clonus (class 3) is the first sign of a generalized convulsion followed by the addition of rearing (class 4). A full motor convulsion (i.e., generalized convulsion) is characterized by the loss of balance that leads the animal to fall over while rearing (class 5). A full motor convulsion typically develops after approximately 15 stimulations in the amygdala-kindling paradigm, although there is lots of variability due to variability in stimulations parameters, rat strains, and nuclei

(Adamec, & Morgan, 1994). Hippocampal kindling is believed to occur more slowly than the amygdala, whereas the piriform and perirhinal cortex kindling are faster (McIntyre, Kelly, & Armstrong, 1993; McIntyre, Kelly, & Dufresne, 1999) and the claustrum is the fastest (Mohapel, Zhang, Gillespie, et al., 2001). This typical convulsive behaviour exemplifies the spread of seizure activity across the brain from a focal stimulation point and represents the characteristics of the progressive motor convulsions that appear specific to limbic and temporal lobe kindling.

Racine (1972b) also described many of the basic electrophysiological properties of kindling that have become reliable markers of the development of kindling. Most important was the description of a stimulation afterdischarge (AD) that consists of high-frequency electrical activity that persists in the focal brain area after the termination of the kindling stimulation. This AD is also described as an epileptic discharge and is necessary for the development of kindling (Racine, 1972b). Several key characteristics regarding ADs include the lowering of AD thresholds over the course of kindling that correspond to the development of motor seizures (Racine, 1972b), an increase in duration, amplitude, spike frequency, and spike morphology (Racine, 1972b; Racine, 1975), and the potentiation of kindling with prior stimulations that mimic the AD pattern (Racine, 1973). Additional EEG components that precede the AD have also been described including the stimulus evoked potential and EEG suppression that precede the AD (Racine, 1972b). Both the motor convulsions and these EEG properties depict how a change in responding occurs when the same stimulus is applied repeatedly.

Despite the usefulness of kindling in the field of epilepsy, many researchers (e.g., Goddard & Douglas, 1975; Racine et al., 1975, etc.) first recognized kindling as a potential model for learning and memory and the neuroplasticity that underlies these phenomena. Currently, kindling is thought of as a useful model of neuroplasticity whereas many epilepsy researchers regard it as a relatively weak model of epileptogenesis compared to other models (Racine, Tuff, & Zaid, 1975; Scharfman, 2002). This is largely because kindling does not result in spontaneous seizures without employing extreme and laborious methods such as extensive kindling (Pinel & Rovner, 1979) whereas other popular models of epileptogenesis, such as the chemoconvulsant models (i.e., pilocarpine and kainic acid), do produce spontaneous convulsions relatively easily. In addition, kindling generally does not result in gross neurological damage, as mentioned above which is in contrast to some aspects of temporal lobe epilepsy such as hippocampal sclerosis (Cook, Fish, Shorvon, et al., 1992). As a result, kindling may be more useful in the study of neuroplasticity rather than epileptogenesis per se.

Neurobiology of Kindling

There are several key structural changes that occur with kindling, particularly within the hippocampus. The hippocampus is the structure that hosts many of the neural changes associated with kindling. For example, the major pathway between the dentate gyrus and the CA3 region of the hippocampus (i.e., the mossy fibre pathway) undergoes axonal terminal sprouting onto the molecular layer of the dentate during kindling (Sutula et al., 1988). Dense sprouting has also been observed in the CA3 stratum oriens (Repressa & Ben-Ari, 1992). Mossy fibre sprouting has been shown to be independent of the site of stimulation (Cavazos et al., 1991). Although mossy fibre sprouting was once postulated as a mechanism of kindling, it now appears to occur after the development of kindling, at least with amygdala kindling (Ebert & Loscher, 1995; Elmer, Kokaia, Kokaia, et al., 1997; Osawa, Uemura, Kimura, & Sato, 2001).

In addition to mossy fibre sprouting, kindling produces other morphological changes such as neurogenesis (Parent, et al., 1998; Scott et al., 1998), synaptogenesis (Pollard, Bugra, Khrestchatisky, et al., 1996; Suemaru, Sato, Morimoto et al., 2000), and astrogliosis (Adams, Von Ling, Vaccarella, et al., 1998; Niquet, Jorquera, Faissner, et al., 1995), each of which has been postulated to be part of the underlying mechanism of kindling. For example, GAP-43 (i.e., growth-associated protein 43) in the hippocampal CA3 region is increased after hippocampal kindling (Bendotti et al., 1993) and synapsin I (a synaptic terminal protein) mRNA expression is also increased in the dentate after amygdala kindling (Morimoto, Sato, Sato et al., 1998). The evidence also shows that astrogliosis results in response to seizures rather than as a mechanism for seizure development (Khurgel & Ivy, 1996).

Similarly, hippocampal cell proliferation has been shown to increase after amygdala kindling (Nakagawa, Aimi, Yasuhara, et al., 2000; Parent, Janumpalli, McNamara & Lowenstein, 1998; Scott, Wang, Burnham, De Boni, & Wojowicz, 1998). However, there is currently less interest in studying kindling-induced cell proliferation because it has been discounted as a mechanism of epileptogenesis. This is because amygdala kindling stimulates hippocampal cell proliferation *after* generalized convulsions have been elicited. In one amygdala kindling study, proliferation was increased after 2-4 generalized convulsions but not after 2-4 subconvulsive stimulations (Scott et al., 1998). In another experiment, hippocampal cell proliferation was increased in groups of rats that displayed 9/10 and 19/20 generalized convulsions but not in those that displayed 4-6 generalized convulsions (Parent et al., 1998). In addition, inhibiting neurogenesis does not prevent the induction of chemoconvulsant seizures (Parent, Tada, Fike, & Lowenstein, 1999), and it actually appears to facilitate the rate of amygdala kindling (Fournier, Wintink, Darnbrough, & Kalynchuk, 2004). The timing of these effects suggests that hippocampal cell proliferation may be a product of seizure activity, rather than a mechanism of epileptogenesis, at least with amygdala kindling. Accordingly, kindling-induced neurogenesis may be either a compensatory mechanism for reducing the future occurrence of seizures or a mechanism by which activity-dependent consequences can occur. This same conclusion was also suggested in a recent major review of the kindling literature (Morimoto et al., 2004). Regardless of the mechanism by which it arises, kindling-induced neurogenesis is involved in the remodelling of the hippocampus that takes places once generalized convulsions have developed and as such, may contribute to hippocampal dysfunction.

Neurochemistry of Kindling

The main neurochemical systems that have been implicated in kindling are mainly the GABAergic (inhibitory) and the glutamatergic (excitatory) systems. Generally, there appears to be an overexcitation and a breakdown of inhibition. For example, high levels of GABA (for e.g., via uptake inhibitors or diazepam) hinder kindling (Gernert, Thompson, Loscher, & Tobin, 2002; Morimoto, Sato, Yamamoto, Watanabe, & Suwaki, 1997), whereas blocking glutamatergic action delays kindling (Loscher, Lehmann, Behl et al., 1999). The GABAergic and glutamatergic systems were first studied because of their inhibitory and excitatory roles, particularly within the hippocampus. For example, the dentate granule region of the hippocampus is highly excitable because the granule cells are mainly glutamatergic cells (Paxinos, 1994). In contrast, the mossy fibre pathway consists largely of inhibitory interneurons (i.e., GABAergic basket cells; Paxinos, 1994); however, excitatory mossy cells are also present. Also, glutamate decarboxylase (the main synthetic enzyme for GABA) has been detected within the granule cells (Sloviter, Dichter, Rachinsky et al., 1996). These data, among others, have contributed to the inhibition-versus-excitation debate in kindling, which is discussed in more detail in the following section.

Effects of Inhibition and Excitation in Kindling

Tuff, Racine, and Adamec (1983) found that kindling stimulations that were spaced too closely in time failed to evoke ADs (i.e., paired-pulse inhibition). Racine suggested that inhibition increased to compensate for the exogenous excitation delivered by the kindling stimulation. Mucha and Pinel (1977) previously showed that kindling stimulations had inhibitory after-effects (i.e., lower mean convulsive class, reduced convulsive duration, and reduced AD duration) if the stimulations were spaced fewer than 90 minutes apart. They also reported that kindled convulsions could not be elicited 24 hours after an extensive series of massed convulsive stimulations. These results suggested that under certain circumstances, kindling stimulations elicit inhibitory compensation to prevent seizures. In contrast, many reports have shown that kindling increases the amplitude of population excitatory post-synaptic potentials (EPSPs) in the limbic pathways (Racine, Gartner, & Burnham, 1972; Douglas & Goddard, 1975; Sutula & Steward, 1986; Maru & Goddard, 1987). It is this change in excitation and inhibition that seems to mostly influence the development of kindling, as discussed below.

Mechanism of Kindling

One of the early postulated mechanisms of kindling was that of monosynaptic potentiation proposed by Racine, Okujava, and Chipashvili (1972). They argued that inter-limbic connections were important for seizure development based on kindling transfer data (i.e., kindling one area to facilitate kindling in a secondary site) and based on lesion data (i.e., severing inter-limbic connections to hinder kindling). However, a monosynaptic potentiation theory was too simplistic to capture the dynamics of kindling. A second generation of mechanistic views incorporated the inhibition/excitation literature. For example, Sloviter (1987, 1991) described the hippocampus as the "gateway" for excitation and suggested that kindling broke down that gateway (by selective loss of inhibitory mossy cells) resulting in a flood of excitation. This theory was based largely on excitatory post-synaptic potential (EPSPs) evidence that the dentate cells became hyperexcitable. Sloviter's gateway hypothesis was criticized because of the evidence described above regarding the build up of inhibition when stimulations occurred too close in time (Mucha & Pinel, 1977; Tuff, Racine, & Adamec, 1983), although the issue continues to be revisited (see Sloviter, Zappone, Harvey et al., 2003). Others have shown that a failure of inhibition occurs in non-dentate regions of the hippocampus such as the CA1 region (Sato, Morimoto, Okamoto et al., 1990). The conflicting results may concern the use of different models (i.e., chemoconvulsants, kindling), the time of sampling (i.e., immediately following the seizure or between seizures), and specific hippocampal region examined (i.e., dentate, CA1, CA3) and make the mechanism of kindling a controversial topic.

The data regarding inhibition and excitation in the development of kindling is overwhelming and conflicting; however, recently, some resolution was attempted. Morimoto, Fahnestock, and Racine, (2004) proposed a glutamatergic-GABA mechanism of kindling-induced epileptogenesis. Upon high-frequency stimulation, glutamate and GABA are both released from presynaptic terminals: Glutamate stimulates postsynaptic AMPA receptors whereas $GABA_A$ receptor-mediated recurrent inhibition counteracts this depolarization immediately. The AMPA stimulation corresponds to the stimulus evoked potential visible with EEG whereas the $GABA_A$-mediated inhibition corresponds to the EEG suppression. If the stimulation amplitude is above threshold (or persists long enough) the $GABA_A$-mediated inhibition fails, resulting in burst firing and synchronization (the beginnings of epileptic activity marked by ADs). However, when stimulations occur too closely in time, the early GABA release prevents seizure induction. NMDA receptors are activated secondarily and facilitate Ca^{++} influx in a voltage-dependent manner allowing for further excitation.

These events lead to a cascade of events resulting in synaptic reorganization in both glutamatergic and GABAergic systems (Morimoto et al., 2004). Many of the changes are compensatory and activity-dependent but not directly related to epileptogenesis. Included in these compensatory results is the failure of $GABA_A$-mediated inhibition in the CA1 region of the hippocampus and the amygdala, whereas recurrent inhibition is strengthened in the dentate and piriform. In the later stages of kindling, extensive remodelling occurs in the form of neurogenesis, axonal sprouting, synaptogenesis, and astrogliosis. However, there is currently less interest in studying some of these effects, including neurogenesis, because they arise after the development of generalized convulsions: They appear to be a product of seizure activity, rather than a mechanism of epileptogenesis, at least with amygdala kindling. Because kindling is often not studied past the development of a few generalized convulsions, little is known about the importance of these secondarily evoked effects on the functional integrity of the temporal lobes.

To date, the biggest obstacle in deciphering the mechanism of kindling is contrasting those changes that result as a by-product of kindling (i.e., compensatory or activity-dependent) from those that contribute to the development of kindling. It seems likely that excitation results in kindled convulsions and that inhibition is a compensatory mechanism. This issue becomes inherent when one begins to consider the factors that contribute to the behavioural consequences of kindling. For example, does kindled-fear result from the same mechanism that produces convulsions (i.e., excitation) or does it result from the activity dependent or compensatory effects (i.e., inhibition, neurogenesis, synaptogenesis, astrogliosis, cell death etc.)?

Mechanisms of the Behavioural Consequences of Kindling

Kindling is an interesting phenomenon because it seems to develop in a manner that leads to many neural changes, only a few of which appear to involve neurological damage. As such, the changes that do occur in kindling seem to be a partially natural phenomenon of the temporal lobe system, or the brain in general. For example some of the activity-dependent effects of the electrical stimulations of kindling, such as hippocampal synaptogenesis and neurogenesis, also occur after environmental stimulation (Brown, Cooper-Kuhn, Kempermann, et al. 2003) and through spatial learning (Kempermann & Gage, 2002; Shors, Miesegaes, Beylin, et al., 2001; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002). Consequently, the question that arises is under what circumstances does the neuroplasticity of the temporal lobe promote the development of pathological outcomes such as convulsions and fear sensitization?

The phenomenon by which kindling increases hippocampal cell proliferation is a potentially important neurobiological outcome that may give rise to the behavioural consequences of kindling such as kindled fear. Hippocampal cell proliferation is interesting because of its potential role in learning and memory (Shors, 2004) and major depressive disorder (Jacobs, [van] Praag, & Gage, 2000; Malberg, 2004). For example, maze learning in rodents increases neurogenesis by facilitating the survival of new hippocampal cells (Shors, Miesegaes, Beylin, et al., 2001) and methods that inhibit neurogenesis (i.e., methylazoxymethanol acetate) impair learning and memory (Shors, Miesegaes, Beylin, et al., 2001; Shors, Townsend, Zhao, et al., 2002). In addition, rearing laboratory animals in complex and enriched environments has been shown to increase neurogenesis by promoting the survival and differentiation of new cells into neurons (Brown et al., 2003; Lu, Bao, Chen, et al., 2003) although voluntary exercise appears to be a significant factor also (van Praag, Kempermann, & Gage, 1999). Furthermore, decreased hippocampal neurogenesis has also been implicated as a mechanism associated with psychiatric disorders such as depression (Jacobs, van Praag, & Gage, 2000). In general, increased hippocampal neurogenesis is thought of as a normal product of hippocampal stimulation or activation, whereas reduced hippocampal neurogenesis has been associated with negative behavioural consequences, such as impaired memory, depression, and cognitive decline. However, this general schema may not be appropriate under all circumstances, as kindling-induced neurogenesis may also have negative behavioural consequences by producing an abnormal or unwarranted amount of newly generated cells. This is one of the major themes of this thesis, and it will be covered in detail beginning in the discussion of Experiment 2.

Behavioural Consequences of Kindling

Fearful Behaviour

The relationship between seizures (or epilepsy) and emotionality has been acknowledged for over a hundred years but it was not until 1977 when it underwent a systematic assessment. Bear and Fedio (1977) reported that individuals with temporal lobe epilepsy displayed heightened interictal (i.e., between seizures) emotionality. Around the same time, these interictal behavioural traits were also studied in animals using the amygdala-kindling model. Ademec (1976) showed that amygdala and hippocampal partial kindling (i.e., kindling that produces ADs but no convulsions) of cats resulted in increased defensive reactions toward rats, mice, and conspecific threat vocalizations. Pinel, Treit, and Rovner (1977) also demonstrated behavioural changes in long-term amygdala and hippocampal kindling (i.e., kindling long after the development of generalized convulsions). Specifically, they showed that kindled rats were hyperesponsive to a pencil tap on the back and reacted with great resistance to being picked up. More recently, Kalynchuk has used the kindling model to develop a model of fear sensitization using both short- (i.e., kindling enough to elicit only a few generalized convulsions but not further) and long-term (i.e., stimulations well passed the point of eliciting the first generalized convulsion) kindled rats (Kalynchuk et al., 1997). With this model, characteristics of fearful behaviour emerged that were not visible with the short-term or partial kindling models, such as panic-like behaviour after many stimulations. These behaviours are described in detail below after a description of how fear is measured in rats.

In rats, fear is often studied by measuring their response to novel objects or environments. Upon exposure to a novel object or novel environment, naïve rats will normally engage in a period of freezing (Walsh & Cummins, 1976). As time passes, the rats become less fearful of the novelty and begin to explore their environment. The act of freezing or exploring is often referred to as the approach-avoidance conflict and is believed to be a basic premise for animal activity in an environment (Montgomery & Monkman, 1955). Eventually, as rats habituate to the environment, they explore less and may engage in grooming, sleeping, or other non-fearful activities (Walsh & Cummins, 1976).

These exploratory behaviours in rodents are often studied in the laboratory using an open-field arena. For example, the amount of time the rat spends moving, rearing, and sniffing are normally considered indicators of the rats' exploration; whereas defecation, urination, and freezing are indications of fearfulness in male rats (Archer, 1975). Furthermore, specific activity patterns in an open field have also been investigated. For example, rats tend to adopt a home base soon after being introduced to a novel environment from which they make slow outbound trips with increasing distance and fast inbound trips (Eilam & Golani, 1989; Golani et al., 1993; Tchernichovski & Golani, 1995). The home base is also where the majority of grooming, rearing, crouching, and long visits occur (Eilam & Golani, 1989). Dead-reckoning navigational strategies have also been observed in open-field exploration of novel environments (Whishaw, Hines, & Wallace, 2001).

Fearful Behavior in Kindled Rats

Open Field: Measures of fearfulness in kindled rats have conventionally included open-field activity. In an open-field test, a kindled rat is placed in a novel arena for five minutes and the rat's activity is assessed based on the number of crosses it makes across a grid floor. Kindled rats typically display fewer line crosses in the first 30 seconds of open-field exposure (indicative of fearful avoidance or freezing) followed by an overall increase in the number of line crosses during the full five minutes (Kalynchuk et al., 2001; Murphy & Burnham, 2003). Interestingly, this latter effect appears to represent the inability to habituate to the novel environment over the course of the 5-minute session rather than a reduction of fear (Young, Wintink, & Kalynchuk, 2004). Others have also observed that kindling increases fear-related behaviours in the open field (Kalynchuk, Pinel, & Treit, 1998; Kalynchuk, Pinel, Treit, Barnes, McEachern, & Kippin, 1998; Nieminen, Sirvio, Teittinen, Pitkanen, Airaksinen, & Reikkinen, 1992) and the holeboard test(Adamec & Morgan, 1994), which is a similar kind of open arena with escape holes (see Barnes, 1979).

Resistance to Capture: The rats' resistance to being picked up from an open field is another measure of fearful behaviour, as was originally used by Pinel, Treit, and Rovner (1977). Each rat is scored on a 7-point scale resistance-to-capture scale modified from Albert and Richmond (1975). This measure has become a highly useful measure of the high levels of fear displayed by amygdala-kindled rats. On average, long-term kindled rats (i.e., approximately 100 kindling stimulations) score high on this test (Kalynchuk et al., 1997; Kalynchuk et al., 1999) corresponding to running away from the experimenter, attempting to bite the hand of the experimenter, and launching defensive jump attacks toward the experimenter's hand. This exaggerated fearfulness is in comparison to sham-stimulated rats that typically show very little resistance to being picked up. Extreme resistance to capture is now considered a reliable and robust measure in long-term amygdala-kindled rats (Kalynchuk, 2000). The resistance to capture shown by kindled rats exemplifies their highly inappropriate fear response. This response is particularly interesting given that kindled rats are easy to pick up from their home cage (Kalynchuk, Pinel, & Treit, 1999). This observation suggests that the novel environment is some sort of trigger for the expression of the fearfulness.

Elevated-Plus Maze: Kindled rats have also been shown to display escape-like behaviour from the elevated-plus maze (Kalynchuk et al., 1999). This apparatus consists of two closed arms and two open arms and is based on the premise that rats avoid the fear-inducing nature of the open space and prefer the closed arms. Kalynchuk et al. (1997) showed that rats that received 20 stimulations spent less time on the open arms compared to sham-stimulated control rats, as would be expected for mildly fearful rats; however, the groups of rats that received 100 kindling stimulations spent more time on the open arms and engaged in purposive jumping from the open arms. The authors interpreted the open-arm activity as indicative of high levels of fear to the point that the rats engaged in escape-like behaviour. Others have also observed fear behaviour in the elevated plus maze (Adamec, 1990a; Helfer, Deransart, Marescaux, & Depaulis, 1996).

Fear-Potentiated Startle: Others have shown that partial amygdala kindling, but not hippocampal kindling, exaggerated conditioned fear-potentiated startle (Rosen, Hamerman, Sitcoske, Glowa, & Schulkin (1996). This has also been examined in two strains of rats that were either fast or slow to kindle. Fear-potentiated startle was greater in fast kindlers compared to slow kindlers; however, slow kindlers had a greater startle response (Anisman, Kelly, Hayley, et al., 2000). Unfortunately, these animals were not compared to sham-stimulated rats.

Features of Kindled Fear

The behaviour of kindled rats has been fairly well characterized. As a result, several key features of the fearful behaviour have emerged. These include defensive behaviour, effects of the number of stimulations, persistence of the effect, site of stimulation, and novelty and habituation. Each of these features is described in detail below.

Defensive behaviour: Kindled fear appears to be a defensive response rather than an aggressive response. This was initially demonstrated by Adamec (1976) when partially kindled cats became defensive when exposed to mice, rats, and conspecific vocalizations. Long-term kindled rats tested as intruders in the resident-intruder paradigm also displayed defensive reactions (Kalynchuk, Pinel, & Treit, 1999). For example, they engaged in more active defensive behaviour (i.e., defensive upright posture, backing and running away), less passive defensive behaviour (i.e., freezing), and less aggressive behaviour (i.e., lateral displays anogenital sniffing, less time on top of the resident) than sham-stimulated rats. These results have also been corroborated using the social interaction test (developed by File & Hyde, 1978) during which time kindled rats that were paired together in a novel arena engaged in more defensive upright boxing than paired sham-stimulated rats (Davis, Gregus, Wintink, & Kalynchuk, 2001). Others have reported increased immobility with short-term kindled rats during the social-interaction test (Helfer, Deransart, Marescaux, & Depaulis (1996).

Number of stimulations: Another feature is that kindled fear intensifies with an increase in the number of stimulations delivered. Most studies of kindled fear focus on rats that have experienced only a few generalized convulsions as is the case with short-term kindling (e.g. Adamec, 1990; Adamec & Morgan, 1994; Helfer, Deransart, Marescaux & Depaulis, 1996); however, Kalynchuk and colleagues have studied the effect of different numbers of kindling stimulations on fearful behaviour (for a review see Kalynchuk, 2000). They found that relative to control rats, rats that receive 20 amygdala stimulations display significant increases in thigmotaxia in an unfamiliar open field, rats that receive 60 amygdala stimulations display significant decreases in open-field activity and increases in resistance to capture from the open field, and rats that receive 100 stimulations display all of these changes plus increases in fleeing behaviour in an open field and escape behaviour in an elevated plus maze (Kalynchuk et al., 1997). These results demonstrate that the number and magnitude of changes in fearful behaviour after amygdala kindling are proportional to the number of stimulations the rats receive. Consequently, amygdala kindling provides a particularly useful method for studying the progression from normal to pathological fear, particularly when employing short-term (i.e., 30 or fewer stimulations) and long-term (i.e., 60 or more stimulations) paradigms.

Site of stimulation: Although kindled-fear behaviour is most intense after amygdala kindling, it is also evident to a lesser extent after hippocampal kindling (Kalynchuk et al., 1998). Kindled fear has also been observed after kindling of the perirhinal cortex, piriform cortex, and bed nucleus of the stria terminalis, but not the caudate and claustrum (Davis, Thorne, Gregus, et al., 2002; Kalynchuk et al., 1998). Kindled fear is also dependent upon the rat strain (McIntyre, 1978), hemisphere of stimulations (Adamec & Morgan, 1994), and site within the amygdala (Adamec & Shallow, 2000).

Persistence: The effects of kindled fear seem to persist long after the final stimulation. For example, Kalynchuk et al. (1998a) reported that resistance-to-capture scores were still significantly higher in the kindled rats compared to the sham-stimulated rats two months after the final stimulation, although they did dissipate somewhat. This result is interesting given that the effects of kindling itself also seem to be permanent (Goddard et al., 1969; Wada & Sato, 1974). The long-lasting effect of kindled fear suggests that it is a result of the long-lasting neural reorganization associated with kindling.

Novelty and habituation: An important feature of kindled fear is that it is manifested primarily in novel environments. Kalynchuk et al. (1999) used the resistance-to-capture test to show that kindled rats were highly resistant to being picked up after five minutes in a novel open field, reacting on average by biting the hand of the experimenter and launching jump attacks at the hand, but these same rats were relatively easy to pick up from their home cage. Similarly, the kindled rats remain fearful following the open-field exposure until returned to their familiar home environment (i.e., rats are able to be picked up and weighed when returned to their home environment immediately following the open-field exposure despite high resistance to capture after the open-field exposure: unpublished observations). Moreover, the other testing environments mentioned above also contained a novelty component. For example, when a kindled rat is tested as an intruder in the resident-intruder test, the environment is novel and when kindled rats are paired together for the social-interaction test, the environment is novel to both rats. The elevated-plus maze is also an example of a novel testing environments. These observations suggest that the manifestation of kindled fear may be specific to novel environments and consequently, it may not represent a generalized elevation in fear. As such, the processing of novel information (i.e., novel spatial environments) may be related to the manifestation of kindled fear.

Related to the novelty component is that kindled rats are less able to habituate to a novel environment. Kalynchuk et al. (1999) repeatedly exposed kindled rats to the same open-field arena for five days in a row and observed a significant decrease in resistance-to-capture scores over the days; however, they failed to reach asymptotic levels comparable to sham-stimulated controls. Accordingly, there appears to be some consequence of kindling that does not allow kindled rats to easily familiarize or habituate to an environment. This may be one reason why kindled rats tend to be more active in the open-field compared to sham-stimulated rats (Kalynchuk et al., 2001; Murphy & Burnham, 2003). In fact, kindled rats' failure to habituate has also been specifically described within the open field (Young, Wintink, & Kalynchuk, 2004). In this experiment the kindled rats were more active in the last minute of a novel open-field session than in the first minute, indicating a failure to habituate. This was in contrast with the sham-stimulated rats that were more active during the first minute of the session and appeared to habituate by being less active during the last minute. Again, this suggests that the processing of spatial information (or the inability to do so) may be related to the development of kindled fear. Furthermore, the seemingly permanence of kindled fear suggests that there might be some sort of permanent neural reorganization that takes place in kindled rats that potentiates their fear response.

These features of kindled fear are important because they suggest that a brain structure that mediates responses to novelty and is involved in the processing of environmental information necessary for habituation may be disrupted in kindled animals. The hippocampus is well known for its role in these types of behaviours. The potential role that the hippocampus might play in kindled fear is considered below.

Hippocampal Changes Associated with Kindled Fear

There is evidence that neural changes within the hippocampus might underlie the development and expression of amygdala-kindled fear. Recently, Kalynchuk et al. (2001) examined FOS protein immunoreactivity in amygdala-kindled and sham-stimulated rats shortly after a 5-minute exposure to a novel open field. FOS immunoreactivity was lower in the dentate granule layer and the CA1 region of the hippocampus and the perirhinal cortex of kindled rats compared to sham-stimulated controls. This decrease in FOS immunoreactivity was correlated with resistance to capture and was interpreted to suggest that the hippocampus of kindled rats was inhibited or less capable of adaptive plasticity. Others have made similar assertions regarding the role of the hippocampus, specifically regarding the change in inhibitory-excitatory balance of the hippocampus as it relates to defensive behavior. Adamec (1991) used a partial-kindling paradigm (i.e., pre-convulsive stimulations) in which animals were kindled in the perforant path. These partially kindled cats had increased CA3 inhibition and decreased CA1 inhibition. Furthermore, these changes were associated with increased defensiveness of a predator to prey (i.e., cats' response to rats). Previously, Tuff, Racine, and Adamec (1983) showed that repeatedly eliciting ADs in the perforant path increased the EPSP components of the dentate gyrus field potentials; however, hippocampal recurrent inhibition accompanied the increased excitation.

Support for Kalynchuk et al.'s (2001) hypothesis that the hippocampus is inhibited was provided by receptor binding data in which kindled rats showed increased hippocampal $GABA_A$/BZ receptor binding that was positively correlated with fear levels (Kalynchuk, Pearson, Pinel, & Meaney, 1999). This may be consistent with the increased $GABA_A$-mediated inhibition suggestion in the mechanism of kindling. Kindled rats are reported to show increased hippocampal $5HT_{1A}$ receptor binding levels, which were also positively correlated with kindled fear levels (Kalynchuk et al., manuscript in preparation). $5HT_{1A}$ mRNA was also increased but it was only within a subpopulation of cells. In contrast, excitatory receptor binding levels of AMPA and NMDA in the hippocampus were decreased (McEachern, Kalynchuk, Fibiger, Pinel, & Shaw, 1995). In general, these results allow for the possibility that reorganization of the inhibition-excitation hippocampal network may mediate kindled fear behaviour in amygdala-kindled rats. A change in inhibition and excitation could arise by an upregulation of inhibitory or a downregulation of excitatory receptors or it could arise by growth or loss of respective cell types. This latter possibility could account for why only a subpopulation of cells in the $5HT_{1A}$ study showed increased mRNA expression. For example, it may be that new cells born as a result of kindling express aberrant numbers of receptors and genes. The cells in the $5\text{-}HT_{1A}$ study that showed increased $5\text{-}HT_{1A}$ mRNA expression could have been cells that were born during the kindling process.

Currently, the majority of research in the kindling field is focused on the development of the initial seizures and little attention is paid to the effects that are not involved in the mechanism of epileptogenesis. However, secondary neural changes arising from kindling, such as cell proliferation and the maturation of new neurons, may be extremely important compensatory mechanisms. For example, after prolonged excitation, $GABA_A$-mediated inhibition fails resulting in seizure activity. These secondary inhibitory mechanisms might involve proliferating cells that are promoted to to integrate into the hippocampal network to impose inhibition right around the time that generalized convulsions have developed. Consequently, the integrity of the hippocampus could be compromised in a manner that leads to impaired hippocampal functioning and consequently, impaired processing of hippocampal-dependent information. By this argument, the behavioural consequences of seizures could arise as a by-product of the brain's attempt to evoke secondary mechanisms that limit the future occurrence of seizures.

Hippocampal-Mediated Exploratory and Fear Behaviour

A well-known theory of hippocampal functioning is O'Keefe and Nadel's (1978) cognitive mapping theory. They suggest that the anatomy and physiology of the hippocampus allow the animal to sort sensory information based on the animal's movement in space. They argue that cells within the hippocampus can be categorized either by the stimuli allowing the animal to navigate in space acquiring lots of spatial movement or by stimuli that limit stimulation from the environment, firing at specific "places" (i.e., place cells). They propose two types of place cells: one type that has a converging place field comprising excitatory input that fire in a given location (i.e., the "I know my location" cells) and a second type that involves inhibition and fires with the removal of previously learned spatial cues (i.e., the "something in my environment is missing" cells). O'Keefe and Nadel propose that the purpose of these cells is to both signal the animal's position and to detect a mismatch between sensory information and previously learned representation, all of which converges in the hippocampus. Accordingly, they offered the hippocampus as the storage unit for the memory of cognitive maps based on excitation and inhibition of place cells.

Gray (1982) and Gray and McNaughton (1983) propose a more basic function of the hippocampus and the surrounding structures, which is based on the conflict between approach and avoidance behaviour that leads to behavioural inhibition. They describe a behavioural inhibition system (BIS) that responds to punishment, nonreward, or novel stimuli with behavioural inhibition, increased attention, and increased arousal, and they recognize the septo-hippocampal system as mediating the inhibition and attentional components. They argue that the septo-hippocampal system acts as a comparator that detects novelty. When a mismatch is detected between the current state of events and preexisting knowledge, the mismatch in neural input results in the behavioural inhibition therein impeding the approach toward a goal.

Gray (1982) first presented this model as a circuit for information gathering, checking, and planning moves. Sensory information from the world is gathered and compared against expected or predicted events. Predictions are generated with sufficient sensory information from the world and also from information regarding past experience. A mismatch among these factors halts the process of generating predictions. At this point, the septo-hippocampal system takes active (as opposed to the previous passive monitoring) control, producing behavioural inhibition and increased attention (as mentioned above). In essence, motor output is inhibited, the system is held in "check", and then the system initiates specific exploratory behaviour (i.e., approach or avoid) to follow up on the checking step.

The role of each structure of the septo-hippocampal system is clearly described by Gray (1982). Sensory information enters via the entorhinal cortex to the hippocampus (i.e., via the dentate, CA3, and CA1) leaving via the fornix toward the mammillary bodies, the anteroventral thalamus, the cingulate cortex, and then to the subiculum. The subiculum relies on stored information concerning the environment: It receives response-independent environmental information from the anteroventral thalamus and response-dependent information from the cingulate. The subiculo-entorhinal projections are considered as the points for the selection of sensory information. The hippocampal circuitry functions to ensure that only important information is circulated through to the subiculum. However, because the subiculum is also receiving direct connections from the entorhinal cortex, the information must also be passed through the hippocampal-gating step, thereby allowing for the matching process. The dentate-CA3-CA1 acts, therefore, as the gate that allows for either habituation (if the information *does not* pass through) or attention/anxiety (if the information *does* pass through).

Gray clearly conceptualized his views when he suggested that an increase in the intensity of attention/anxiety could occur when the gate opens (i.e., the dentate-CA3 gate) so that more stimuli become important and checked. This is an important concept in comparing O'Keefe and Nadel's theory to that of Gray and McNaughton. The former theory argues for the role of the hippocampus in spatial mapping, based primarily on lesion studies, whereas the latter theory was used to argue against the spatial mapping theory as being too specific in function and instead suggests that when the hippocampus is lesioned, too much information is retained and competing information cannot be suppressed. Gray and McNaughton believe that lesions or other means of interfering with hippocampal functioning impair response inhibition and result in increased goal approach behaviour. Accordingly, spatial memory deficits may not be memory deficits per se but rather, an inability to appropriately navigate through space because of a failure to inhibit cues that do not provide information about the goal.

Both Gray and McNaughton's and O'Keefe and Nadel's theory, offer an interesting framework in which to consider the behaviour of amygdala-kindled rats. In general, an animal's ability to navigate through space with the appropriate balance of approach-avoidance might be compromised if the hippocampus undergoes extensive reorganization and results in altered inhibition-excitation. Given that kindling does affect the hippocampus in such a manner, this suggests that kindled fear may arise through hippocampal-dependent changes. The data and theoretical framework provided by the two theories thus far provides ample reason for suspecting that changes to the hippocampus are involved in the fearful behaviour produced by kindling. Kindled rats demonstrate their exaggerated fearfulness in novel environments. The theories of Gray and McNaughton and of O'Keefe and Nadel would both predict a hippocampal involvement in the expression fear. Therefore, the hippocampus is a particularly enticing region for examining the neural changes associated with kindled fear. In fact, a limited amount of hippocampal-dependent behaviour has been examined in kindled rats.

Effects of Kindling on Spatial Navigation

In addition to the effects of amygdala kindling on fear (described above), spatial memory impairments have also been observed particularly after hippocampal kindling. In general, hippocampal kindling impairs spatial memory in the Morris water maze (MWM; Hannesson, Wallace, Pollock, Corley, Mohapel, & Corcoran, 2004) and the radial-arm maze (RAM; Sutula, Lauersdorf, Lynch et al., 1995). Both tests require animals to navigate through the apparatus for a goal of either a hidden escape platform (in the MWM) or the food-baited enclosed arm (in the RAM) and are generally considered to require a functioning hippocampus for successful navigation (Hodges, 1996). These results have been replicated in perforant-path kindled paradigms (McNamara, Kirkby, dePage, et al., 1993), which stimulates the major pathway that terminates on the granule cell layer of the hippocampal dentate gyrus. In contrast, spatial memory appears to be intact after amygdala kindling (Nieminen, Sirvio, Teittinen, et al., 1992), although acquisition impairments that are not specifically hippocampal-mediated have also been observed (Anisman & McIntyre, 2002; McNamara, Kirkby, dePage, & Corcoran, 1992). Unfortunately, the majority of amygdala-kindling studies have failed to fully consider the development of kindled fear over the course of kindling. Currently, the effects of amygdala kindling on hippocampal-dependent memory tasks have not been adequately assessed and have never been assessed in female kindled rats.

Sex Differences and Kindling

An important issue that has not been addressed is the effect of amygdala kindling in female rats. The majority, if not all, of the kindling data described thus far has been derived from male rats. All of the data on the behavioural consequences of kindling have been derived from male rats only. There are several reasons why sex differences need to be addressed in kindled fear. First, sex differences clearly exist in the development and expression of anxiety: Women are more often diagnosed with anxiety-related disorders than men (reviewed in Palanza, 2001). Therefore, any model that attempts to appropriately model anxiety should be based on data that includes female behaviour. Second, as the hippocampus is being tested as a main structure involved in the development of kindled fear, sex differences may become apparent given the well-known sex differences in hippocampal-mediated behaviours. For example, males are well known to outperform females on spatial tasks (e.g., Postma, Jager, Kessels, et al., 2004).

There has been some attempt to examine the relationship between amygdala kindling and the hormonal status of both male and female rats. For example, estrogen has been shown to exhibit proconvulsant properties whereas progesterone has anticonvulsant properties. For example, estradial and testosterone in male and female rats, respectively, lowers ADT (Edwards, Burnham, & MacLusky, 1999). Edwards, Burnham, Mendonca, et al. (1999) have also shown that ADT varies with the estrous cycle: ADT are lower during high estrogen phases. Kindling has also been reported to cause female rats to cease their normal estrous cycle resulting in constant proestrus (Edwards, Burnham, Ng, Asa, & MacLusky, 1999), an estrous phase associated with high estrogen levels. In fact, the animal data mimics the clinical data (Morrell & Montouris, 2004). These results are consistent with clinical reports of higher incidents of seizures in girls as they begin to menstruate (Klein, van Passel-Clark, & Pezzullo, 2003) and higher incidents of seizure activity when women are pregnant (Kilpatrick & Hopper, 1993) when circulating estrogens are high. Epilepsy is also associated with a higher rate of amenorrhea in women (Herzog, 1996). In male rats, kindling increases serum testosterone and estradiol (Edwards, Burnham, & MacLusky, 1999)

The exact mechanism by which sex hormones exert their effect is not known but it has been suggested that the hypothalamic-pituitary-adrenal (HPA) axis is involved (Edwards, Burnham, Ng, et al., 1999). The HPA axis mediates many physiological responses but it is particularly known for its role in stress response by the release of corticosteroid hormones into circulation. Indeed, glucocorticoids have been shown to be proconvulsant in humans (Odeh, Lavy, & Stermer, 2003); however, andrenocorticotropic hormone has anticonvulsant effects in 15-day old laboratory rat pups (Edwards, Vimal, & Burnham, 2002). There has been suggestion that a lack of inhibitory control via the amygdala-hippocampal pathway of the HPA axis may promote seizures (and, incidentally, comorbid clinical depression; Zobel, Wellmer, Schulze-Rauschenbach, et al., 2004). The hippocampus is involved in the HPA response by providing the negative feedback necessary to discontinue the stress response (Jacobson & Sapolsky, 1991). Consequently, a dysfunctional hippocampus may promote fearful behavior by interfering with the negative feedback of the stress response. These considerations may prove important in the future; however, a full examination of these influences is beyond the current scope of this thesis. Including females in the experiments of this thesis provides the first step in identifying specific sex differences and similarities in kindled fear. The inclusion of females may also allow for the detection of sex difference under certain circumstance that result may not otherwise have been detected. Consequently, male and female rats were included in every experiment.

The Purpose of this Dissertation

The purpose of this dissertation is twofold: To investigate sex differences in the behavioural consequences of amygdala kindling and to evaluate the hypothesis that kindling-induced fear is mediated by neural changes within hippocampal circuits. There are still several aspects of kindled fear behaviour that are not well understood yet are important elements. One major element in the field is the extent to which the model applies to female rats: Most of the behavioural and neural data thus far has been generated using males, especially in the extended kindling model and to a large extent in the conventional models. Some exceptions to this exist that concern the role of reproductive hormones in afterdischarge thresholds described above (e.g. Edwards, Burnham, Mendonca, Bowlby, & MacLusky, 1999; Edwards, MacLusky, & Burnham, 2000) and cortical kindling paradigms (Teskey, Hutchinson, & Kolb, 1999), none of which include behavioural measures. This is an important consideration given that sex differences do exist on many tests of anxiety and spatial tasks in both human and non-human animals. For example, females tend to be more active under anxiogenic conditions (see Archer, 1975 for a review) and perform worse on spatial tasks, as mentioned. These sex differences are described in detail in Experiment 1 and 2, respectively.

The second purpose of this dissertation is to identify specific hippocampal changes that might be important for the development of kindled fear. This is an important step for understanding how pathological fear and anxiety might arise, and for identifying new strategies that can treat these pathologies or even prevent their development in the first place. There are a plethora of changes that occur in the hippocampus that are currently unexplored correlates of kindled-fear behaviour; for example, mossy fibre sprouting, synaptogenesis, and astrogliosis are some. However, an interesting hypothesis is that kindled fear results from an abnormal amount of new cells within the hippocampus. An increase in hippocampal cell proliferation has already been described above (Scott et al., 1889; Parent et al., 1998). This phenomenon could lead to a variety of outcomes including an excess of cells, misplaced cells, an excess of inhibitory cells, or generally dysfunctional cells, all of which could affect the integrity of the hippocampus in a manner that results in increased fear. Furthermore, hippocampal cell proliferation and subsequent neurogenesis is similarly increased by exposure to a spatial learning task (see Prickaerts, Koopmans, Blokland, & Scheepens, 2004; Shors, et al., 2001). Given that kindled fear emerges in situations in which spatial information processing is required (i.e., in a novel open field) it is conceivable that kindling-induced changes to the normal rate or fate of hippocampal cell proliferation might interfere with normal spatial processing. Accordingly, exploring the role of hippocampal neurogenesis in the development of kindled fear is a logical step.

The following four experiments comprise this thesis: In Experiment 1, males and females were compared on several measures of fear behaviour in order to address whether males and females display similar levels of fear after kindling; In Experiment 2, spatial memory performance was assessed repeatedly throughout the kindling phase in both male and female kindled rats using a hippocampal-dependent version of the Morris water maze; In Experiment 3, the rate of hippocampal cell proliferation was examined as a correlate of kindled fear; In Experiment 4, an attempt was made to curtail the development of kindled fear by activating the hippocampus with environmental stimulation and subsequently assessing the fate of hippocampal cell proliferation.

The hypotheses were that: 1) males and females would display similar levels of fear but could display sex-specific differences on some measures, 2) kindled rats would display disrupted performance in the Morris water maze, 3) hippocampal cell proliferation would be increased as a result of kindling, 4) environmental stimulation would curtail the development of kindled fear and also alter the survival of cells proliferating in the hippocampus. These four experiments provide a comprehensive examination of the behavioural consequences of amygdala-kindled male and female rats and provide evidence that amygdala-kindled fear behaviour is hippocampal-mediated.

Chapter 2: Experiment 1

Fear Behaviour in Amygdala-Kindled Male and Female Rats

The purpose of Experiment 1 was to assess potential sex differences in the effects of kindling on fear behaviour. Surprisingly, there have been no previous studies of the behavioural consequences of kindling in female rats. Characterizing these behavioural effects of kindling in female rats is important for several reasons. There are unequivocal reports of sex differences in human anxiety (see Palanza, 2001): Women are more often diagnosed with anxiety disorders than men and the manifestation of symptoms differs between men and women. This is paralleled in animal models of anxiety. Female rats are more likely to display increased locomotor activity and rearing whereas male rats are more likely to show increased freezing and defecation when anxious (for a review see Archer, 1975).

Sex differences in the manifestation of anxiety-related behaviours have traditionally posed a problem when attempting to interpret fear levels across both males and females. For example, male rats have been considered more fearful than females by some researchers (e.g., Gray, 1971). However, others have suggested that the level of fear does not necessarily differ between males and females but that traditional tests of anxiety have been validated with males and are therefore not entirely applicable for testing females (Fernandes, Gonzales, Wilson, & File, 1999; Johnston & File, 1991; Palanza, 2001). A major problem in this area is that there have been relatively few studies of fear and anxiety in female compared to male rats. Characterizing the development and progression of kindled fear in female rats will thus provide important information about the fundamental nature of fearful behaviour, including the motivation of sex-specific fear-related behaviours. Furthermore, using measures like the resistance-to-capture test might reduce some of the problems associated with comparing males and females on conventional tests of anxiety, which often rely on activity levels.

The purpose of the present experiment was to compare the behavioural consequences of short- and long-term amygdala kindling in male and female rats. For this experiment, separate groups of male and female rats received 99, 30, or sham stimulations and were subsequently tested on measures of fear (i.e., open-field and resistance-to-capture) and learned helplessness (i.e., forced-swim test). The measures of fear have been used in previous experiments and were discussed in detail in the general introduction. The Porsolt forced-swim test (Porsolt, 1979) is commonly used as a measure of learned helplessness in studies of animal depression. The major measure of the test is the latency for the animal to adopt an immobile posture. Although the test is used to measure learned helplessness, it has been criticized because the exact motivation for an animal to discontinue movement is not agreed upon. Some people have argued that the animals are experiencing learned helplessness whereas others argue that the animals are conserving energy rather than adopting a helpless posture (West, 1990). This test has been used as a stress challenge to examine rats' responses to a highly stressful situation irrespective of antidepressant action (e.g. Campbell, Lin, DeVries, & Lambert, 2003; Molina, Wagner, & Spear, 1994). Accordingly, as a stress challenge, this test may be a useful in measuring kindled rats' response to a novel stressful environment.

Methods

Subjects

The subjects were 36 male and 34 female adult (approximately 250 and 200 g respectively at the time of arrival) Long Evans rats (Charles River Canada, St. Constant, PQ). They were individually housed in rectangular polypropylene cages with wood shavings as bedding. Food and water was available *ad libitum*. Lights in the colony room were set on a 12:12-hr light:dark cycle with lights on at 8:00 a.m. All experimental manipulations were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the Dalhousie University Committee on Laboratory Animals.

Surgery

Each rat underwent stereotaxic surgery approximately one week after arrival and was handled daily prior to surgery. At the time of surgery, males rats weighed between 275-300g and female rats weighed between 240-275g. A single bipolar electrode (MS-303-2, Plastics One, Roanoke, VA) was aimed at the left basolateral amygdala of each rat under sodium pentobarbital anaesthesia (Somnotol, MTC Pharmaceuticals; 65 mg/kg i.p.) using the following coordinates from bregma: A: 2.8mm, M-L: 5.0mm, V: 8.5mm, with the incisor bar set at –3.3mm (Paxinos & Watson, 1998). The electrode was secured to the skull with four stainless steel screws and dental acrylic. Flamazine (1% silver sulfadiozine) antibiotic was used to facilitate healing around the incision as needed.

Kindling

After a postoperative recovery period of at least 5 days, the male and female rats were divided into three conditions each: One group of male and one group of female rats received 99 kindling stimulations (99-stim males, n = 12; 99-stim females, n = 12), one group of male and one group of female rats received 69 sham stimulations followed by 30 kindling stimulations (30-stim males, n = 12; 30-stim females, n = 12), and one group of male and one group of female rats received 99 sham stimulations (sham-stim males, n = 12; sham-stim females, n = 10). To facilitate the kindling and behavioural testing of such a large number of rats, about half the rats in each condition were placed into one large squad, and the other half were placed into a second large squad. The second squad began the kindling and behavioural testing phases of the experiment one week after the first squad.

Kindling occurred in the following manner. Three stimulations were delivered each day, 5 days per week, with a minimum of 2 hr between consecutive stimulations. A few seconds prior to each stimulation, each rat was placed in a plastic box (60 cm L X 25 cm W X 10 cm H) containing a thin layer of wood shavings, the stimulation lead was attached, and the stimulation was delivered. Each stimulation consisted of a 1 second, 60 Hz train of pulses. Each pulse had a biphasic amplitude of 800 μA peak-to-peak and a duration of 1 ms. After all convulsive activity had ceased, the rat was returned to its home cage. Rats receiving sham stimulations were treated in exactly the same manner except that no current was delivered.

The measure of seizure severity was the convulsion class elicited by kindling each stimulation. Convulsion class was scored according to Pinel and Rovner's (1978) extension of Racine's (1972b) widely used 5-class scale (class 1: head nodding only; class 2: head nodding and jaw clonus; class 3: head nodding, jaw clonus, and forelimb clonus; class 4: head nodding, jaw clonus, forelimb clonus, and rearing; class 5: head nodding, jaw clonus, forelimb clonus, rearing, and falling once; class 6: a class 5 with multiple falling episodes; class 7: a class 6 with running fits; class 8: any of the preceding symptoms with periods of tonus). The rate of kindling was assessed by the mean number of stimulations required for rats to reach the first class 5 convulsion.

Vaginal Smears

The influence of the female estrous cycle was a consideration in both kindling development and behavioural testing, therefore, vaginal swabs were taken daily from the female rats starting approximately five days prior to kindling and ending the last day of behavioural testing. On stimulation days, swabs were taken prior to the third stimulation of the day, approximately mid-day of their light phase (between 12h00 and 16h00). On non-stimulation days, swabs were done during the same time interval. The contents of the swabs were smeared onto slides, dipped in 70% ethanol, and later stained using the Giemsa (Sigma-Aldrich) method for cell type (i.e., leukocyte) identification for estrous phase classification. To control for handling effects, the males were also picked up by the tail and their genitals were briefly exposed in a similar way to the treatment of the females. Analysis of cycling in female rats was measured by counting the number of errors in the cycle during the 33 days of the kindling phase. Errors included more than one consecutive day in an estrous phase, backward cycling, and missed phases according to staining characterization. When possible, ambiguous smears were estimated based on the phase characterization of adjacent days.

Behavioural Testing

One behavioural test per day began the day following the final stimulation. All behavioural testing was done during the light phase in a testing room separate from the colony room.

Open field. The open field was used to measure fearful behaviour. The open field consisted of a square wooden box with a Plexiglas bottom (70 x 70 x 60 cm). Lines marked a grid on the floor (for a total of 36 squares). Rats were placed individually in the open field for 5 min in a brightly-lit room. The number of lines crossed was counted for each minute of the open-field test. The frequency of side-wall jumping was also scored.

Resistance-to-capture test. At the end of the 5-min open-field test, an unfamiliar experimenter (blind to the experimental condition of the rat) wearing a leather glove attempted to pick up the rat from its position in the open field. The resistance to being picked up was measured on a 7-point resistance-to-capture scale: 0 = easy to pick up, 1 = vocalizes or shies away from hand, 2 = shies away from hand and vocalizes, 3 = runs from hand, 4 = runs away and vocalizes, 5 = bites or attempts to bite, 6 = launches jump attack (see Kalynchuk et al., 1997). Some rats were highly resistant to being captured and eluded the gloved hand when the experimenter tried to pick them up. In these cases, the experimenter persisted and the rat was given a score based on the highest degree of resistance to capture that it displayed. The open field was cleaned thoroughly with Fantastik solution in between each rat.

Forced swim. The forced swim test was used to measure swimming activity in response to a stressor. Rats were placed in a square cylinder (60 cm high, 25 x 25 cm wide) filled with water at 27 $\pm$ 2 ° C for 10 min. The water was maintained at a level 30 cm from the bottom of the cylinder, which prevented the rat from touching the bottom with its hind legs while its head was above the water. The main behavioural measures that were recorded were the latency to immobility and the time spent immobile. Immobility was recorded when the rat made only enough movement to stay afloat with no directional swimming. Latency to immobility was determined the first time the rat became immobile for 5 s or more. Thereafter, each time the rat adopted that position time spent immobile was immediately recorded. This was a conservative estimate of latency to immobility to avoid scoring swimming pauses. The number of head shakes, sinks, and dives displayed by each rat was also scored.

Histology

At the conclusion of the behavioural testing, all rats were sacrificed by overdose with sodium pentobarbital (Somnotol, MTC Pharmaceuticals) according to the Canadian Council on Animal Care guidelines. Their brains were removed and preserved in formalin. The brains were then frozen and sliced at 40 μm along the coronal plane through the amygdala. Every fifth slice was mounted on a slide and stained with cresyl violet. The position of each electrode tip was estimated from the stained slides by a naive observer using the Paxinos and Watson (1998) stereotaxic atlas.

Data Analysis

The data were analyzed using SPSS 10.1. Simple effects analysis of variance (ANOVA) was used to examine the effect of kindling group and sex on kindling acquisition and emotional behaviour. Repeated measures ANOVA and polynomial trend analysis was used to analyze the patterns of behavior in open-field line crosses. The Kruskal-Wallis non-parametric ANOVA was used for the resistance-to-capture test. F ratios less than 1.0 (i.e., not significant) are indicated as such with no probability value reported.

Results

Histology

Rats were removed from the behavioural testing and/or statistical analyses if the electrode-skull apparatus became disassembled at any point during the experiment or if rats had electrodes terminating outside the amygdala as observed with cresyl violet histological staining. Four males (two 99-stim, one 30-stim, one sham-stim) and four females (one 99-stim, three sham-stim) were removed for these reasons. Thus, the data analyses were based on a total of 59 rats: 99-stim males = 10, 99-stim females = 11, 30-stim males = 10, 30-stim females = 11, sham-stim males = 10, and sham-stim females = 7.

Kindling

Figure 1.1 illustrates the mean number of stimulations to the first class 5 convulsion for the males and females in the 30-stim (i.e., 69 sham stimulations followed by 30 electrical stimulations) and 99-stim groups. The number of stimulations required to elicit the first class 5 convulsion was not significantly different between 30-stim and 99-stim females, $F(1, 38) = 1.95, p = .170$, but the 99-stim males required more stimulations

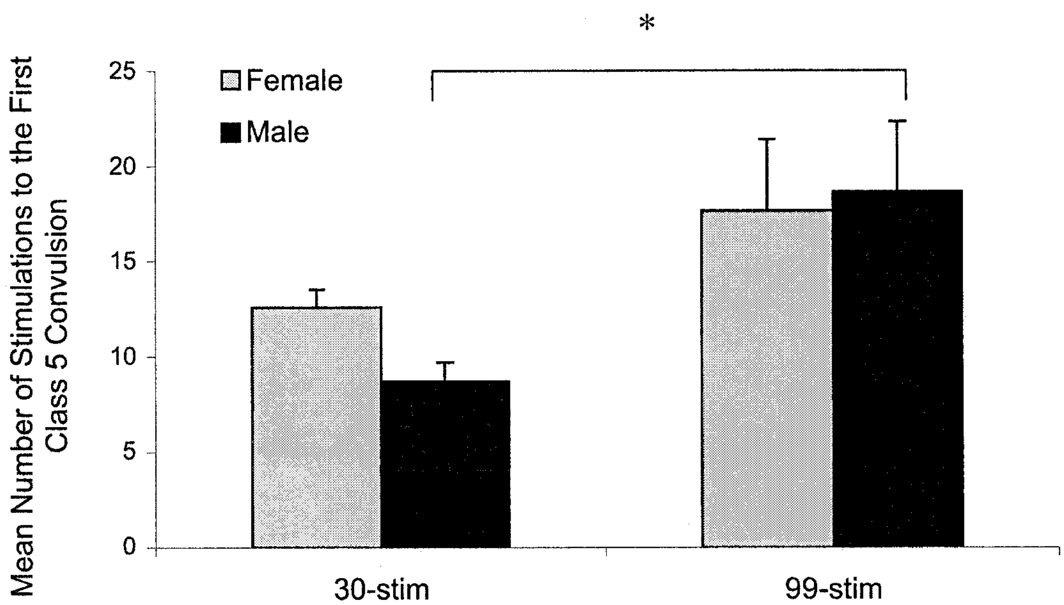


Figure 1.1. The acquisition of kindling in the male and female rats. Mean (± SEM) number of stimulations required to elicit the first class 5 convulsion for male and female rats in both the 30-stim and the 99-stim kindling conditions. * $p < .05$

to elicit the first class 5 convulsion than did the 30-stim males, $F(1, 38) = 6.71, p = .013$. There were no significant sex differences in either the 30-stim, $F(1, 38) < 1.0$, or the 99-stim kindling groups, $F(1, 38) < 1.0$.

Vaginal Smears

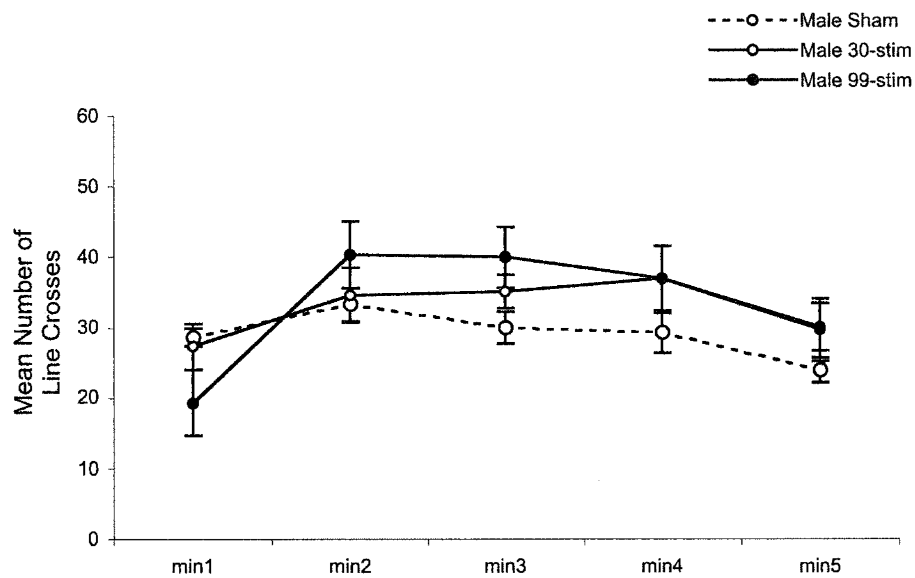
The effect of kindling on the female rats' reproductive cycle was assessed by estimating the mean number of cycling errors for each condition of female rats. Kindling had no significant effect on estrous cycling, $F(2,26) < 1.0$, (sham-stim $M = 10.57$, $SD = 6.78$, 30-stim $M = 9.64$, $SD = 8.18$, 99-stim $M = 9.36$, $SD = 5.57$).

Open-Field Activity

Figure 1.2 illustrates the mean number of lines crossed in each minute in the open field for the male and female rats in each group. The 99-stim males and females displayed a clear difference across the 5 minutes compared to the sham-stimulated groups. Specifically, the 99-stim rats exhibited fewer line crosses during the first minute and more line crosses in subsequent minutes of the open-field session. The 30-stim rats showed a similar pattern without exhibiting a reduced mean number of line crosses in the first minute.

Statistically, this pattern was assessed using simple effects repeated measures ANOVAs separately for males and females of each kindling group. These analyses revealed a statistically significant change across minute for the sham-stim females, $F(4, 212) = 4.55, p = .002$, the 30-stim females, $F(4,212) = 5.99, p < .001$, the 99-stim females, $F(4,212) = 11.33, p < .001$, and the 99-stim males, $F(4,212) = 11.13, p < .001$.

A



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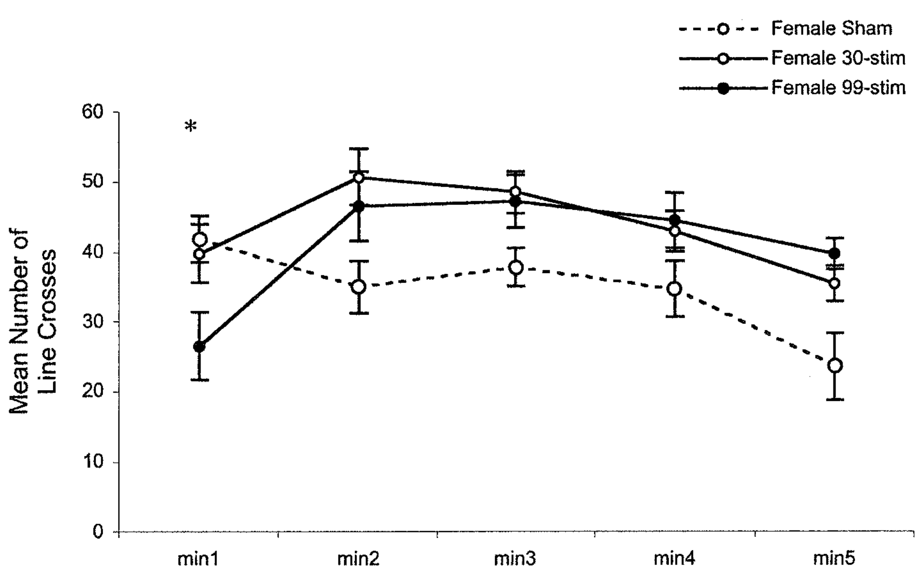


Figure 1.2. Mean number of line crosses across each minute of the open field for males (A) and females (B) of each kindling condition. See text for statistical analayses of the pattern differences among kindling conditions.

The sham-stim males showed no statistically significant difference across minute, $F(4,212) = 1.61, p = .174$. The 30-stim males failed to show a statistically significant difference across minute, although the effect approached statistical significance, $F(4,212) = 2.27, p = .063$. Simple main effects of group and sex, and simple interactions were also assessed at each minute. In each minute there was a statistically significant, or nearly significant, simple main effect of kindling group, $F(2,53) = 5.80, p = .005$ (minute 1), $F(2,53) = 2.83, p = .068$ (minute 2), $F(2,53) = 4.56, p = .015$ (minute 3), $F(2,53) = 2.85$, $p = .067$ (minute 4), and $F(2,53) = 6.19, p = .004$ (minute 5). In each minute there was also a statistically significant, or nearly significant, simple main effect of sex, $F(1,53) = 11.37, p = .001$ (minute 1), $F(1,53) = 5.51, p = .023$ (minute 2), $F(1,53) = 12.41, p = .001$ (minute 3), $F(1,53) = 3.92, p = .053$ (minute 4), $F(1,53) = 3.73, p = .059$ (minute 5). There was no statistically significant interaction between sex and kindling at any minute, $F(2,53) \leq 1.26, p \geq .291$.

Polynomial trend analysis was done across all conditions except for the sham-stim males who showed no statistically significant change across minute of the open field. This analysis revealed that the 30-stim males displayed a statistically significant quadratic trend, $F(1,9) = 9.270, p = .014$, and the 99-stim males displayed significant quadratic and cubic trends, $F(1,9) = 48.960, p < .001$ and $F(1,9) = 10.608, p = .010$. The sham-stim females displayed significant linear and cubic trends, $F(1,6) = 6.493, p = .044$ and $F(1,6) = 10.651, p = .017$. The 30-stim females displayed a significant quadratic trend, $F(1,10) = 16.213, p = .002$. The 99-stim females displayed significant linear and quadratic trends, $F(1,10) = 6.310, p = .031$ and $F(1,10) = 15.529, p = .003$, respectively.

Finally, during the open-field test it was noted that some rats would jump up at the Plexiglas ridge of the open field situated approximately 1/3 of the distance up the wall from the floor. The percent of rats in each group that engaged in this sidewall jumping is shown in Figure 1.3. The majority of jumping rats were female, especially kindled females. A chi square analysis confirmed the overall statistical significance of this effect, χ^2 (5) = 13.977, p = .016. Sex differences in each kindling group were investigated but the effects did not reach statistical significance: sham-stim, χ^2 (1) = 1.518, p = .218; 30-stim, χ^2 (1) = 2.651, p = .104; 99-stim, χ^2 (1) = 2.376, p = .123.

Resistance-to-Capture Test

Figure 1.4 illustrates both the mean and median resistance-to-capture scores displayed by the rats in each group. The highest resistance to capture scores were observed in both the 99-stim male and female rats, with intermediate levels in the 30-stim male and female rats, and low levels in the sham-stim rats. A Kruskal-Wallis median test for nonparametric data revealed an overall statistically significant effect, $H(5) = 23.092$, $p < .001$. Follow-up analyses were done with Kruskal-Wallis multiple comparisons one-tailed post hoc tests. The data were collapsed across sex because of identical median values for both males and females in each kindling group, and consequently, no sex differences emerged. Analysis of the collapsed male and female data revealed a significant difference between the 99-stim and the sham-stim groups, $H(3) = 26.06$, $p < .05$, and between the 99-stim and the 30-stim group groups, $H(3) = 14.61$, $p < .05$, and a nearly significant difference between the 30-stim group and the sham-stim group, $H(3) = 11.45$, $p < .075$.

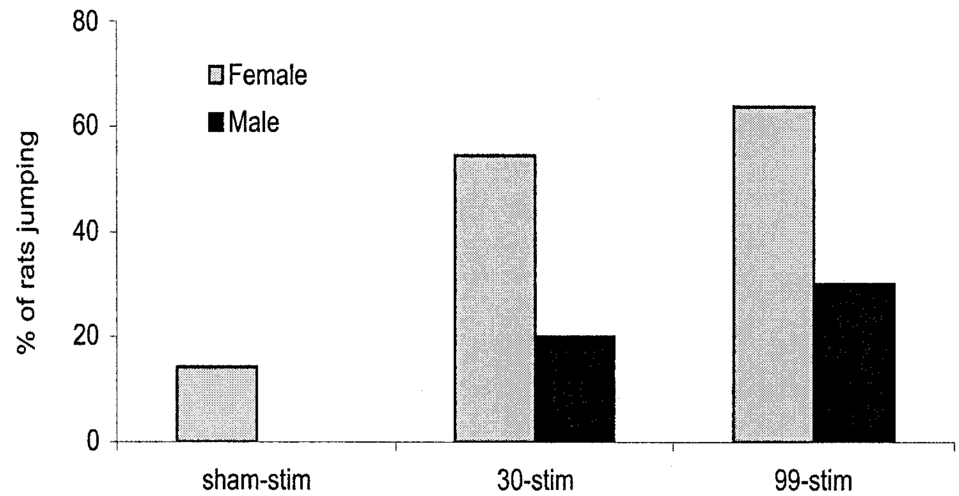


Figure 1.3. The percentage of male and female rats in the sham-stim, the 30-stim, and the 99-stim kindling conditions that engaged in sidewall jumping during the 5-min open-field test. Chi-square (5) = 13.977, p =.016

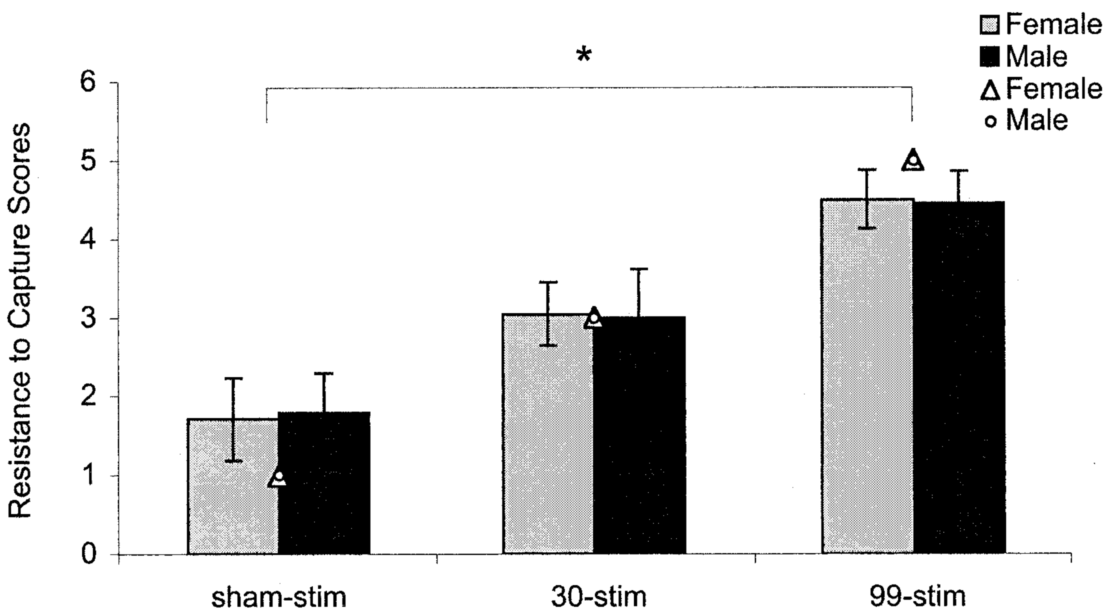


Figure 1.4. Mean (bars + S.E.M.) and median (△,○) open field resistance-to-capture scores for male and female rats across kindling conditions. * p <.05

Forced Swim

Due to video equipment failure, the data from five rats were lost, resulting in slightly smaller group sizes for this test (i.e., sham-stim female = 7, sham-stim male = 8, 30-stim female = 10, 30-stim male = 9, 99-stim female = 11, 99-stim male = 9). Immobility measures for males and females in each kindling group are shown in Figure 1.5. The mean latency to immobility is illustrated in Figure 1.5a and shows that for both males and females kindling produced an increased latency to immobility, $F(2, 49) = 3.34$, $p = .044$ and $F(2, 49) = 5.51, p = .007$, respectively. Significant sex differences were not observed in the sham-stim group, $F(1, 49) < 1.0$, or in the 30-stim group, $F(1, 49) < 1.0$, but females had a significantly longer latency to immobility than males in the 99-stim group, $F(1, 49) = 4.65, p = .036$.

A similar pattern of results was observed for the percent time spent immobile in the forced-swim test, although the effect was greater in the male kindled rats (see Figure 1.5b). The kindled males spent significantly less time immobile than the sham-stim males, $F(2, 49) = 13.90, p < .001$, but for females, only a tendency toward a decrease in time spent immobile was observed across kindling groups, $F(2, 49) = 2.70, p = .078$. In addition, males spent significantly more time immobile than females in the sham-stim group, $F(1, 49) = 14.12, p < .001$, and in the 99-stim group, $F(1, 49) = 4.03, p = .050$, but there was no significant difference between males and females in the 30-stim group, $F(1, 49) < 1.0$.

The number of head shakes and sinks during the forced-swim test were not influenced by kindling group or sex (data not shown). Diving was only observed in eight

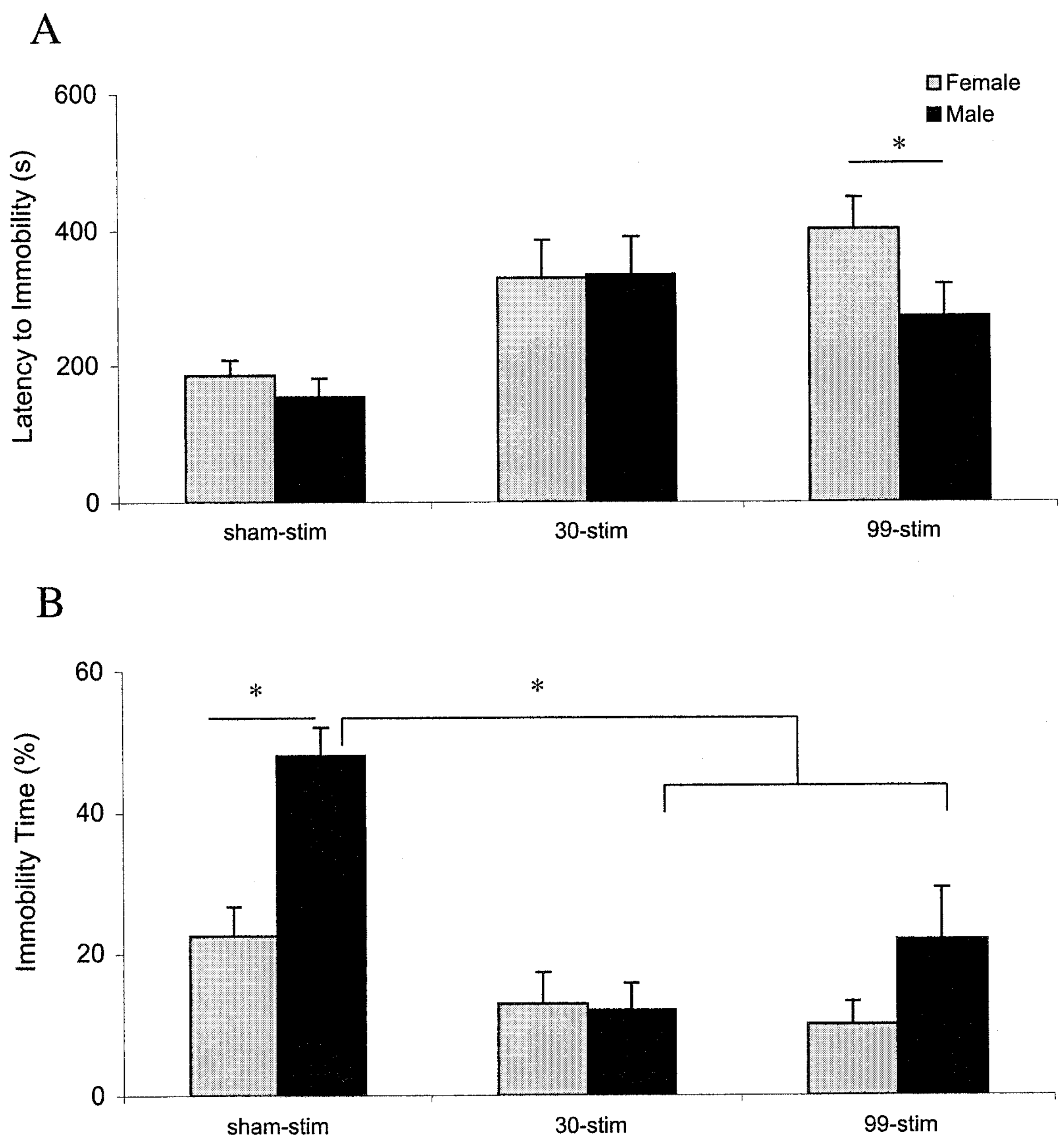


Figure 1.5. Mean (± SEM) latency to immobility (*A*) and mean (± SEM) percent time spent immobile (B) for male and female rats across kindling conditions in the forced-swim test. See text for statistically significant difference across kindling groups and between males and females.

rats, and the number of diving rats was equally dispersed across groups (approximately two per group) except that no diving was observed in the 99-stim males (data not shown).

Discussion

Four important results emerged from this experiment. First, in general, kindling increased fearful behaviour to the same degree in both female and male rats. The magnitude of these effects increased with increasing numbers of kindling stimulations. These results are consistent with previous observations in male kindled rats (e.g., Helfer et al., 1996; Kalynchuk et al., 1997; Kalynchuk et al., 2001) and provide the first demonstration of kindling-induced fear in female rats after both short- (i.e., 30 stimulations) and long-term (i.e., 99 stimulations) amygdala kindling. Second, kindling (both 30-stim and 99-stim) produced a distinct pattern of activity in both male and female rats across each minute of the open field that differed from the pattern of activity seen in the sham-stim rats. Third, although kindling increased fear in both the male and female rats, females were generally more active in the number of line crosses and sidewall jumps, both of which are discussed in more detail below. Fourth, kindling did not produce learned helplessness in the forced-swim test. In fact, the effect was just the opposite: The kindled rats displayed increased mobility in this test. This latter finding suggests that the effects of kindling on emotional behaviour may be specific to fear.

Fear/Anxiety Measures

Male and female rats reacted with similar resistance-to-capture scores from the open field in each kindling condition, which increased proportionally with the number of kindling stimulations. Both the 99-stim males and females exhibited greater resistance to capture than did the 30-stim males and females, which in turn displayed greater resistance to capture than the sham-stim males and females. The increase in resistance to capture in the 99-stim rats was dramatic. These rats engaged in defensive jump attacks and fleeing with loud vocalizations when the experimenter attempted to remove them from the open field. These data are consistent with previous reports of proportional increases in fear according to the number of kindling stimulations (Kalynchuk et al., 1997; Kalynchuk et al., 2001; Kalynchuk, Pearson, et al., 1999), and reveal a similar effect in amygdala-kindled females. These observations confirm that kindling provides a useful model for studying fear sensitization in both male and female rats.

In addition, fear-related activity was also evident in the open field as the male and female 99-stim rats displayed fewer line crosses in the first minute compared to the sham-stim rats. The reduced number of line crosses likely demonstrates fear-induced freezing or hesitation to move upon initial exposure to the novel environment. This same initial fear-related behaviour was not present in the 30-stim males or females. Furthermore, a distinct pattern of activity emerged in the open field as a result of kindling that has not previously been reported in either males or females. The 99-stim male and female rats crossed fewer lines in the first minute of the open field, but they displayed an increased number of line crosses in the second and subsequent minutes. The 30-stim male and female rats did not show the same reduced activity in the first minute, but they did display an elevated number of line crosses in the second and subsequent minutes. However, the sham-stim rats displayed either a constant rate of activity (males) or a mild reduction in line crosses (females) across the five minutes in the open field. The pattern of activity in the open field seen in the kindled rats is quite interesting. As mentioned above, it is quite likely that the reduced number of line crosses during the first minute is a fear response triggered by the novelty of the open field. The fact that this effect is larger in the 99-stim rats than the 30-stim rats is consistent with the resistance-to-capture data, and probably reflects the fact that the magnitude of kindled fear increases with an increasing number of stimulations. However, the hyperactivity seen in both the 30-stim and the 99-stim kindled rats after the first minute is more difficult to explain. It is unlikely that kindling produces a general increase in locomotor activity, because if this were the case then the kindled rats would have shown increased line crosses during minute 1 as well. One possibility is that the increase in activity represents a failure to habituate to the open field. Interestingly, rats with hippocampal lesions show a similar lack of habituation in an open field (Bannerman, Gilmour, Norman, et al., 2001; Coutureau, Galani, Jerrard, & Cassel, 2000). Another possibility is that activity during the first minute of novel open-field exposure might represent a component of fear that parallels the subsequent resistance to capture levels, whereas activity in subsequent minutes of novel open-field exposure may represent a different component of fear. This possibility is discussed in the general discussion.

Some sex differences in open-field behaviours emerged from the present study. For example, the female rats engaged in more sidewall jumping in the open field than did the male rats. This difference was particularly apparent in the 30-stim and 99-stim conditions. This sex difference is not likely due to the females weighing less than the males because weight seems to be proportional to jumping behaviour: Kindled rats typically weigh significantly more than sham-stimulated rats (Adamec & Shallow, 2000;

and unpublished observations). The observed sidewall jumping, especially in kindled females, is particularly interesting because similar behaviours have been previously reported in females in response to electric shock (Beatty & Beatty, 1970). These authors interpreted the sidewall jumping as indicative of escape behaviours. Moreover, the notion that kindling may lead to escape-related behaviour has been previously proposed by Kalynchuk et al. (1997), who observed purposive jumping from the open arms of an elevated plus maze in kindled male rats subjected to 60 and 100 stimulations. The sidewall jumping observed in the present experiment may represent an example of kindling-induced escape-related behaviour that is particularly prevalent in female rats.

A second interesting sex difference emerged with respect to overall activity in the females in the open field. This difference may represent a general tendency for females to be more active than males, particularly under mild levels of stress such as the initial exposure to an unfamiliar open field. This conclusion is consistent with previous findings in non-kindled rats. Naïve female rats have been shown to reliably spend more time engaged in active behaviours in an open field, such as moving, rearing, and sniffing (Archer 1975; Brotto et al., 2000; Johnston & File, 1991). In contrast, naïve male rats tend to display more freezing and defecation in the open field (Archer, 1975). Interestingly, this baseline tendency for females to be more active than males did not confound the resistance to capture levels of fear. This suggests that the resistance to capture scale and assessment of the pattern of activity in the open field may be a useful measure to assess fear behaviour in both males and females.

In summary, the results of this experiment add to the large body of literature that shows that amygdala kindling substantially increases fear on many different behavioural measures (e.g., Mohapel & McIntyre, 1998; Helfer et al., 1996; Barnes, Pinel, Francis, & Gig, 2001; Adamec & Morgan, 1994; Rosen, Hamerman, Sitcoske, et al., 1996; Nieminen et al., 1992; Depaulis et al., 1997). The present study confirms the effect in kindled female rats while also depicting fear-related sex differences in the open field.

Forced Swim

The fact that the kindled rats in the present experiment had a longer latency to immobility and spent less time immobile than the sham-stim rats in the forced swim test is interesting when considered in the context of the effect of kindling on fearful behaviour. This active response to the acutely stressful nature of the forced swim test may be suggestive of high levels of fear such that passive adaptation does not or cannot occur. Such an interpretation would be consistent with the heightened fear shown by long-term kindled rats in novel situations (Kalynchuk, Pinel, & Treit, 1999). Furthermore, long-term kindling has been reported to specifically increase active defensive behaviours. For example, when tested as intruders in a resident-intruder paradigm, kindled rats spent less time engaged in aggressive behaviour (i.e., lateral attacks, anogenital sniffing, back biting) and passive defensive behaviour (i.e., freezing, lying on back), and more time engaged in active defensive behaviour (i.e., defensive upright posture, running away, defensive jump attacks), compared to sham-stim rats (Kalynchuk, Pinel, Treit, 1999). Kindled rats also display increased resistance to capture from an open field and escape behaviour in an elevated plus maze (Kalynchuk et al., 1997; Kalynchuk et al., 2001). Taken together, these observations suggest that kindled rats engage in active, albeit defensive, responses when confronted with stressful situations. The decrease in immobility time shown by the kindled rats in the forced-swim test may be a further indication of such active responses.

Sex differences in immobility measures on the forced swim test were apparent in the sham-stim and 99-stim conditions. Both the sham-stim and 99-stim females spent less time immobile than the sham-stim males and 99-stim males respectively. In addition, the 99-stim females had a longer latency to immobility than the 99-stim males. This heightened activity in females is consistent with previous reports that females are more active than males in an open field (Archer, 1975). Even within the context of the forced swim test itself, naïve males have been reported to exhibit greater immobility than naïve females (Brotto, Barr, & Gorzalka, 2000). However, this should be interpreted with caution because others have reported greater immobility in female rats (Brotto, Gorzalka, & Barr, 2001), and still others have reported no sex differences (Papaioannou, Gerozissis, Prokopiou, Bolaris, & Stylianopoulou, 2002) in terms of immobility time.

Kindling effects

An interesting result from this experiment was that in general, the 30-stim rats kindled faster than the 99-stim rats. This effect was statistically significant in the male rats but it failed to reach statistical significance in the females rats, although a trend was present. This differential rate of kindling may occur because the rats in the 30-stim rats received 69 sham stimulations prior to the 30 kindling stimulations. These sham stimulations made the kindling box very familiar to the 30-stim rats prior to the first kindling stimulation. As a result, the kindling box may have been less stressful for the 30-stim rats than it was for the 99-stim rats. This may have facilitated the development of kindling in the 30-stim rats. Although a curious result in itself, the differential kindling rates in the 99-stim and 30-stim rats probably did not alter the behavioural results because kindling itself has previously been dissociated from the expression of kindling-induced fear (Adamec, 1990b).

Conclusion

The present experiment is the first to demonstrate that kindled fear develops in short- and long-term kindled female rats. These results suggest that the amygdala-kindling model of fear sensitization can be generalized to female rats. Using both males and females could assess a greater scope of factors that contribute to the development of kindled fear. This model also offers a means to investigate the neural mechanisms underlying anxiety that could have implications for both men and women suffering from anxiety-related disorders. Furthermore, this experiment is foundational for further investigating the neural mechanisms of kindled fear as it relates to both male and females. As such, behavioural treatments that reduce the magnitude of kindled fear can be considered according to both male and female rats and according to treatments of anxiety for men and women.

Chapter 3: Experiment 2

Hippocampal Functioning in Amygdala-Kindled Male and Female Rats

Specific neural changes within the hippocampus that are associated with kindled fear have already been described. These include an increase in $5HT_{1A}$ receptor binding and mRNA expression (Kalynchuk et al., in prep) and increased $GABA_A$ and benzodiazepine receptor binding (Kalynchuk, Pearson, et al., 1999). Excitatory neurotransmitter receptor changes in the opposite direction have also been observed such as decreased NMDA and AMPA receptor binding (McEachern et al., 1996). This pattern of results suggests that kindling may produce an imbalance in the excitatory-inhibitory nature within the hippocampus. Furthermore, significant group differences in FOS immunoreactivity have been observed between kindled and sham-stimulated control rats specifically within the hippocampus: Kindled rats showed decreased FOS immunoreactivity following exposure to a novel open field (Kalynchuk et al., 2001). Because FOS immunoreactivity is thought to indicate stimulus-induced neural activity, the authors argued that reduced FOS might be a result kindled rats having a less plastic hippocampus. In fact, Kalynchuk et al., (2001) argued that less activity within the hippocampus of kindled rats might affect their behaviour in an unfamiliar environment, leading to increased fearful behaviours. This hypothesis has not yet been tested.

Evidence that the hippocampus plays an important role in fear behaviour and in determining whether a particular context requires a fearful response was also reviewed earlier (see Gray, 1982; McNaughton & Gray, 2000). Generally, the hippocampus is known for its role in mediating the processing of spatial information required for navigation within an environment and as such, many tests of hippocampal functioning are spatial in nature. Upon initial exposure to an unfamiliar environment, rats display characteristic exploratory behaviour (Eilam & Golani, 1989; Tchernickovski & Golani, 1995; Golani, Benjamini, & Eilam, 1993). Within a few minutes, rats develop spatial representations of the environment that they are exploring (discussed in O'Keefe & Nadel, 1978). With time, they habituate to the environment and both their fear of unfamiliarity and their desire to explore lessens (Walsh & Cummins, 1976). When the hippocampus is disrupted via lesion or drug treatments, rats are impaired at spatial tasks either because the hippocampus is directly responsible for creating spatial maps (Morris, 1984; O'Keefe & Nadel, 1978; Steele & Morris, 1999) or because rats become hypersensitive to the environmental stimuli and are unable to filter out extraneous information (Gray & McNaughton, 1983, 2000). In either case, the fear behaviour expressed by kindled rats in unfamiliar environments may be mediated by the kindling-induced changes in hippocampal functioning. Examining rats' performance on a hippocampal-dependent task would be one way to functionally test this possibility.

Hannesson and Corcoran (2000) recently reviewed the data on spatial memory deficits associated with kindling. On hippocampal-dependent spatial tasks, amygdala-kindled rats are generally spared from deficit, in contrast to other kindled sites in which deficits are obvious. The majority of the studies employed either the Morris water maze or the radial-arm maze tasks in which spatial navigation to a location (i.e., place learning) was required. For example, amygdala-kindled rats were able to exhibit place learning in the Morris water maze (McNamara, Kirkby, dePape, & Corcoran, 1992; Nieminen, Sirvio, Teittinen, Pitkanen, Airaksinen, & Riekkinen, 1992) and in the radial-arm maze

(Letty, Lerner-Natoli, & Rondouin, 1995) whereas hippocampal-kindled rats showed impairments on both of these tasks (Hannesson, Wallace, Pollock, et al., 2004; Gilbert, McNamara, & Corcoran, 2000; Leung, Brzozowski, & Shen, 1996). These tests were conducted in rats that were subjected to a short-term kindling paradigm; however, when amygdala-kindled rats were tested in the water maze approximately a week after extended kindling (i.e., 300 stimulations) place learning was delayed (Cammisuli, Murphy, Ikeda-Douglas, et al. 1997). The rats in the latter study were eventually able to learn the place of the hidden platform after repeated trials, suggesting that long-term kindling interferes with kindled rats' ability to acquire spatial information, at least during the early acquisition phase of water maze testing. However, the point at which acquisition begins to fail is not clear.

The fact that spatial deficits observed in amygdala-kindled rats are dependent upon the extent of kindling emphasizes the need to examine spatial performance at multiple points throughout the course of amygdala kindling. When spatial tasks are conducted at the end of a kindling phase – as is often the case – the testing environment is usually unfamiliar to the rats and consequently would be fear provoking. Consequently, the acquisition deficits observed in rats after 300 amygdala kindling stimulations could reflect a deficit that only manifests itself under the highly fearful circumstances of an unfamiliar testing environment, therein mimicking fear in the open field. Testing long-term kindled rats under familiar conditions with pre-exposure to the testing paradigm would clarify the nature of hippocampal involvement. Following training, spatial performance could be assessed in familiar spatial environments in which reconfiguration of static cues is necessary for accurate place navigation. Using such methods, a change in spatial performance could be observed throughout kindling as rats receive increasing numbers of kindling stimulations with little concern for fear of novelty. Furthermore, because it is well-known that kindled fear intensifies with increased number of kindling stimulations (Kalynchuk, 2000), testing spatial performance at multiple points over the course of kindling would clarify the time at which spatial deficits develop in relation to kindling stimulations and the development of kindled fear.

The purpose of the present experiment was to determine if and when amygdala-kindled male and female rats exhibit hippocampal-dependent behavioural deficits using a delayed-matching-to-place (DMTP) version of the water maze. Steele and Morris (1999) used a similar version of the water maze and showed that an intact hippocampus was necessary for correctly performing the task. Using similar methods, all rats in the present study received training days prior to kindling that included new platform locations each day of training followed by matching trials 1 hour after introduction of the new location. The training phase was done prior to the onset of kindling in order to familiarize the rats to the testing environment and to the spatial arrangement of the room. Subsequently, the rats were tested weekly throughout kindling for their ability to recall the new place of the platform after a 1-hour delay. This method of testing spatial performance was ideal because the rats were permitted to form a spatial representation of the room and acquire the information about how to perform the task prior to the kindling. Thus, spatial cues remained stable whereas new spatial configurations were required to locate the platform during each water maze session. This experiment was designed to specifically test whether amygdala-kindled rats displayed hippocampal-mediated deficits. Both males and females were tested because amygdala-kindled females have not yet been examined on hippocampal-dependent tasks.

Method

Subjects

The animals were 33 male and 34 female adult (approximately 250-300 and 200-250 g, respectively at the time of arrival) Long Evans rats (Charles River Canada, St. Constant, PQ). The rats were individually housed in rectangular polypropylene cages with wood shavings as bedding. Food and water was available *ad libitum*. Lights in the colony room were set on a 12:12-hr light:dark cycle with lights on at 8:00 a.m. All experimental manipulations were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the Dalhousie University Committee on Laboratory Animals.

Surgery

Each rat underwent stereotaxic surgery (according to methods described in Experiment 1) approximately one week after arrival and was handled daily prior to surgery. At the time of surgery, males rats weighed between 275-300g and female rats weighed between 240-275g.

Experimental Groups & Schedule

There were three general experimental conditions in this experiment. One group of male and female rats was pre-trained in the water maze, then subjected to kindling and water maze testing, and finally tested in an unfamiliar open field at the end of the kindling phase (kindle-WM: female N = 11, male N = 12). Another group of male and female rats was pre-trained in the water maze, then subjected to sham kindling stimulations and water testing, and finally tested in an unfamiliar open field at the end of the kindling phase (sham-stim WM: female N = 13; male N = 12). A final group of male and female rats was subjected to kindling and tested in an unfamiliar open field at the end of the kindling phase, but they did not undergo pre-training or testing in the water maze (kindle-only: female N = 10; male N = 9). The details of these manipulations are given below. The experimental schedule of these manipulations are described in Figure 2.1.

Water maze

Water maze training began after a 1-week postoperative recovery period. Spatial memory was assessed in a circular tank (diameter: 1.5 m; depth 90 cm) in a dimly lit (i.e., two 60 W light bulb in each corner of the room) room (3 m wide X 7.5 m long). The tank was filled with water (22 $\pm$ 1 °C) made opaque by white non-toxic tempura paint. The water maze was filled to a depth of 72 cm and contained a movable white platform (70 cm high, 12 cm in diameter) submerged 2 cm below the water line. Spatial cues were placed around the room on the walls, ceiling, and doors, and on a table and sink counter in the room. Examples of cues included 2D shapes and patterns on the walls, distant light sources, two distinctly different doors at either end of the room, an artificial plant, the back of a television monitor, and a water bucket and hose. The spatial cues were stationary for the duration of the experiment. The swim path taken by each rat was monitored with an overhead camera suspended from the ceiling.

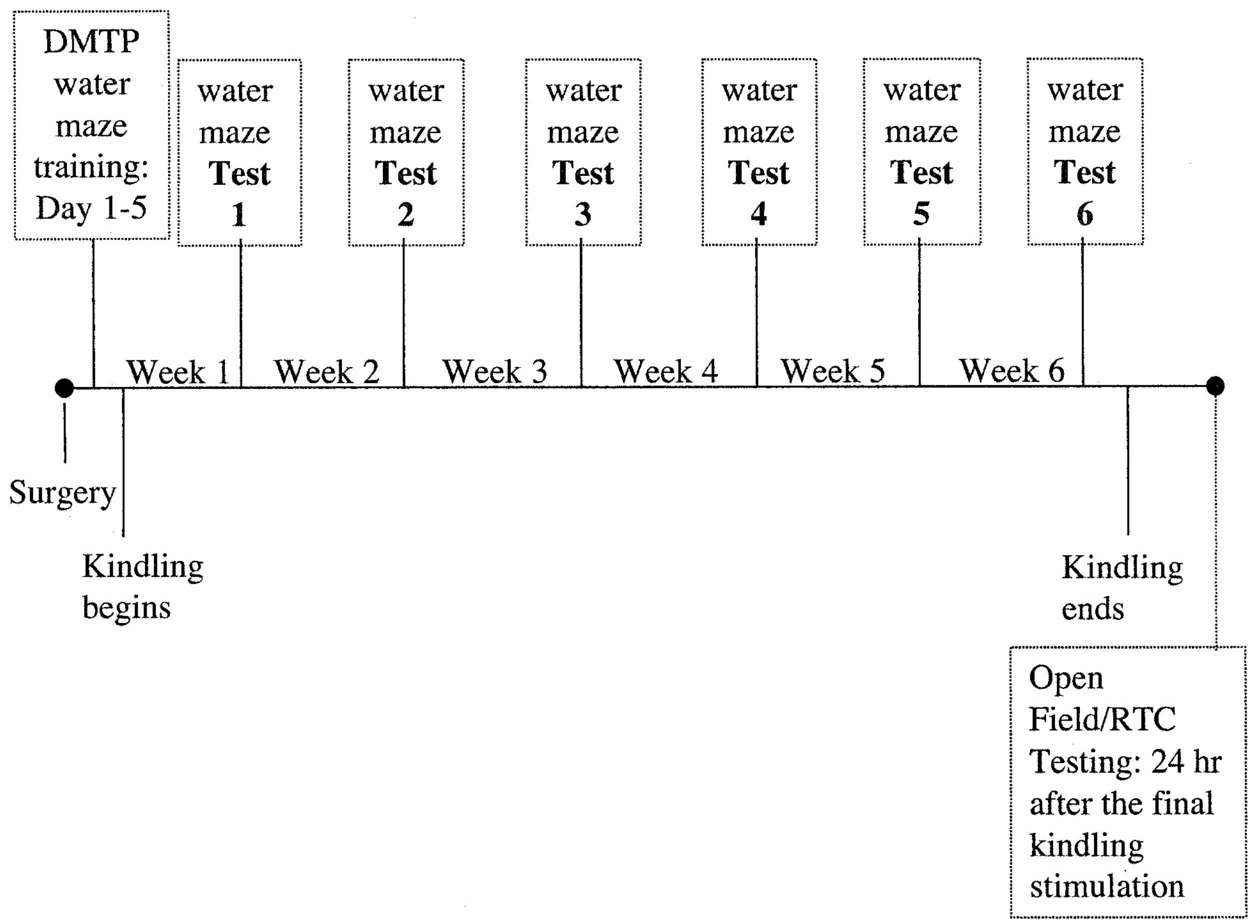


*Figure 2.1.*A schematic representation of the experimental schedule. The rats underwent stereotaxic surgery followed by the delayed-match-to-place (DMTP) training in the Morris water maze 1 week later. Kindling began on the second day following the water maze training. Rats were tested on the DMTP version of the water maze each week (1-6) of kindling with a new test platform location each week. The rats received 3 days of kindling stimulations following Week 6 and were then tested in the open field 24 hr after the final kindlgn stimulation.

The training and testing procedures in the water maze were similar to those used by Steele and Morris (1999), using a delayed-match-to-place (DMTP) version of the task. The platform location was based on direction (i.e., N S E W) and circumference (i.e., internal/external) as depicted in Figure 2.2. For the training phase, the platform location was always placed directly along the directional axis (N S E W) and for the testing phase, the platform location was always placed within a quadrant (NE NW SE SW). Location was also altered between internal and external circumferences. The platform was never placed in the same location more than once throughout the entire experiment.

Each pre-training and testing day comprised five trials. Each trial was a maximum of 60 s followed by 15 s on the platform. There was a 1-hour delay between trial 1 (i.e., place trial) and trial 2 (i.e., first matching trial). The rats were each released into the water maze from the same locations (i.e., N S E W). The release locations were randomly selected (without replacement) for each day and trial.

Training. Training in the water maze began after a 1-week postoperative recovery period. The rats underwent five days of training in order to habituate to the water maze and to learn the task requirements. An important task requirement was being able to retain information about the platform location from the place trial (i.e., trial 1) to the matching trials (i.e., trials 2 – 5) over the 1-hour delay period. Each training day consisted of gently releasing the rat into the water maze with its ventral surface facing and touching the wall. The rat was allowed to swim in the water maze for a maximum of 60 seconds. If the rat did not find and climb onto the platform within 60 seconds, it was picked up from its position within the maze and placed onto the platform. Once the rat was on the platform,

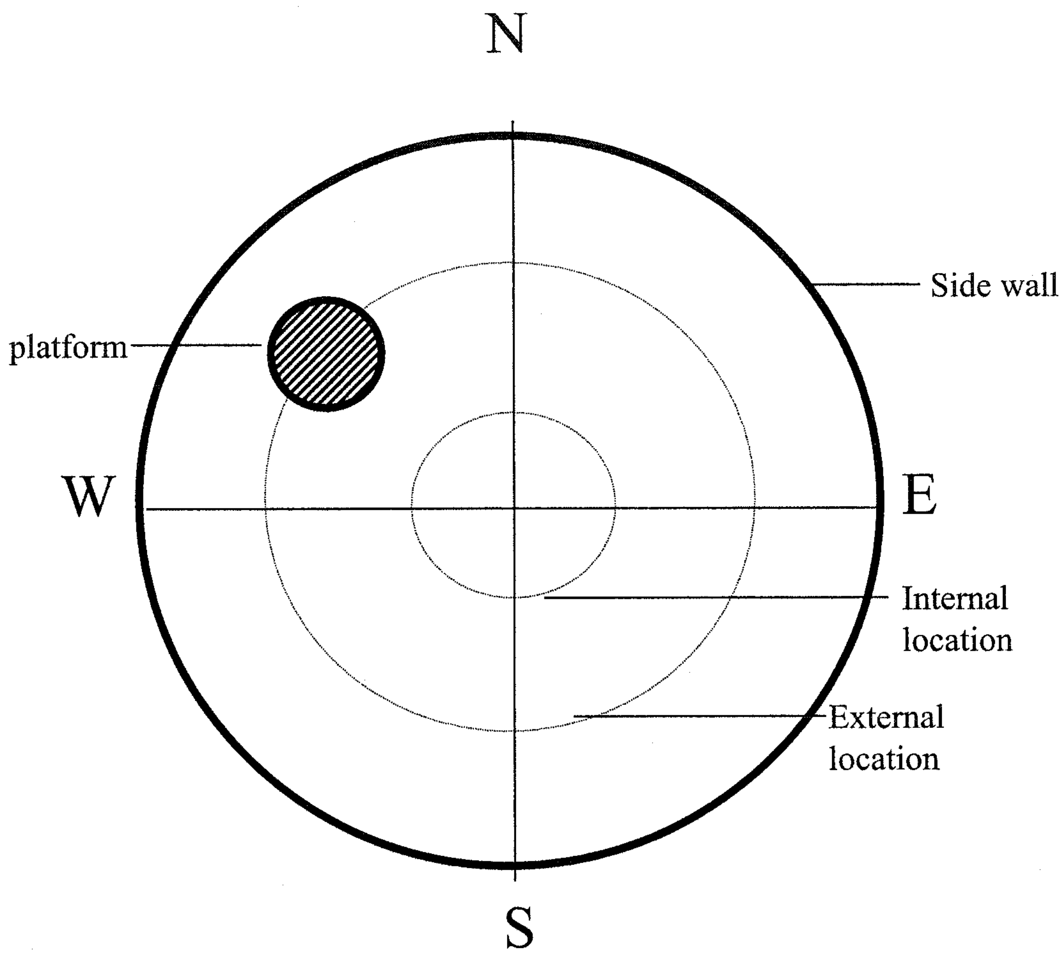


*Figure 2.2.*A picture representation of the water maze and the platform location. The platform location was based on direction (I.e., N E S W) and circumference (I.e., internal/external). For example, a platform placed in the NW-external location would be places as visible in the diagram above.

it remained there for 15 s before being removed. Following trial 1, the rat was picked up from the platform, returned to its home cage, and placed under a heat lamp for at least 10 minutes. One hour later, the rat was released back into the water maze from a location different from the beginning of trial 1, and allowed to find the platform according to the same conditions described above. The rats received five days of training within a 6-day period (i.e., four consecutive training days followed by a rest day and then a final training day).

Performance in the water maze during training on the task was measured by the difference in latency to find the platform between trial 1 and trial 2. A reduction in latency to find the hidden platform on trial 2 reflects the rats' memory of the platform location learned on trial 1. All trial data (i.e., trials 1 – 5) are depicted in the data figures below.

Kindling

After the water-maze training phase, the male and female rats were divided into two groups: One group of male and one group of female rats received 99 kindling stimulations (kindle-WM males, n = 12; kindle-WM females, n = 11), and one group of male and one group of female rats received 99 sham stimulations (sham-stimWM males, n = 12; sham-stimWM females, n = 13). The rats were divided into groups based on their water maze performance during the last two days of training in order to balance the number of strong and weak performers between the kindled and sham-stim groups (i.e., the group selections had similar means and standard deviations on the last two training days). Two additional groups of male and female rats did not undergo the any water-maze training or testing but received 99 kindling stimulations (kindle-only males, n = 9, kindle-only females, n = 10) and served as a control for the kindled-water maze group during the open field testing.

Kindling and sham-stimulations occurred in the same manner as described in Experiment 1, which included three daily stimulations, five days a week. The rate of kindling was assessed by the mean number of stimulations required for rats to reach the first and third class 5 or higher convulsion and the mean number of class 5 or higher convulsions per week of kindling.

Testing in the Water Maze

During each week of the kindling phase, the rats received kindling or sham stimulations for five consecutive days (i.e., Monday to Friday), followed by a day off (i.e., day 6), followed by the water maze testing (i.e., day 7). The procedures for water maze testing occurred in the same fashion as the training phase. Each rat's performance during the water maze testing was measured by the latency to find the hidden platform on trial 2. Differences in the mean latency on trial 2 between groups of rats were compared. All trial data (i.e., trials 1-5) are shown in the figures.

Open-field and Resistance-to-Capture Tests

The open-field testing was done on the day following the final stimulation in the same manner as that described in Experiment 1.

Histology

Electrode placement verification was done according to the methods described in Experiment 1.

Data Analysis

The data were analyzed using SPSS 11.0.1. Statistical significance was set at $p \leq .05$. F ratios < 1.0 are not statistically significant and do not include p value in text.

Kindling. The kindling data were analyzed with a two-by-two analysis of variance (ANOVA) with sex (male/female) and water maze condition (kindle-only/kindle-WM) as between-subjects factors to examine the number of stimulations required to display the first generalized convulsion (i.e., class 5 of higher). The same two-by-two analysis was also conducted on the number of generalized convulsions for each week of kindling.

Water maze. Water maze performance was measured by the latency-to-platform trial data. During water-maze training, acquisition was determined using a mixed-design ANOVA with trial as the repeated measure (trial 1 vs. trial 2) and sex (male/female) and kindling condition (kindle-WM/sham-stimWM) as the between-subjects factors. Performance during weekly testing was measured with a mixed-design ANOVA of the latency to reach the platform on trial 2 across all testing sessions (i.e., 6 in total) with kindling condition (kindle-WM/sham-stimWM) as the between-subjects factor. This latter analysis was done separately for males and females.

Open-Field and Resistance to Capture. The mean number of line crosses for each minute of the open field was assessed using simple effects repeated measures ANOVA to examine each condition and sex separately. Repeated measures ANOVA and polynomial trend analysis was also conducted in the same manner as Experiment 1. Resistance to capture was statistically assessed using the Kruskal-Wallis nonparametric test, as was done in Experiment 1.

Results

Histology

Rats were removed from the statistical analyses if the electrode-skull apparatus became disassembled at any point during the experiment or if rats had electrodes terminating outside the amygdala as observed with cresyl violet histological staining. Consequently, five rats were removed from the study: One kindle-only female, one kindle-WM female, and three kindle-WM males. Thus, the data analyses were based on a total of 30 males (i.e., kindle-only males = 9, kindle-WM males = 9, sham-stimWM males = 12) and 32 females (i.e., kindle-only females = 9, kindle-WM = 10, sham-stimWM females = 13).

Kindling

Kindling acquisition is represented in Figure 2.3. As seen in Figure 2.3a, both male and female rats exposed to the water maze (i.e., kindle-WM group) required more stimulations to display the first class 5 or higher generalized convulsion compared to the kindle-only males and females, $F(1,33) = 4.135, p = .050$. There was no significant difference between males and females on this measure, $F(1,33) = 0.025, p = .874$, and there was no significant sex by group interaction, $F(1,33) = 0.002, p = .968$.

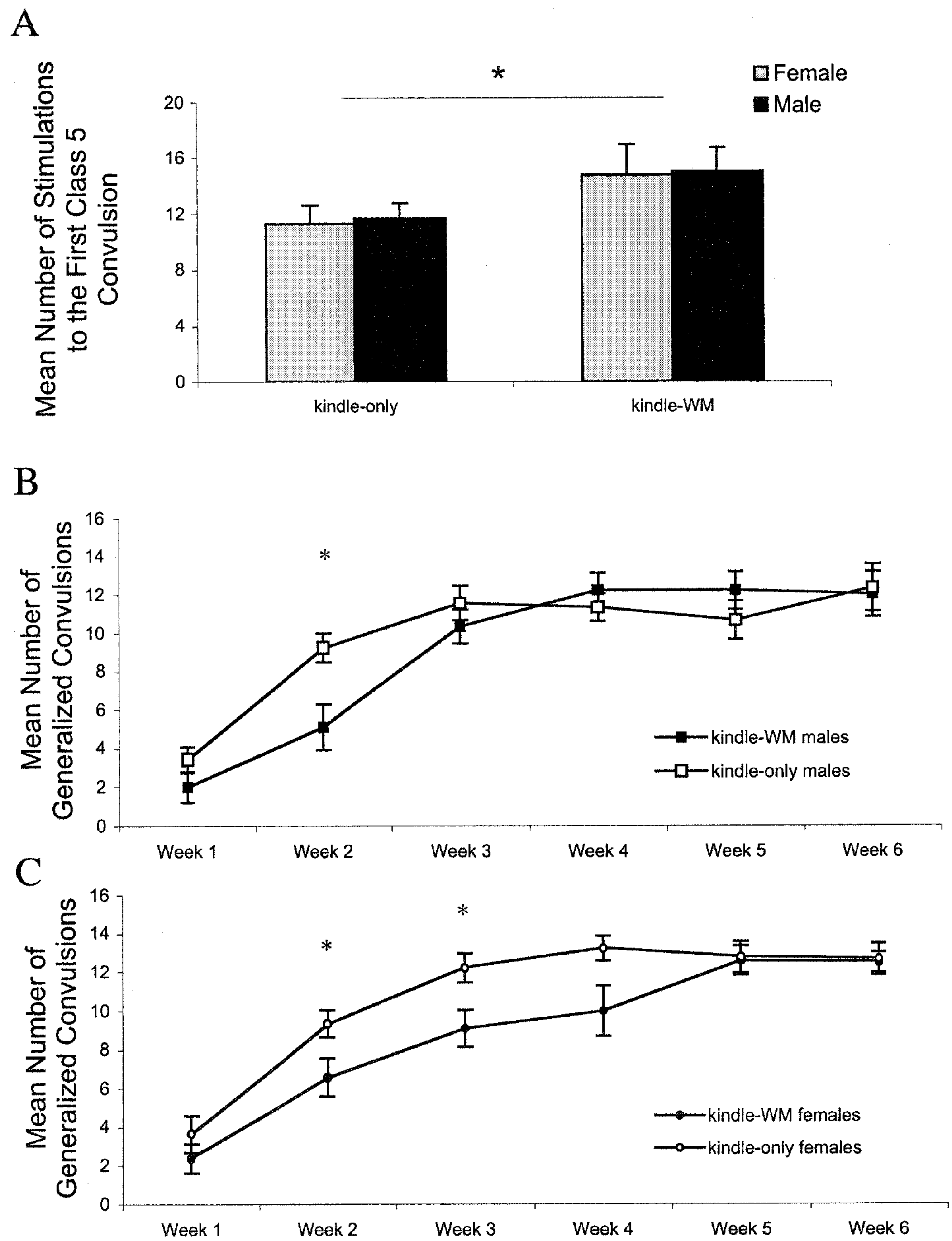


Figure 2.3. The acquisition of kindling in the male and female rats. A) Mean (± S.E.M.) number of stimulations required to elicit the first class 5 convulsion for male and female rats in both the 30-stim and the 99-stim kindling conditions. B) & C) Mean ± (S.E.M.) number of generalized convulsions during each week of kindling for males (B) and females (C). * $p < .05$

The kindle-WM males and females both also displayed fewer generalized convulsions within the first few weeks of kindling (Figure 2.3b). An ANOVA of the data at each week of kindling with water maze condition (kindle-only or kindle-WM) and sex as the between-subjects factors revealed no significant difference between the kindle-WM rats and the kindle-only rats in the first week of kindling, $F(1,33) = 2.99, p = .093$; however, the kindle-WM rats experienced significantly fewer generalized convulsions than the kindle-only rats in week 2, $F(1,33) = 13.573, p = .001$, and week 3, $F(1,33) = 5.972, p = .020$. There was no significant difference between the groups during any other week, $p \geq .446$. In addition, there was no significant main effect of sex during any week of kindling, $p \geq .173$, and no significant sex by water maze condition interactions during any week, $p \geq .293$, except during week 4, $F(1,33) = 4.783, p = .036$.

Water Maze Performance: Latency to Hidden Platform

Water maze performance is displayed as the latency to find the hidden platform across trials 1 through 5 in Figure 2.4. Figure 2.4 shows the performance during the 5-day training period for males (Figure 2.4a) and females (Figure 2.4b) separately. Figure 2.5 shows the performance during each week of the 6-week kindling phase for males (Figure 2.5a) and females (Figure 2.5b).

Training: As seen in the Figure 2.4a, both the sham-stimWM and the kindle-WM males acquired the task by the final training day. They had shorter latencies to reach the hidden platform on the matching trial (i.e., trial 2) compared to the place trial (i.e., trial 1) while also displaying decreasing group variability (i.e., S.E.M.) across the 5 days of the

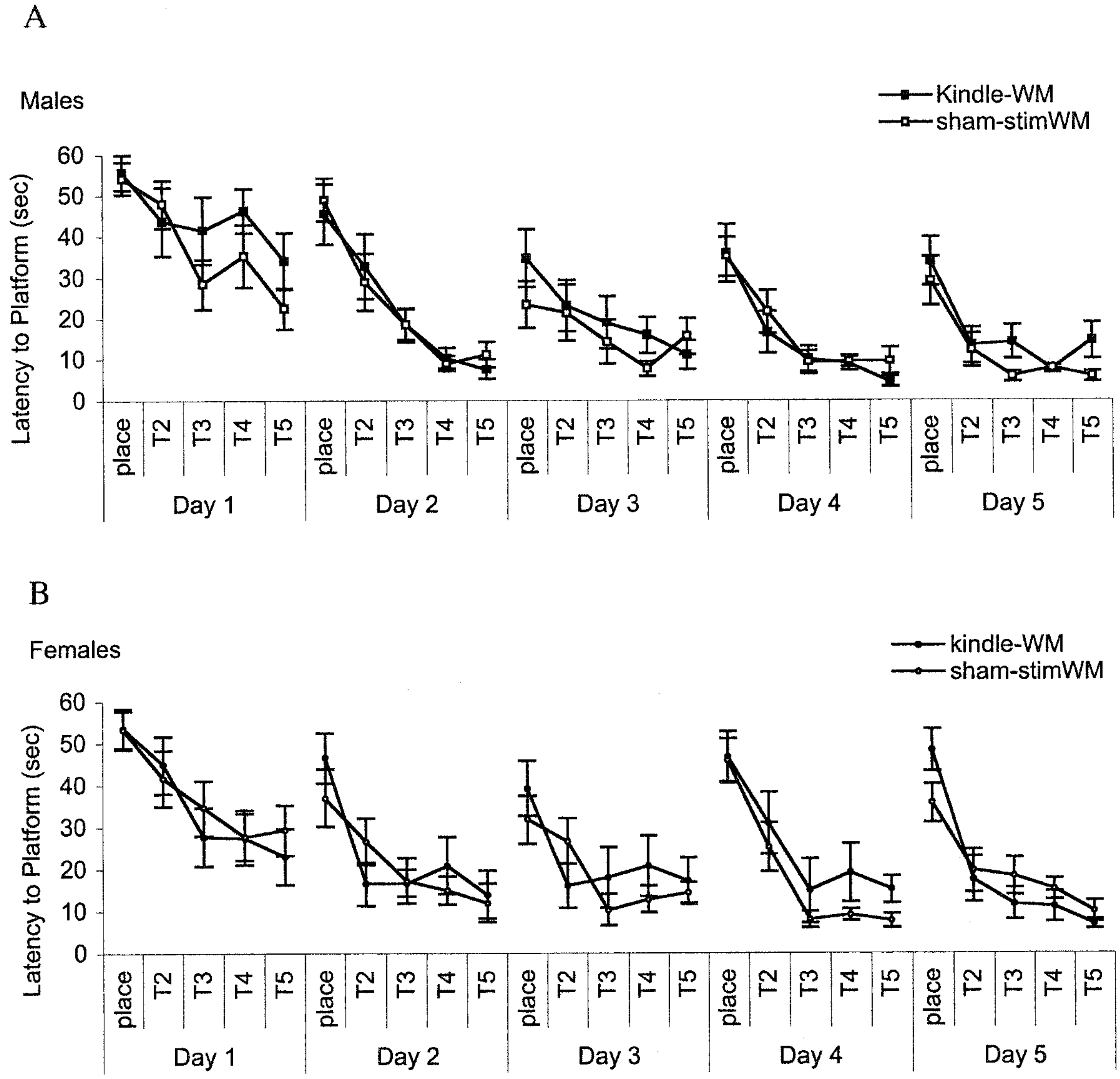


Figure 2.4. Mean (± S.E.M.) latency to reach the platform for each place and matching trials across each training day (Day 1 - 5) for kindle-WM and sham-stimWM males (A) and females (B).

acquisition phase. This pattern of acquisition was also evident in both the sham-stimWM and the kindle-WM females (Figure 2.4b).

The difference between trial 1 and trial 2 on Day 5 was assessed statistically using a mixed-design ANOVA. The only statistically significant effect that emerged was a main effect of trial, $F(1, 40) = 48.598, p < .001$. Females showed a trend toward overall longer latencies to reach the platform; however the effect did not reach statistical significance, $F(1, 40) = 3.693, p = .062$. All other main effects and interactions were not statistically significant (p values $\geq .140$).

Testing: Performance during the testing phase was measured as the latency to reach the platform on trial 2 (i.e., first matching trial; see Figure 2.5). During kindling, the sham-stimWM males continued to perform with short latencies to reach the hidden platform accompanied by further reduced group variability. However, the kindle-WM males displayed longer latencies to find the hidden platform and increased group variability, particularly during weeks 3 – 5, followed by a return to asymptotic levels during week 6. The sham-stimWM females and kindle-WM females both continued to display short latencies to find the hidden platform and their behaviour was not altered as a result of kindling; however they were less efficient at reaching the platform than sham-stimWM males. A transient difference was observed during the third week of testing in which the kindle-WM females outperformed the kindle-WM males.

The latency to reach the platform on trial 2 during testing was statistically assessed by a mixed-design ANOVA with trial 2 as the repeated measure (i.e., across 6 weeks) and group (i.e., kindle-WM vs. sham-stimWM) as the between-subjects factor. This was done separately for males and females. The analysis resulted in a significant

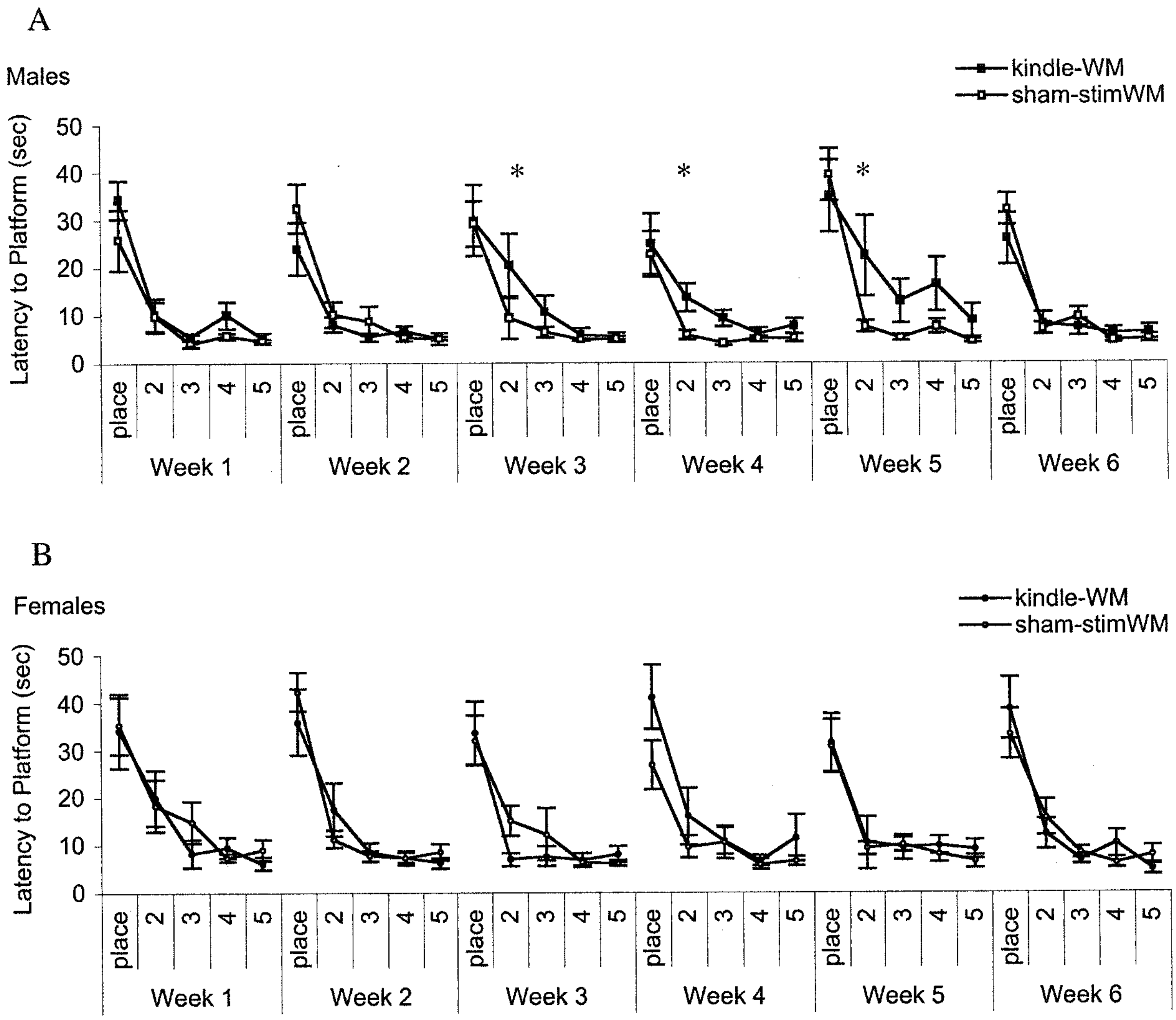


Figure 2.5. Mean (± S.E.M.) latency to reach the platform for each place and matching trials across each week of testing (Week 1 -6) for kindle-WM and sham-stimWM males (A) and females (B). *indicates significant differences

main effect of kindling group for males, $F(1, 19) = 7.374$, $p = .014$, showing that kindle-WM males took longer to reach the platform than sham-stimWM males overall. There was no statistically significant main effect of Trial, $F(5, 95) = 1.459$, $p = .227$, and no significant interaction between trial and kindling group, $F(5, 95) = 1.724$, $p = .159$. The separate analysis for females showed that there was no statistical difference between kindle-WM and sham-stimWM females. There was no main effect of Trial, $F(5, 105) = 1.336$, $p = .255$, no main effect of kindling group, $F(5, 105) < 1.0$, and no interaction between the two factors, $F(1, 21) < 1.0$.

Sex differences in the latency to reach the platform were statistically assessed within each kindling condition (i.e., kindle-WM and sham-stimWM). Within the kindle-WM condition, there was no statistically significant main effect of Sex, $F(1,17) < 1.0$, and no statistically significant trial difference, $F(5,85) < 1.0$; however, there was a nearly significant interaction between Sex and Trial, $F(5,85) = 2.122$, $p = .071$ (using Huynh-Feldt's correction). Within the sham-stimWM condition, a significant sex difference was observed. The males reached the platform significantly faster than the females, $F(1,23) = 6.011$, $p = .022$. Sex did not interact with Trial, $F(5,115) < 1.0$, nor was there a statistically significant main effect of Trial, $F(5,115) = 1.431$, $p = .228$.

Open-Field Line Crosses and Resistance-to-capture

Open-field line crosses. As seen in Figure 2.6, the kindle-only conditions displayed the characteristic pattern of reduced line crosses in the first minute of the open field, followed by an increased number during the subsequent minutes. In contrast, the sham-WM conditions displayed consistent activity across the five minutes. The kindle-

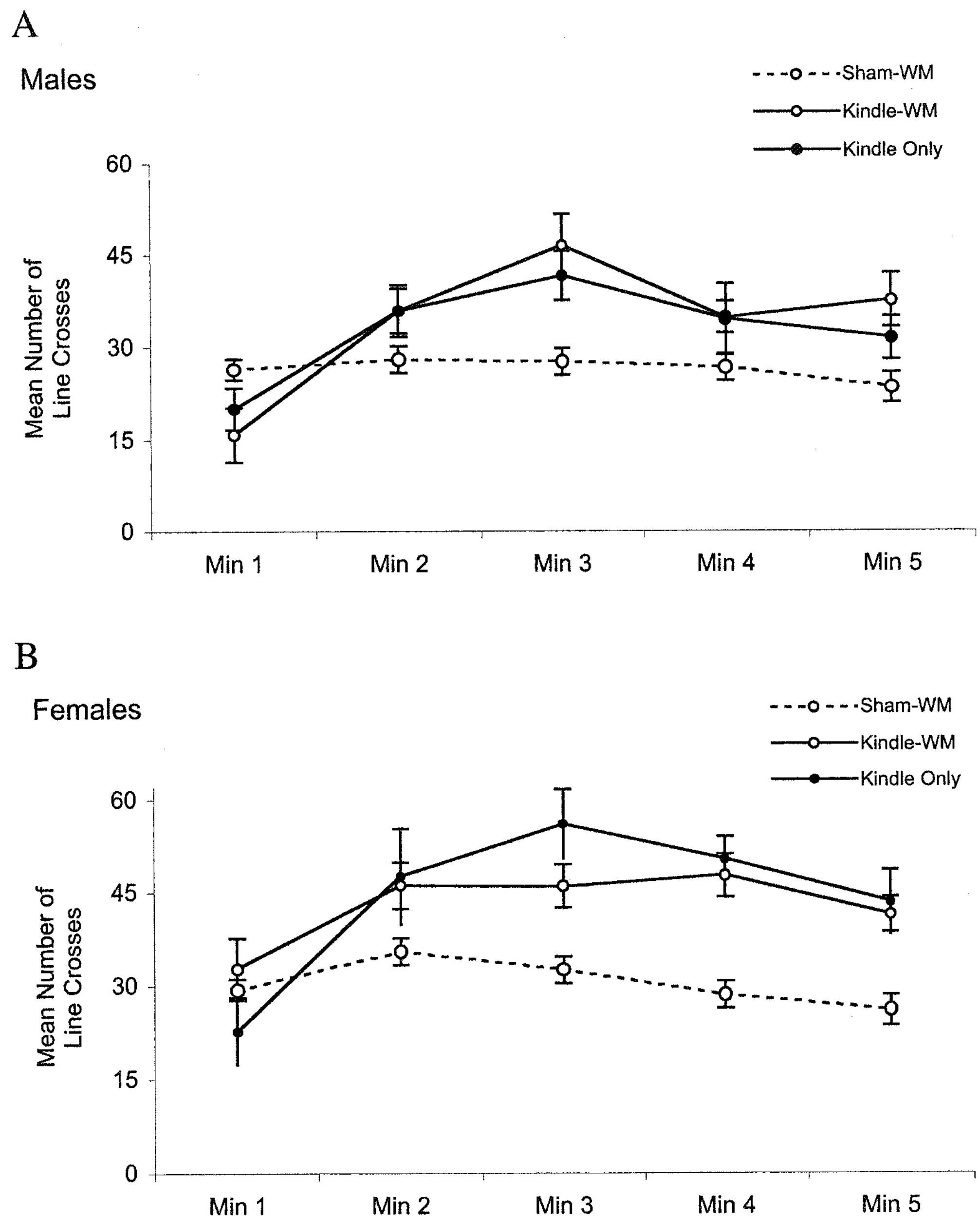


Figure 2.6. Mean number of line crosses across each minute of the open field for males(A) and females (B) of each kindling condition. The patterns of activity were assessed for each condition and the statistically significant patterns are described in text.

WM males displayed a similar pattern as the kindle-only males. The kindle-WM females displayed similar activity as the kindle-only females although they did not display the reduced activity in the first minute.

Statistically, the number of line crosses for each minute of the open field was assessed using simple effects ANOVA and polynomial trend analysis. The results showed that the sham-WM males and females did not differ significantly in the mean number of lines crossed over across the five minutes, $F(4,224) < 1.0$ and $F(2,224) = 1.91, p = .110$, respectively. In contrast, both the kindle-only and kindle-WM conditions did vary across the five minutes, $F(4,224) = 12.52$ $p < .001$ (kindle-only males), $F(4,224) = 6.30$ $p < .001$ (kindle-WM males), $F(4,224) = 16.00$ $p < .001$ (kindle-only females), and $F(4,224) = 5.61$ $p < .001$ (kindle-WM females).

The activity of the kindle-only males followed a statistically significant quadratic pattern, $F(1,8) = 31.305, p = .001$, whereas the activity of the kindle-WM males followed statistically significant linear, $F(1,8) = 9.104, p = .017$, quadratic, $F(1,8) = 31.878, p < .001$, and cubic trend, $F(1,8) = 11.322, p = .010$. The trend for the kindle-WM and the kindle-only females were both quadratic, $F(1,9) = 15.583, p = .003$ and $F(1,8) = 67.366$, $p < .001$, respectively.

Resistance to Capture. The mean and median resistance to capture scores displayed by the rats in each group are shown in Figure 2.7. Both kindled conditions (i.e., kindle-only and kindle-WM) of males and females displayed higher levels of resistance to capture compared to the sham-stim WM rats. Although the kindle-WM and kindle-only males did not differ in resistance to capture scores, the kindle-WM females had lower RTC scores than the kindle-only females.

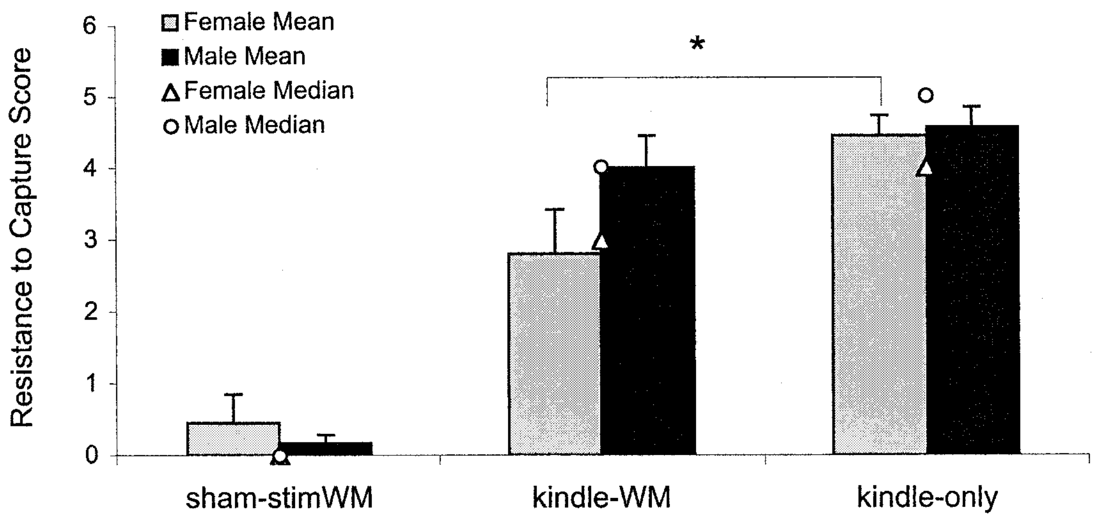


Figure 2.7. Mean (bars + S.E.M.) and median (△ ○) open field resistance-to-capture scores for male and female rats across kindling conditions. * p < .10

Statistically, a Kruskal-Wallis nonparametric ANOVA was used to assess the resistance-to-capture scores across condition separately for males and females. The results indicated a significant effect of condition for males, $H(2) = 22.176, p < .001$, and females, $H(2) = 16.664, p < .001$. Post hoc tests for males revealed that both the kindle-WM and the kindle-only rats had significantly higher resistance-to-capture scores than the sham-stimWM rats, $H(3) = 9.90, p < .05$ (for both comparisons), and that the kindle-WM did not differ significantly from the kindle-only rats, $H(3) = 10.58, p > .05$. Post hoc test for females revealed that both the kindle-WM and the kindle-only rats had higher resistance-to-capture scores than the sham-stimWM rats, $H(3) = 9.27$, $\mathrm{p} < .10$, $H(3) = 9.738, p < .05$, although the difference between the kindle-WM and the sham-stimWM was marginal. The lower resistance-to-captures scores in the kindle-WM females compared to the kindle-only females was also marginal and did not reach statistical significance, $H(3) = 10.58, p <.10$.

Discussion

The primary purpose of this experiment was to assess hippocampal functioning in amygdala-kindled male and female rats using the hippocampal-dependent version of the water maze task (i.e., DMTP). The results revealed three important findings. First, the kindled males (i.e., kindle-WM) displayed impaired performance in the water maze as was hypothesized, but a similar effect was not observed in the kindled females. Second, the kindled females exposed to the water maze displayed lower levels of fear in the open field and during the resistance-to-capture test compared to the kindle-only females, whereas a similar effect did not emerge in the kindled males. Third, both groups of kindled males and females exposed to the water maze (i.e., kindle-WM) displayed a delayed onset of generalized convulsions compared to the kindled males and females that were not exposed to the water maze (i.e., kindle-only conditions). These results reveal an interesting relationship between hippocampal-mediated behaviour and fear that is dependent upon the sex of the rat. Kindling disrupted the males' performance on the hippocampal-dependent task and these males were also characteristically fearful; however, kindling did not disrupt the females' performance in the water maze yet exposure to the task appeared to reduce the magnitude of fear behaviour. The nature of this relationship among hippocampal performance, fear behaviour, kindling, and sex is discussed in more detail below.

Water Maze Performance

The main purpose of this experiment was to test the integrity of the hippocampus in amygdala-kindled rats using a hippocampal-dependent behavioural task. The procedure used for this experiment was similar to the procedure shown by Steele and Morris (1999) to be hippocampal dependent. This procedure allowed the rats to learn the task requirements and the spatial arrangement of the room (i.e., during a training phase) prior to any experimental manipulations (i.e., kindling in the present case) in order to reduce the stress of an unfamiliar testing environment and allow for a more valid assessment of hippocampal function. Spatial memory was assessed by varying the location of the hidden platform in a manner that required the rats to relearn new platform coordinates during each testing session within a static spatial environment. Using this hippocampal-dependent version of the water maze task, the amygdala-kindled males were shown to have impaired performance. This important result supports the hypothesis that kindling is accompanied by disrupted hippocampal-dependent behaviour in a manner that relates to kindled fear, at least in kindled males. The sex differences are discussed later.

There have been previous attempts to assess spatial memory performance in amygdala-kindled rats; however, in all of these studies except for one, the authors neglected the issue of the inherent fear associated with the novelty of the testing. Unfortunately, the one study in which fear behaviour was taken into account is mentioned in a review paper, and the spatial memory results were not provided (unpublished data described by Hannesson & Corcoran, 2000). To my knowledge, the results have not been published elsewhere. Generally, amygdala kindling has been shown to produce no significant impairments in spatial memory (Letty, et al., 1995; McNamara, et al., 1992; Nieminen, et al., 1992) except when kindling is extended for a long period of time such as 300 stimulations (Cammisuli, et al., 1997). The acquisition deficits reported in this latter study may not be surprising given that long-term amygdala-kindled rats are extremely fearful of novel situations. Accordingly, the observation here that amygdala kindling disrupts male rats' performance under conditions when fearfulness is minimized is a new and significant contribution to our understanding of the behavioural effects of amygdala kindling.

Several other features of the observed spatial memory deficits in kindled males are noteworthy. First, the deficit in the kindled males' performance was evident after the 31 – 45 block of kindling stimulations (i.e., during week 3 of testing). At this point during the kindling phase, amygdala-kindled rats have experienced multiple generalized convulsions. According to the analysis of the kindling data presented above, the amygdala-kindled rats (kindle-WM and kindle-only) began displaying generalized convulsions during the first week of kindling on average (i.e., after approximately 12 – 15 stimulations). This timing is important because it shows that spatial memory deficits do not correspond directly with the onset of the kindled convulsions. Alternatively, the memory deficits are more likely associated with neural reorganization or neural changes occurring subsequent to epileptogenesis and closer to the time that fear behaviour is developing. Interestingly, an interrelationship also exists because exposure to the water maze delayed the rate of kindling in this experiment.

The time point at which spatial performance deficits became evident is similar to the time point in which fear behaviour begins to appear in amygdala-kindled rats. For example, amygdala-kindled rats do not display significant increases in fear behaviour after 20 stimulations (Kalynchuk et al., 1997) but do begin to display elevated fearful behaviour after 30 stimulations (see Experiment 1). This suggests that the two observations are related phenomena and may arise from the same neural mechanisms.

The water maze performance is also interesting with regard to sex differences. Although kindling did not appear to alter the spatial performance of the female rats, it is important to consider their behaviour in relative terms. For example, there was no difference between the sham-stim WM and the kindle-WM females in the latency to reach the platform; however, the sham-stim WM females took visibly longer than the sham-stim WM males. This likely reflects the commonly observed sex difference in spatial tasks of this nature in which males outperform females. Alternatively, there was no visible difference between the kindle-WM males and the sham-stim females. This suggests that the kindle-WM males were behaving in a manner similar to females. Therefore, although the results do not suggest that amygdala kindling completely abolishes the ability of males to find a hidden platform (i.e., they were able to locate the platform in a delayed manner), they do suggest that the kindled males approximate the relatively poorer performance of normal females. It is also interesting to note that during week 3 when kindled males began to show disruptions on the task, the kindled females showed an apparent enhancement of performance approximating the performance of normal males. Unfortunately, it is difficult to comment on the significance of this effect without further testing; however, in general, changes occurring around the third week of kindling (i.e., after 30 stimulations) may be a significant developmental point.

The role of the hippocampus in the water maze task used in this experiment was clearly demonstrated by Steele and Morris (1999). They showed that when the hippocampus was lesioned, the rats were not able to find to the hidden platform after a one-hour delay between the place and matching trials. The rats in the Steele and Morris study were almost completely impaired. The amygdala-kindled male rats in the present study were much less impaired in comparison; however, they still showed significant deficits during three weeks of testing. The design of the present study was conservative and done in a manner to reduce the novelty of the testing situation. Given the significant change in kindled males' performance observed under these conservative conditions, one can conclude that hippocampal dysfunction does exist. However, given that the kindled males were not completely impaired, in a manner shown by hippocampal-lesioned rats, one can also conclude that the hippocampal dysfunction produced by kindling is more subtle than the dysfunction produced by a lesion, as one would expect given that there is little obvious hippocampal damage per se. Further hippocampal-dependent tests (behavioural and electrophysiological) are now warranted to corroborate the anatomical and molecular changes reported in the hippocampi of amygdala-kindled rats.

Open Field and Resistance-to-Capture

Kindled male rats (kindle-WM and kindle-only) displayed characteristically high levels of fear in the open field and during the resistance-to-capture test and their fear behaviour was not affected by exposure to the water maze task. This finding supports our initial hypothesis that hippocampal dysfunction is involved in fear behaviour. Similarly, kindled females not exposed to the water maze (i.e., kindle-only) showed the same characteristically high levels of fear that were previously observed in kindled male and female rats (Experiment 1). In contrast, fear behaviour was reduced in the kindled females that were exposed to the water maze (i.e., kindle-WM). This finding was somewhat surprising and suggests that even limited exposure to a hippocampal-dependent task throughout the kindling paradigm (i.e., once per week) served to protect the kindled females from some of the negative behavioural consequences of kindling.

The reduction of fearful behaviour in the kindle-WM females, although statistically marginal, highlights a potential niche to investigate how exposure to a hippocampal-dependent task might reduce the development of kindling-induced fear. However, because this was only observed in females, the intensity of the exposure may be a significant factor to consider. For example, exposure to the present task may differ in the intensity by which it activates the hippocampus between males and females. For females, the DMTP task may have been more difficult, representing the well-known tendency for females to under-perform on spatial tasks that require the use of spatial cue configuration to navigate to a location, as described in the introduction. Thus, water maze exposure in females may have provided a greater hippocampal challenge that altered the development of kindling-induced fear behaviour. For males, the task may have been less difficult at the onset of kindling and consequently provided no effect in reducing kindling-induced fear behaviour. Clearly, this possibility is speculative, yet it is worthy of investigation. Furthermore, proposing an explanatory mechanism for the observed sex difference is difficult given that the only prior account of kindled fear in females was reported in this thesis (i.e., Experiment 1) and there are currently no post-mortem data from the brains of kindled females. Knowledge of the neural changes in hippocampal regions of kindled females is necessary before the role of the hippocampus in females' kindled fear can be fully assumed.

Kindling

The kindled rats that were exposed to the water maze (kindle-WM) required more kindling stimulations to elicit the first generalized convulsion and had fewer generalized convulsions in the second and third weeks of kindling than the kindled rats that were not exposed to the water maze (kindle-only). These effects were observed in both males and females and stand in contrast to the sex differences observed in the water maze performance and the fear behaviour. The kindling result is important because it provides further confirmation that the onset and number of generalized convulsions during kindling do not necessarily predict the behavioural consequences of kindling, as was briefly discussed in Experiment 1 with regard to the finding that the 30-stim rats kindled faster but were less fearful than the 99-stim rats. In other experiments, we have also shown that environmental enrichment *facilitates* the development of kindling in male rats but reduces the magnitude of fear behaviour in the same rats (Young, Wintink, & Kalynchuk, 2004). Therefore, although the number of stimulations a kindled rat receives predicts the severity of subsequent behavioural consequences, the development of generalized convulsions does not appear to have any predictive value in this regard. This same notion has been suggested elsewhere (Adamec, 1990b).

The kindling data are also interesting because a delay in the onset of generalized convulsions could suggest that exposure to some aspect of the water maze served to interfere with epileptogenesis. For example, simply being exposed to a novel environment weekly provided a neuroprotective effect against the deleterious effects of kindling. Alternatively, the hippocampal challenge of learning the spatial task could have also provided a protective effect. This same conclusion was offered for the kindled females' reduced fear behaviour after exposure to the hippocampal challenge. These possibilities provide an interesting theoretical framework for targeting the hippocampus in preventative therapies for epileptogenesis and the behavioural consequences of seizures. This hypothesis suggests that providing a hippocampal-dependent challenge alters the development or expression of kindling-induced neural changes within the hippocampus. For example, exposure to a hippocampal-dependent challenge during the course of kindling might reverse the alterations in hippocampal receptor and gene expression that have been associated with kindling-induced fear. Interestingly, kindling, spatial learning, and environmental environment are all known to promote neurogenesis within the adult hippocampus as discussed in the introduction. It may be that cells born under conditions of kindling mature to express abnormal number of inhibitory and excitatory receptors, as has been observed in the brains of fearful kindled rats (see Kalynchuk, 2000). However, exposure to a hippocampal-dependent challenge during kindling also might induce newly born cells to express normal levels of receptors and genes. This idea will be addressed in Experiment 4 of this thesis.

Sex Differences

Kindling disrupted males' water maze performance but did not alter that of the females. Exposure to the hippocampal-dependent task reduced females' fear behaviour but did not reduce that of the males. Both males and females exposed to the water maze kindled slower than the rats that were not exposed to the water maze. Collectively, these findings show that sex is an important consideration when studying the role of the hippocampus in kindling and kindled fear. One possible interpretation of these results is that male rats are more susceptible than females to the effect of kindling on hippocampal function and the behavioural consequences. For example, the kindled male rats were impaired on the memory task, but the kindled female rats were not. This suggests that kindling had more of a negative effect on hippocampal function in the males than the females. Similarly, the kindled males were more fearful than the kindled females, in that exposure to the hippocampal-dependent task reduced fearful behaviour in the kindled females but not the kindled males. If kindling had more of a negative effect on hippocampal function in male rats than in female rats, then exposure to a stimulus that might reduce these effects would be more effective in females than in males. It may be that the hippocampal-dependent task used in the present experiment, although appropriate and necessary for the primary purpose of this experiment (i.e., assessing hippocampal function in kindled rats), was not strong enough to protect male rats against the negative consequences of kindling. Thus, exposure to another, more sustained hippocampal stimulus might also provide a protective effect in kindled male rats. This idea is tested in Experiment 4.

It is also important to mention that the present results might have occurred because the neural changes arising from kindling and the water maze task might differentially activate or organize male and female brains. Sex hormones have been shown to alter seizure threshold previously (Edwards et al., 1999) and kindling has been shown to alter the estrous cycle in females (Edwards, et al., 2000). These same hormones have also been shown to alter spatial behavior (Galea, Kavaliers, Ossenkoo, & Hampson, 1995; Williams, Barnett, & Meck, 1990). Ideally, the estrous phase of each female during her spatial performance and prior to each kindling stimulation should be assessed. These data were partially collected during this experiment but given the limited numbers of females that would be represented in each estrous phase (i.e. 9-10 per group divided by 4-5 possible estrous phases) it would be difficult to make any conclusions that would not be too speculative. However, the fact that sex difference did emerge provides the necessary foundation to further examine the difference, which should include a consideration of hormonal status.

Unfortunately, as mentioned above, there are fewer data regarding the neural changes associated with epileptogenesis and kindling in female rats. It is currently not known whether kindled females display similar alterations in hippocampal receptor and gene expression to those observed in kindled males. Thus, a definitive statement about the neural mechanisms underlying behavioural changes in kindled rats cannot be made until a more data are available.

Conclusion

The major findings of this experiment are that 1) amygdala-kindled males showed impaired hippocampal-dependent behaviour after 3 weeks of kindling but the kindled females were not similarly impaired, 2) exposure to the hippocampal-dependent task reduced fear behaviour in kindled-female rats, and 3) exposure to the hippocampal-dependent task delayed the development of kindling. These results support the hypothesis suggested elsewhere (Kalynchuk et al., 2000) that the integrity of the hippocampus is compromised by amygdala kindling in a manner that relates to fear behaviour. They also suggest that treatments that activate the hippocampus might be useful in hindering the development of fear sensitization and the development of kindling. The results reveal a mutually dependent relationship among amygdala kindling, fear behaviour, and hippocampal functioning that now incorporates the sex of the animal as an additional factor. This latter point begs the need for data on the neural correlates of fear behaviour in female rats.

Chapter 4: Experiment 3

Hippocampal Cell Proliferation in Amygdala-Kindled Male and Female Rats

The purpose of this experiment was to examine the effect of different numbers of kindling stimulations on hippocampal cell proliferation. As mentioned in the general introduction, short-term amygdala kindling has been shown to increase hippocampal cell proliferation (Scott et al., 1998; Parent et al., 1998). However, it is not known whether long-term kindling also increases cell proliferation, nor is it clear whether kindling-induced increases in cell proliferation persists once the kindling stimulations are terminated. This is an important issue for determining the neural mechanisms underlying kindling-induced behavioural changes. In the discussion of Experiment 2, it was hypothesized that cells born under conditions of kindling may express aberrant numbers of inhibitory and excitatory receptors, which might compromise normal information processing in the hippocampus. This could lead to increased fear and altered cognitive abilities, as was shown in Experiment 1 and 2 of this thesis.

To begin to test the idea that hippocampal cell proliferation might play a role in kindling-induced behavioural changes, the rate of cell proliferation was examined under several conditions. These conditions mimicked those from Experiment 1 (i.e., 99-stim, 30-stim, or sham-stim) so that the rate of cell proliferation could be compared with the behavioral observations of Experiment 1. The rate of cell proliferation was also assessed at two time points (i.e., during kindling and following a stimulation-free period) in order to examine the extent to which it is dependent the recent occurrence of seizures (i.e., activity dependent). Finally, this experiment was conducted using male and female rats because of the scarcity of data on the neural changes associated with kindling and kindled fear in female rats. As mentioned above, little is known about the neural changes associated with kindling or kindling-induced behavioural changes in female rats.

Methods

Subjects

The subjects were 42 male and 41 female adult (approximately 250 and 200 g respectively at the time of arrival) Long Evans rats (Charles River Canada, St. Constant, PQ). They were individually housed in rectangular polypropylene cages with wood shavings as bedding. Food and water was available *ad libitum*. Lights in the colony room were set on a 12:12-hr light:dark cycle with lights on at 8:00 a.m. All experimental manipulations were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the Dalhousie University Committee on Laboratory Animals.

Surgery

Each rat underwent stereotaxic surgery approximately one week after arrival and was handled daily prior to surgery. At the time of surgery, males rats weighed between 275-300g and female rats weighed between 240-275g. The surgical methods were the same as those used in Experiment 1.

Kindling

Figure 3.1 shows the design of this experiment. All rats were subject to kindling or sham-stimulations prior to being sacrificed for immunohistochemical determination of

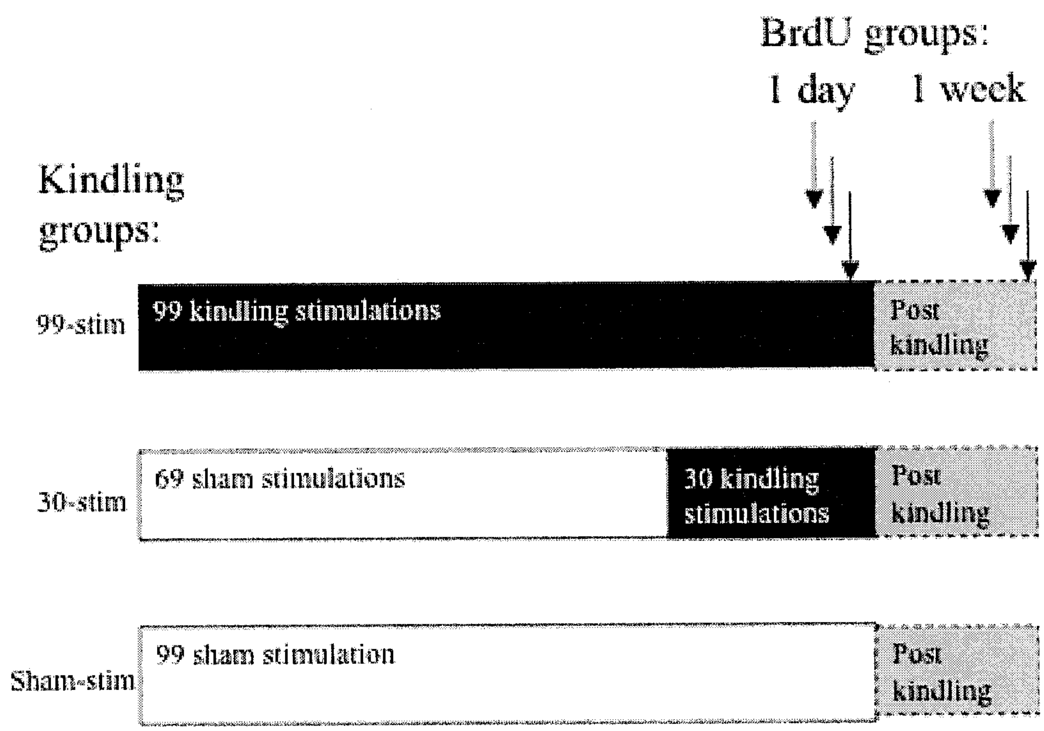


Figure 3.1. A schematic representation of the experimental groups and schedule.

hippocampal cell proliferation. The kindling paradigm was the same as the one used in Experiment 1. After a post-operative recovery period of at least 5 days, the male and female rats were divided into three kindling groups each: One group of male and one group of female rats received 99 kindling stimulations (99-stim males, n = 16; 99-stim females, n = 16), one group of male and one group of female rats received 69 sham stimulations followed by 30 kindling stimulations (30-stim males, n = 14; 30-stim females, n = 13), and one group of male and one group of female rats received 99 sham stimulations (sham-stim males, n = 12; sham-stim females, n = 12).

BrdU Immunohistochemistry and Tissue Preparation

The mitotic label bromodeoxyuridine (BrdU; dissolved in warm saline) was administered to each rat in three separate intraperitoneal injections (50 mg/kg per injection). The injection was given 36 h, 24 h, and 12 h prior to perfusions. Half the rats were transcardially perfused 24 h after the last kindling stimulation and half were perfused 1 week following the last kindling stimulation. The rats were anaesthetized with a lethal dose of sodium pentobarbital (100 mg/kg, i.p.) and perfused first with 0.9% saline followed by 4% paraformaldehyde (dissolved in 0.1 M phosphate buffer (PB)). The brains were removed and stored in the same paraformaldehyde solution for 1 week and then stored in 0.1M phosphate-buffered saline (PBS) with 0.5% sodium azide (PBS/azide; to inhibition bacterial growth) until the brains were sectioned at 40 μm through the entire hippocampus using a vibrating microtome (Leica Microsystems Inc.). The hippocampal sections were divided into 12 series, such that each series contained approximately 8-10 40 μm thick sections spaced 440 μm, or 11 sections, apart. During sectioning, the brains were bathed in 0.1 M PB and once it was cut, the tissue was returned to the PBS/azide solution until it was processed for immunohistochemistry.

The immunohistochemistry protocol for BrdU determination of cell proliferation was as follows. Free-floating tissue sections were rinsed twice for 15 min each in 0.1M PB to remove the azide. The tissue was then incubated in 50% formamide/50% 2X saline sodium citrate (SSC) for 2 hr at 65°C. The tissue was then rinsed in 2X SSC for 5 minutes and incubated in 2N HCl for 30 min at 37°C. The tissue was then rinsed in 0.1M boric acid (pH 8.5) for 10 min followed by 3 washes in 0.1 M tris buffered saline (TBS) for 5 min each. Following the washes, the tissue was rinsed in 1% H_2O_2 for 30 min to reduce endogenous peroxidase activity and then washed again 3 times in TBS (10 min each). The tissue was then blocked with a solution of 10% normal horse serum (NHS, Vector Laboratories) and 0.1% triton (Sigma-Aldrich) in TBS (TBSt) for 1 hr. The tissue was rinsed twice in TBSt for 5 min each and then incubated in the primary antibody (mouse anti-BrdU, 1:500, Roche Diagnostics) for 72 hr at 4°C. After the 72-hr incubation, the tissue was rinsed twice in TBSt for 15 min each and then incubated in the secondary antibody (biotinylated horse, anti-mouse, 1:200, Vector Laboratories). After incubation, the tissue was washed once in TBSt (15 min), once in TBS (15 min), and then incubated in the avidin-biotin complex (ABC standard elite, 1:200, Vector Laboratories) for 3 hr. The tissue was then removed, rinsed in TBS 3 times (5 min each), and then reacted with diaminobenzidene (DAB: 10 mg pellet in 20 ml TBS) and 1% H_20_2 as the catalyst agent. The reaction was stopped after 15 minutes with 3 washes in TBS (5 min each) and then stored and mounted in 0.1M PB.

After the tissue was mounted, it was left to air dry for 48 hours and counter stained with 1% organic cresyl violet (Sigma-Aldrich Organics), dehydrated with ethanols, and cleared using xylenes. The slides were cover slipped using Entallen (Fisher Scientific) and cleaned once they had dried.

Cell Counting & Sampling

BrdU-labelled cells were counted by an observer who was blind to the experimental conditions of each rat. Three sections along the anterior-posterior plane of the hippocampus were sampled according to the Paxinos and Watson (1998) stereotaxic atlas referenced to bregma: -2.12 mm, -3.14 mm, and –6.04 mm (see Figure 3.2). All BrdU-labelled cell profiles were counted using a Nikon light microscope under 40X and 100X magnification. Pyknotic cells were identified based on examples depicted and used in Cameron and McKay (2001). These cells were not included in the count.

Experimental Groups

The experimental design was a 3 (kindling group: 99-stim, 30-stim, sham-stim) by 2 (time point: 1 day (Day) and 1 week (Week) post kindling) by 2 (sex: males, females) between-subjects design for a total of 6 groups per sex. The experimental conditions and numbers of subjects at the start of the experiment were as follows for males: 99-stim Day = 8; 99-stim Week = 8; 30-stim Day = 7; 30-stim Week = 7, sham-stim Day = 6, and sham-stim Week = 6. For the females, the conditions and numbers

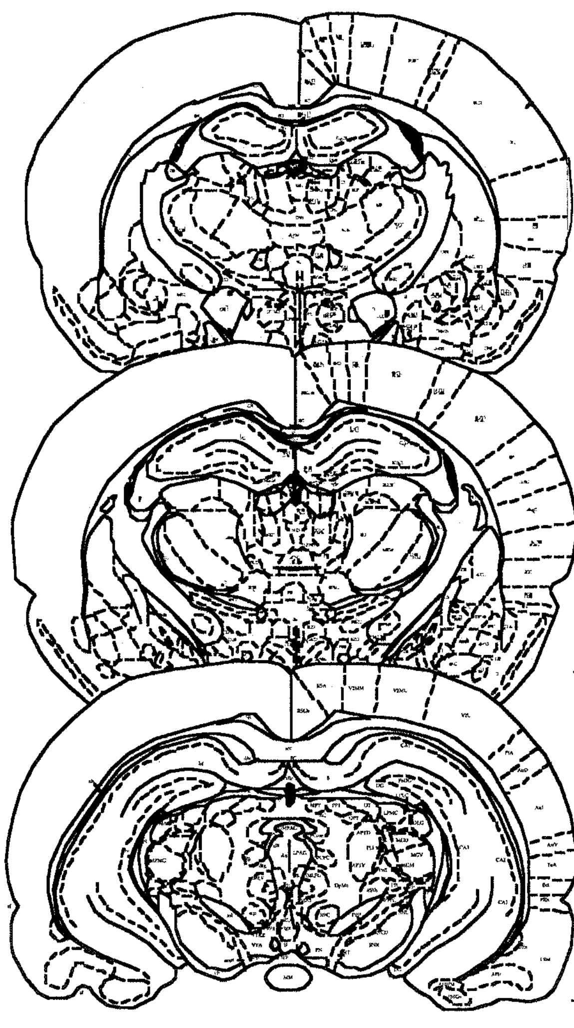


Figure 3.2. Sections through the hippocampus that were sampled for BrdU-labeled cell along according to Paxinos and Watson's stereotaxic atlas referenced to bregma.

were as follows: 99-stim Day = 8, 99-stim Week = 8, 30-stim Day = 6, 30-stim Week = 7, sham-stim Day = 6, sham-stim Week = 6.

Data Analysis

The mean number of BrdU-labelled cells for each kindling group and time point was examined in each of the three sections (i.e., -2.12 mm, -3.14 mm, and –6.04 mm) and statistically analyzed with multivariate analysis of variance (MANOVA). Student Newman Keuls post hoc tests were done to follow-up any statistically significant interactions that emerged. Statistical significance was set at $p \leq .050$.

Results

Kindling

Figure 3.3 illustrates the mean number of stimulations to the first class 5 convulsion for the males and females in the 30-stim (i.e., 69 sham stimulations followed by 30 electrical stimulations) and 99-stim groups, collapsed across time-point condition. The number of stimulations required to elicit the first class 5 convulsion was not significantly different between 30-stim and 99-stim females, $F(1, 23) = 2.170, p = .154$, but the 99-stim males required more stimulations to elicit the first class 5 convulsion than did the 30-stim males, $F(1, 23) = 5.834, p = .024$. However, there were no significant sex difference in either the 30-stim, $F(1, 19) = 2.462$, or the 99-stim kindling groups, $F(1, 38) < 1.0$.

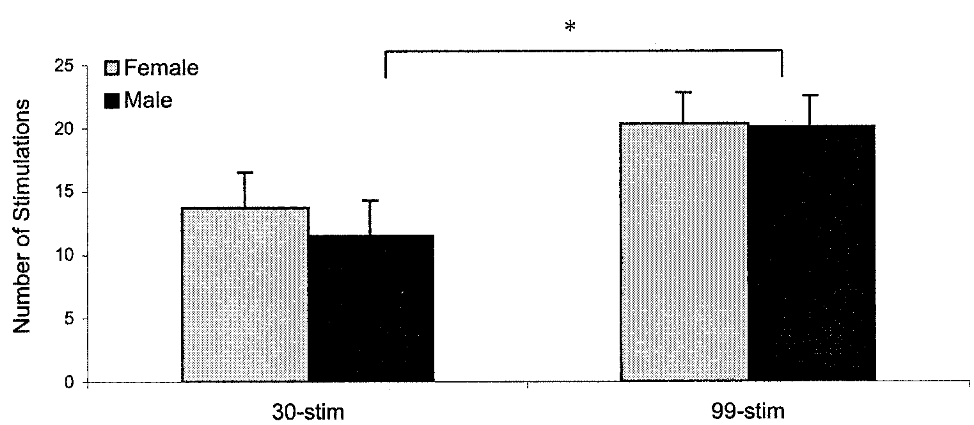


Figure 3.3. The acquisition of kindling in the male and female rats. Mean (± SEM) number of stimulations required to elicit the first class 5 convulsion for male and female rats in both the 30-stim and the 99-stim kindling conditions. * $p < .05$

Histology

As in Experiments 1 and 2, rats that had lost their electrode head apparatus at any point during the experiment or had electrode placements that terminated outside the amygdala were removed from the experiment. Therefore, the data analyses was based on the following numbers for males: 99-stim Day = 7; 99-stim Week = 7; 30-stim Day = 6; 30-stim Week = 7, sham-stim Day = 6, and sham-stim Week = 6. For the females, the numbers of rats were as follows: 99-stim Day = 8, 99-stim Week = 7, 30-stim Day = 5, 30-stim Week = 5, sham-stim Day = 6, sham-stim Week = 6.

BrdU Immunohistochemistry

The mean number of BrdU-labelled cells in each of the three brain sections was compared among the kindling and time-point groups. The data from all three brain sections were statistically analyzed. There was a similar pattern of cell proliferation in all three brain sections (see Figure 3.4). The results show that the number of BrdU-labelled cells was greatest in the 30-stim Day condition relative to all other kindling conditions and time-point conditions.

The number of BrdU-labelled cells was quantified and the results are depicted in Figure 3.5. Statistically, for each brain section (i.e., anterior: -2.12 mm, mid: -3.14 mm, and posterior: -6.04 mm) there was a statistically significant kindling condition by time point interaction, $F(2,48) = 6.550$ (anterior), 9.326 (mid), and 6.105 (posterior), p's < .001. This effect was not dependent upon sex: i.e., the 3-way interaction between sex, kindling condition, and time point was not statistically significant, $F(2,48) = 0.196$ (anterior), 0.662 (mid), and 0.318 (posterior), p's $\geq$ .520. In addition, there was no

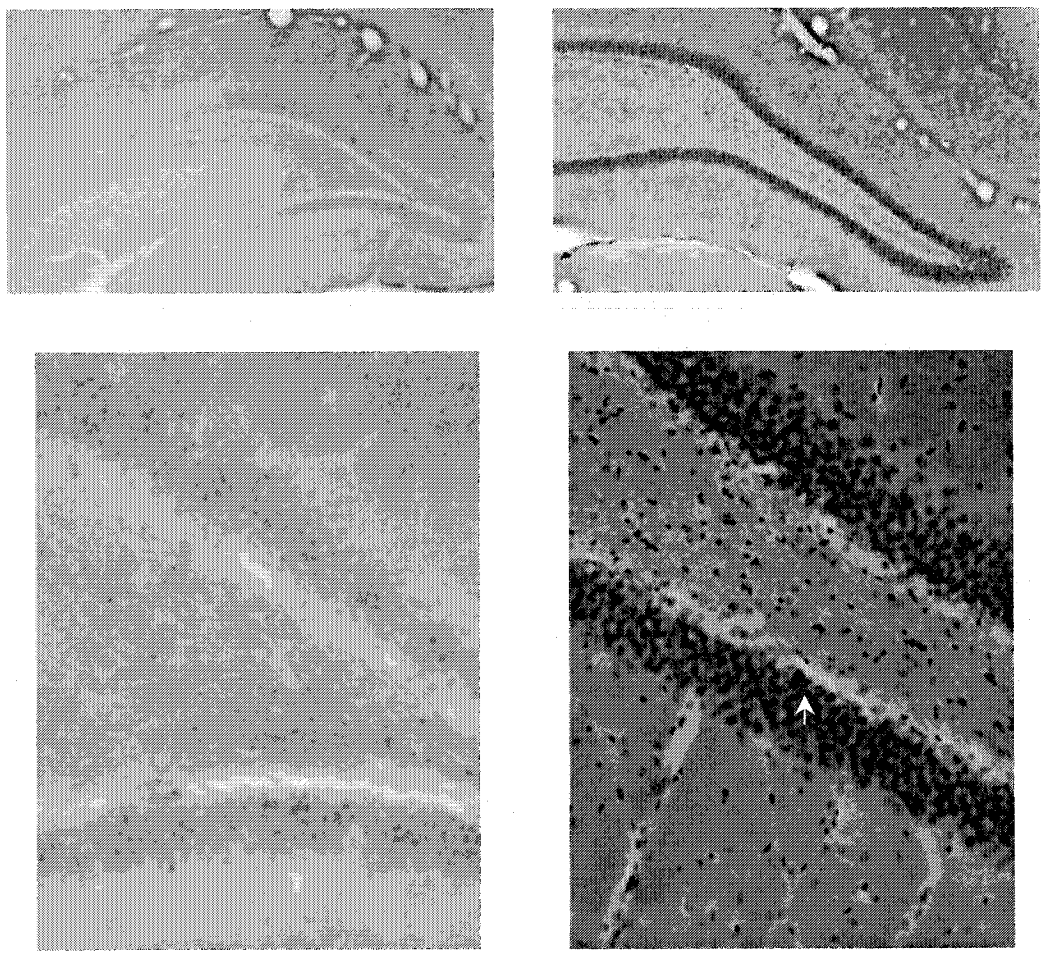


Figure 3.4a. Photographs of BrdU-labeled cells in the sham-stim males at 4X magnification (top) and 20X magnification (bottom) in the 1-day condition (left) and the 1-week condition (right).

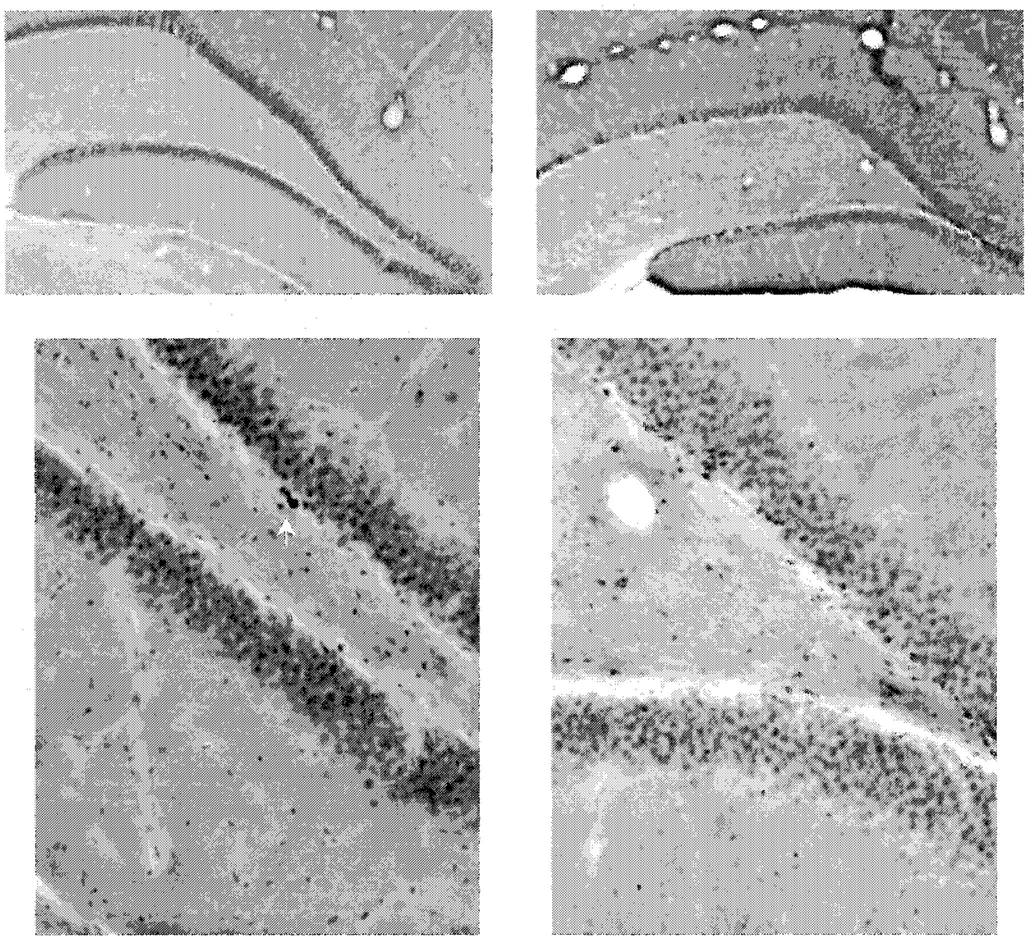


Figure 3.4b. Photographs of BrdU-labeled cells in the sham-stim females at 4X magnification (top) and 20X magnification (bottom) in the 1-day condition (left) and the 1-week condition (right).

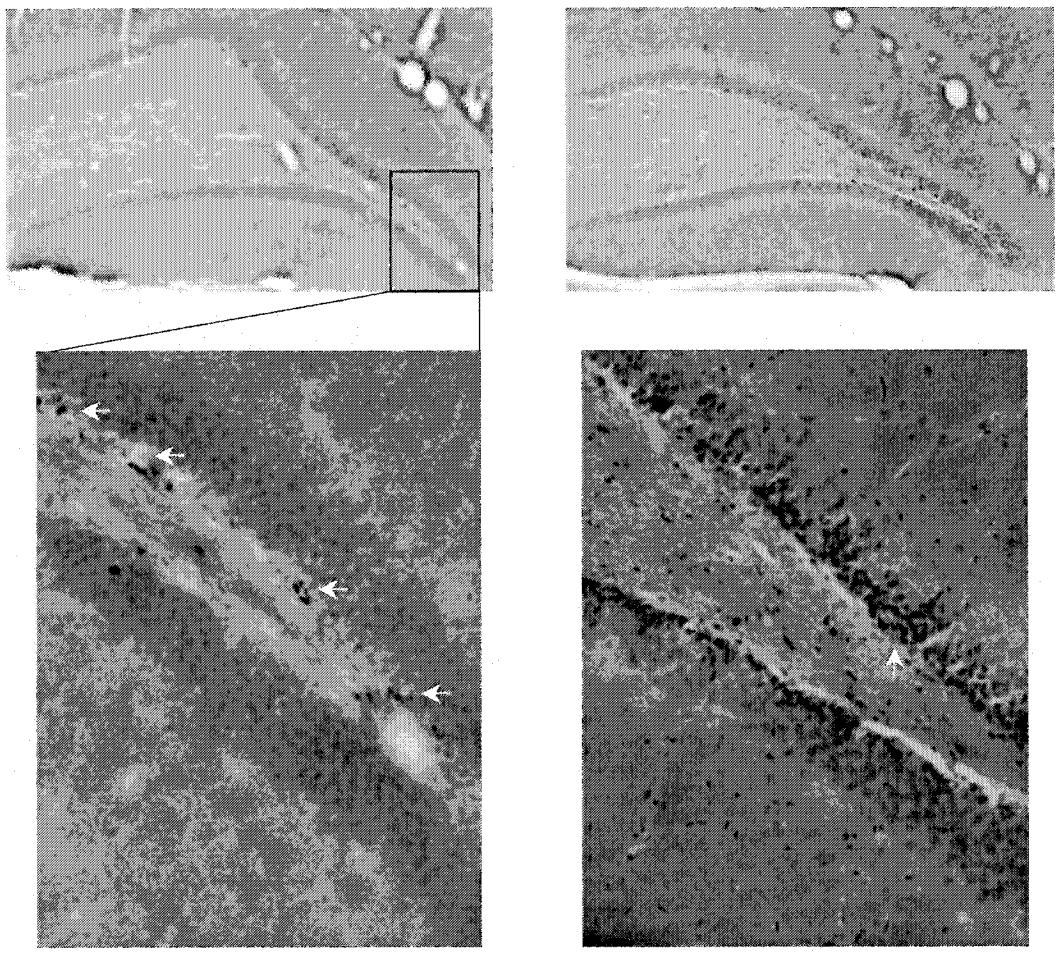


Figure 3.4c. Photographs of BrdU-labeled cells in the 30-stim males at 4X magnification (top) and 20X magnification (bottom) in the 1-day condition (left) and the 1-week condition (right).

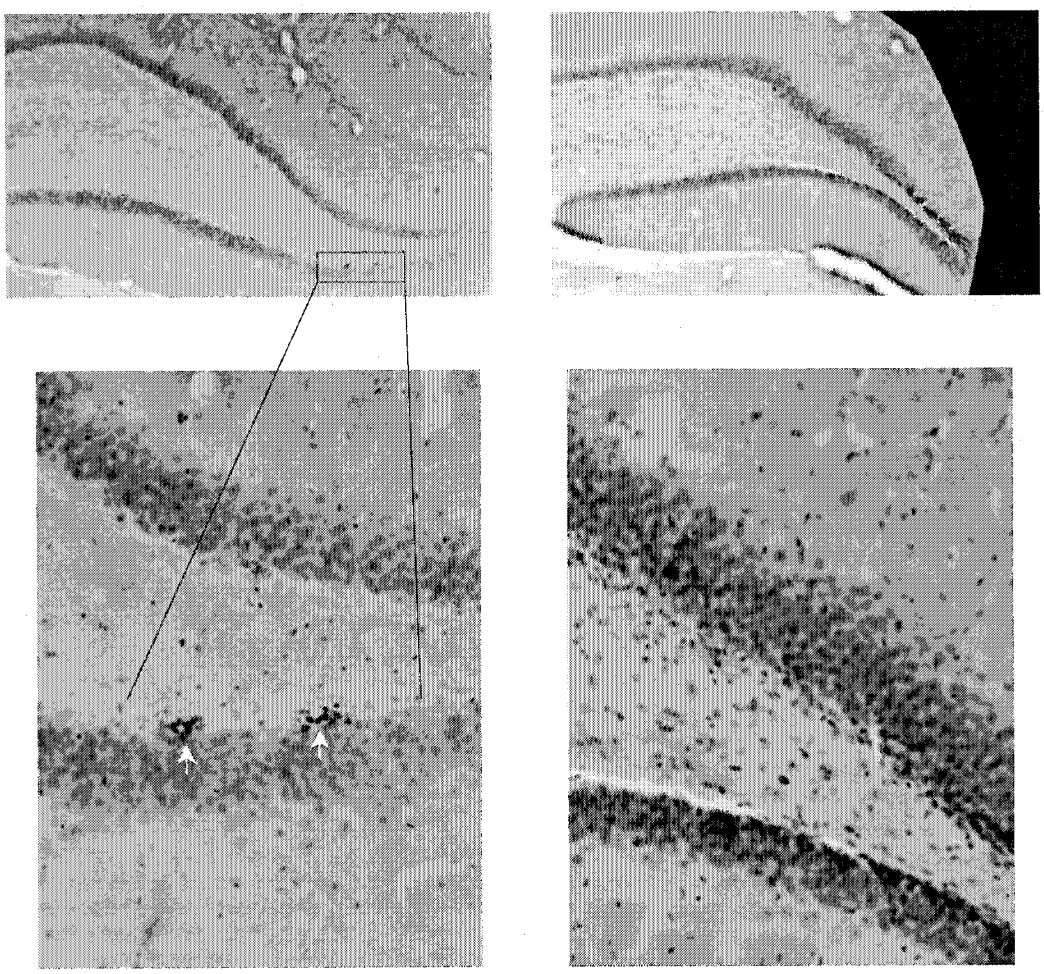


Figure 3.4d. Photographs of BrdU-labeled cells in the 30-stim females at 4X magnification (top) and 20X magnification (bottom) in the 1-day condition (left) and the 1-week condition (right). Arrows indicate two distinct masses of labeled cells.

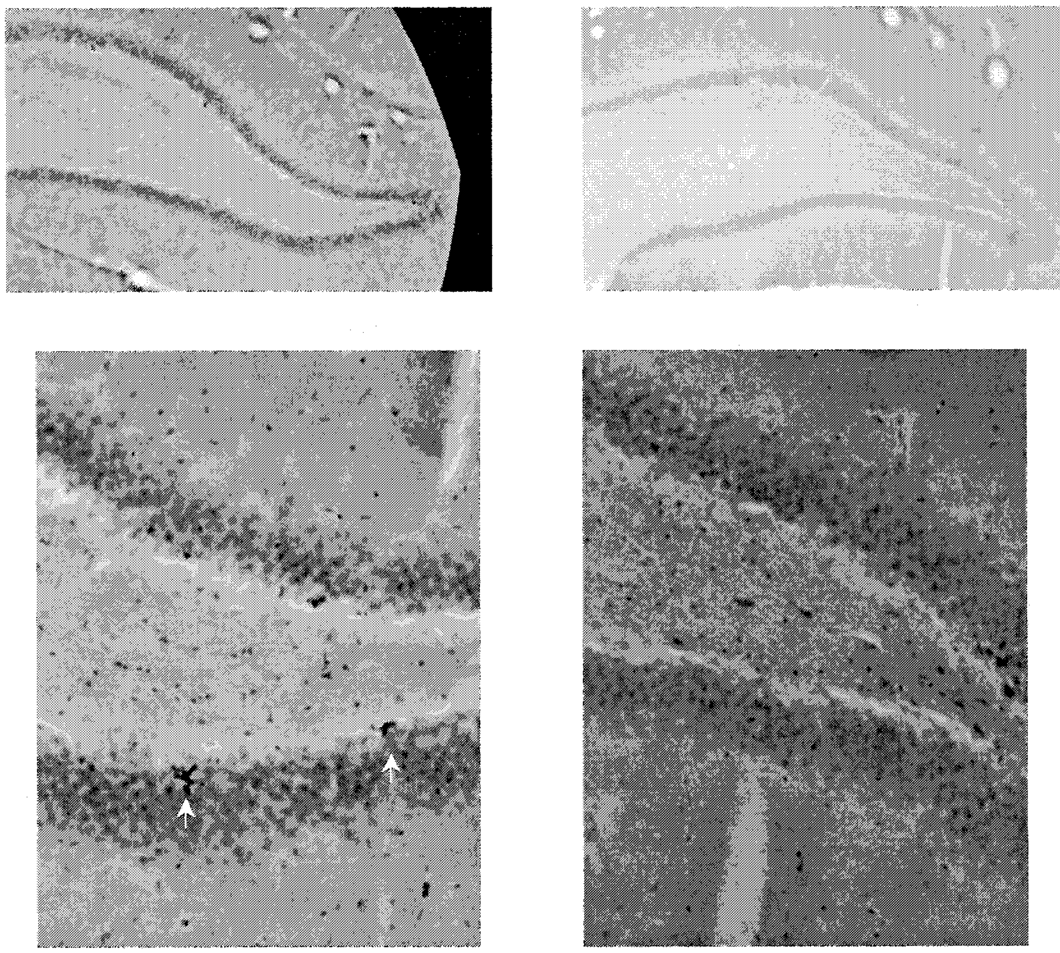


Figure 3.4e. Photographs of BrdU-labeled cells in the 99-stim males at 4X magnification (top) and 20X magnification (bottom) in the 1-day condition (left) and the 1-week condition (right).

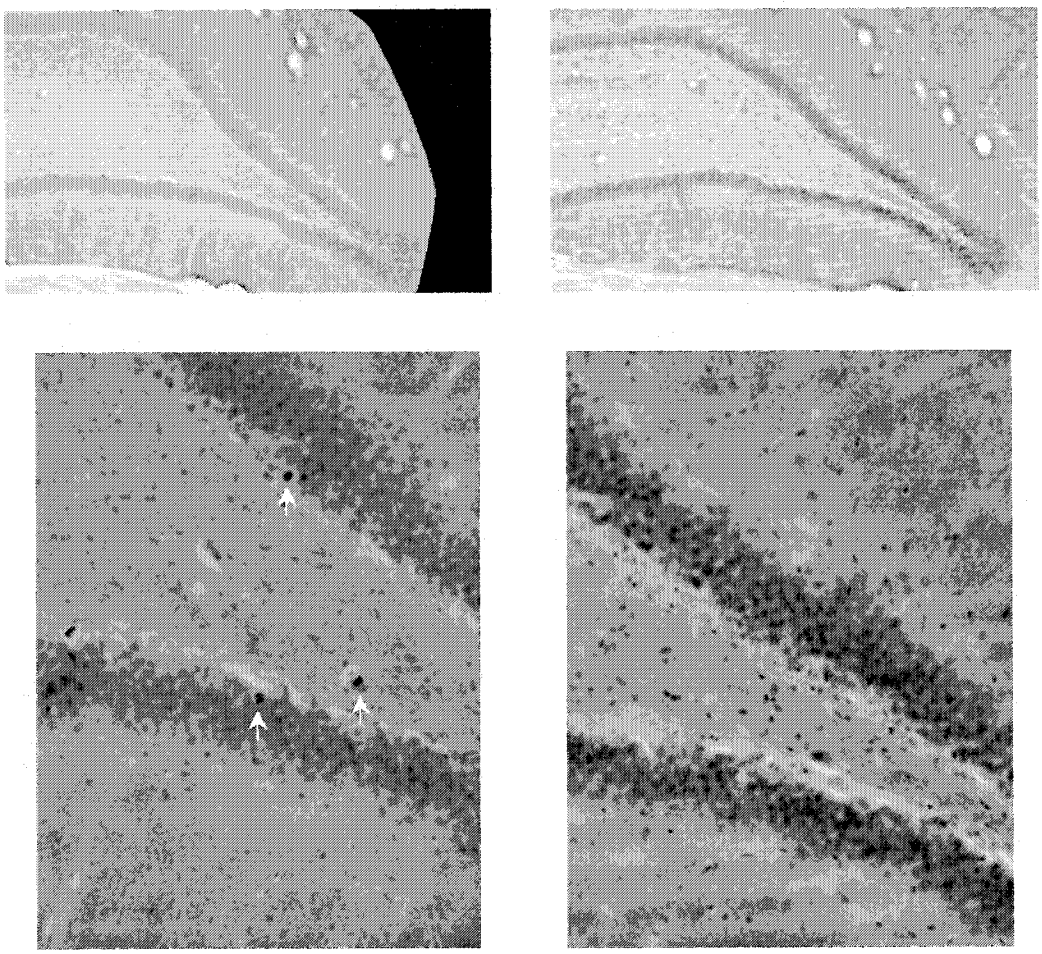


Figure 3.4f. Photographs of BrdU-labeled cells in the 99-stim females at 4X magnification (top) and 20X magnification (bottom) in the 1-day condition (left) and the 1-week condition (right).

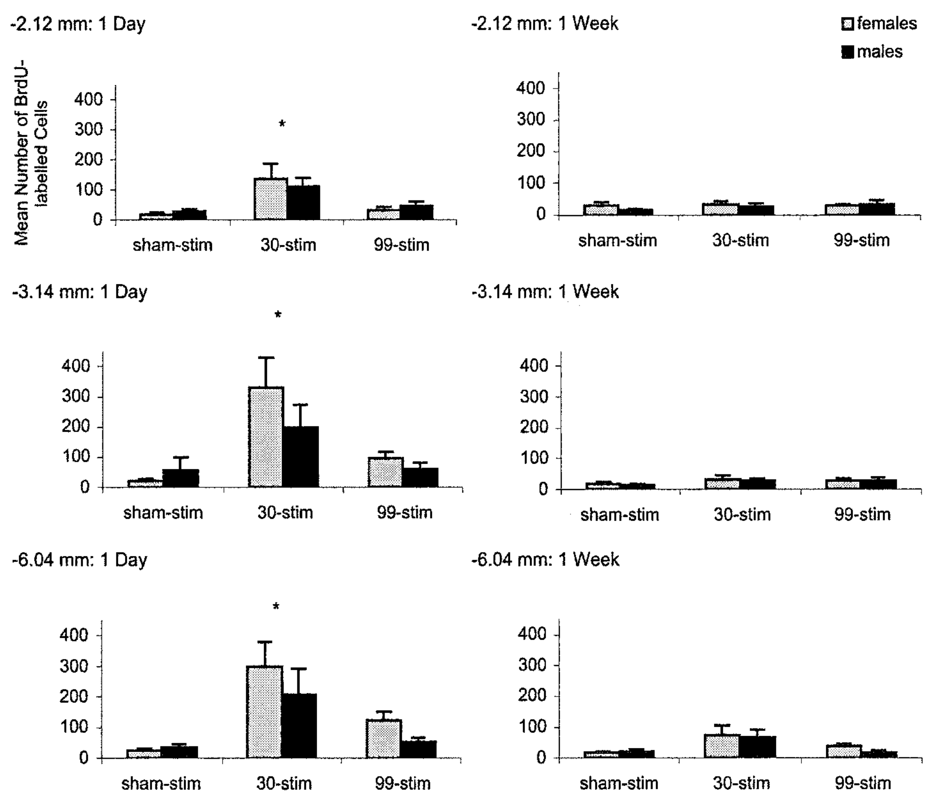


Figure 3.5. Mean number of BrdU-labeled cells sampled at three regions anterior to posterior through the hippocampus (-2.12, -3.14, -6.04 from bregma) 1 day or 1 week after the last kindling stimulation for males and females. * $p < .05$

statistically significant interaction between sex and kindling condition (p's $\leq$ .284) or between sex and time point (p's $\leq$ .498). Similarly, there was no main effect of sex (p's $\leq$ .137). The only significant main effects that emerged were for kindling, $F(1, 48) = 7.459$ (anterior), 11.057 (mid), 16.168 (posterior), p's < .001, and time point, $F(1,48) = 9.964$ (anterior), 22.556 (mid), 17.605 (posterior), p's < .001, both of which are qualified by their interaction.

Student-Newman Keuls post hoc tests for males and females confirmed that the 30-stim Day condition had significantly more BrdU-labelled cells compared to all other conditions in each brain section (i.e., anterior – posterior) for both males and females ($p < .05$). The other conditions did not differ significantly from each other, $p = .996$ (females, anterior), .696 (males, anterior), .652 (females, mid), .806 (males, mid), .347 (females, posterior), .662 (males, posterior).

Discussion

The purpose of this experiment was to examine the effect of different numbers of kindling stimulations on hippocampal cell proliferation in male and female rats. The results provide two important and novel findings. First, kindling-induced cell proliferation is significantly higher after 30 stimulations in both male and female rats than it is after 99 stimulations. In fact, the rate of cell proliferation after 99 stimulations is comparable to sham-stimulated levels, suggesting a return to baseline. Interestingly, this effect was evident in a previous study, but the authors did not acknowledge it. Parent et al., (1998) reported that amygdala kindling increased the rate of cell proliferation after 9/10 and 19/20 generalized convulsions; however, cell proliferation in the group of rats that displayed 19/20 convulsions was visibly lower than that of the rats that displayed 9/10 convulsions (see Figure 1d in Parent et al., 1998). Therefore, it appears that the greatest effect of amygdala kindling on cell proliferation occurs early in the kindling process and subsequent kindling stimulations do not have any significant effect on the rate of hippocampal cell proliferation.

The second important finding from this experiment was that the high rate of cell proliferation observed after 30 stimulations does not persist once the stimulations cease. Indeed, the level of cell proliferation was at baseline levels by one week after the final stimulation. This is an interesting observation because it suggests that the kindling stimulations themselves regulate the rate of hippocampal cell proliferation. Conceivably, kindling might also regulate the fate of the proliferating cells. For example, cells born during kindling may differentiate and form pathological connections that become functional around the time when kindled fear is high (i.e., around 99 stimulations). For example, in the kindling paradigm used in these experiments, the rats receive 15 stimulations per week for a total of six and a half weeks. The rats would thus reach the 30-stimulation mark after two weeks. Importantly, Gage and colleagues have found that at least 4 weeks is required for newly born granule cells to differentiate, migrate, and extend axons into the perforant path so that they can functionally alter existing hippocampal processing (van Praag, Schinder, Christie, Toni, Palmer, & Gage). This timeline corresponds closely with the fact that the highly fearful 99-stim kindled rats receive about 4 additional weeks of stimulations after the 30-stimulation mark. Furthermore, the fact that the cells are developing while kindling is ongoing is probably important for the type of molecular triggers that are present and the subsequent connections that the cells form with existing cells. This will be addressed in the next experiment.

Sex Differences

The effect of kindling on hippocampal cell proliferation followed the same pattern for males and females. Assessing cell proliferation rates in both males and females is important for considering the potential role of cell proliferation in kindled fear. In Experiment 1, kindled fear was shown to develop similarly in males and females. Consistent with those behavioural results is the observation that cell proliferation in relation to kindling is also similar between males and females. However, it is interesting to note that the females showed a trend toward a higher rate of cell proliferation, especially in the middle and posterior sections, although this trend was not shown statistically.

Conclusion

Kindling-induced hippocampal cell proliferation is a transient phenomenon that is evident after generalized convulsions have developed but returns to baseline levels after extensive kindling and within a week after the kindling stimulations are terminated. The increase in cell proliferation therefore, does not appear to be a mechanism of kindling nor is it an absolute by-product of kindling stimulations. The pattern of results suggests that kindling-induced cell proliferation precedes extreme fear levels. Consequently, kindling-induced cell proliferation could contribute to the development of kindled fear. This is likely, given that newly born cells need time to differentiate and form synaptic connections with other cells.

Chapter 5: Experiment 4

Environmental Enrichment, Hippocampal Cell Proliferation, and Fear in Amygdala-Kindled Male and Female Rats

The results of Experiment 2 unexpectedly revealed that exposing kindled rats to a hippocampal-dependent task reduced the magnitude of fearful behaviour in female rats. One possible explanation for this effect is that exposure to a hippocampal-dependent task partially reverses the pathological neural changes that underlie the expression of kindling-induced fear. The logic for this hypothesis is as follows. The results of Experiment 3 and previous studies (i.e., Parent et al., 1998; Scott et al., 1998) showed that hippocampal cell proliferation is dramatically increased by 30 stimulations but not after 99 stimulations. What happens to the cells born within the 30 stimulations? Estimates suggest that it takes about 4 weeks for proliferating cells to differentiate into neurons, migrate to the granule cell layer, and extend axons into neighbouring regions (van Praag et al., 2002). More time is likely needed for these cells to then form connections with existing cells. This is an intriguing timeline given that kindled fear begins to be significantly different from sham levels after 30 stimulations. It is possible that new cells that are born around 30 stimulations can be induced to form either pathological connections with other cells, or more normal, beneficial connections with other cells, depending on the environment at the time they are migrating. For example, under conditions of continuing kindling stimulations, newly born cells might aberrantly survive and disperse within the hippocampus to form pathological connections with other cells that lead to the expression of kindled fear. Furthermore, when exposed to a hippocampal-dependent task along with kindling, newly born cells might mature in a manner that results in more beneficial connections that promote learning rather than inappropriate fear behaviour.

The present experiment was designed to begin to assess this hypothesis. It served two purposes that derived from the results observed in Experiments 2 and 3. The first purpose was to determine whether exposure to a more extensive behavioural activation of the hippocampus could reduce the magnitude of kindled fear in both male and female kindled rats. It is possible that the male rats in Experiment 2 did not benefit from exposure to the hippocampal-dependent task because brief exposure to the water maze once per week during the course of kindling was not a strong enough stimulus. Therefore, in this experiment, the rats were subjected to a longer period of environmental enrichment with repeated exposure to novel and complex environments during kindling. In general, the environments consisted of opportunities for the rats to engage in hippocampal-dependent information processing.

The second purpose of this experiment was to determine whether the survival and dispersal of proliferating hippocampal cells are related to the behavioural outcomes (i.e., kindled fear as measured previously) associated with kindling and environmental enrichment treatment. For example, if exposure to the enriched environment during the course of kindling reduces fear, as hypothesized, will it also facilitate survival of cells born early in kindling and form more beneficial neuronal connections? For this experiment, rats first received 21 stimulations to induce kindling and increase the baseline rate of hippocampal cell proliferation. The rats were then injected with BrdU and either subjected to additional kindling stimulations alone or kindling plus environmental enrichment. It was important to begin the enrichment sessions at the time when kindling had significantly elevated cell proliferation but not yet produced significant increases in fear. Another advantage of waiting until the 20-stimulation mark to begin the enrichment sessions was that this avoided potential confounding effects of enrichment itself on cell proliferation. Because environmental enrichment affects cell proliferation, rats that received kindling + enrichment from the beginning would presumably have higher levels of cell proliferation after 20 stimulations than rats that received kindling stimulations alone. This would make it impossible to compare cell survival and dispersal in those groups. Instead, this method ensured that all the kindled rats had similar levels of cell proliferation at the beginning of the enrichment/isolation manipulation. The number and dispersal of surviving cells within various hippocampal regions was assessed following the final kindling/enrichment session.

Method

Subjects

The subjects were 50 male and 50 female adult (approximately 250 and 300 g respectively at the time of arrival) Long Evans rats (Charles River Canada, St. Constant, PQ). They were individually housed in rectangular polypropylene cages with wood shavings as bedding. Food and water was available *ad libitum*. Lights in the colony room were set on a 12:12-hr light:dark cycle with lights on at 8:00 a.m. All experimental manipulations were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the Dalhousie University Committee on Laboratory Animals.

Surgery

Each rat underwent stereotaxic surgery approximately one week after arrival and was handled daily prior to surgery. At the time of surgery, males rats weighed between 275-300g and female rats weighed between 240-275g. All surgical procedures were the same as those used in Experiment 1. Rats were left to recover for approximately one week before being kindled.

Experimental Groups & Schedule

There were five general experimental conditions in this experiment (see Figure 4.1). The treatments are described below and briefly explained here. One group of male and female rats received 99 kindling stimulations (Kindle-Kindle, n = 20). Another group of male and female rats received 99 kindling stimulations plus environmental enrichment (Kindle-Kindle/Enrich, n = 20). A third group of male and female rats received 21 kindling stimulations followed by sham stimulations and environmental enrichment (Kindle-Sham/Enrich, n = 20). A fourth group of male and female rats received 21 kindling stimulations followed by sham stimulations only (Kindle-Sham, n = 20). A final group of male and female rats received 21 sham stimulations followed by an additional 78 sham stimulations and no environmental enrichment (Sham-Sham, n = 2).

Kindling

All stimulation parameters were the same as those used in the previous experiments. After a post-surgical recovery period of at least 5 days, 40 male and 40 female rats first received 21 kindling stimulations. Another 10 male and 10 female rats

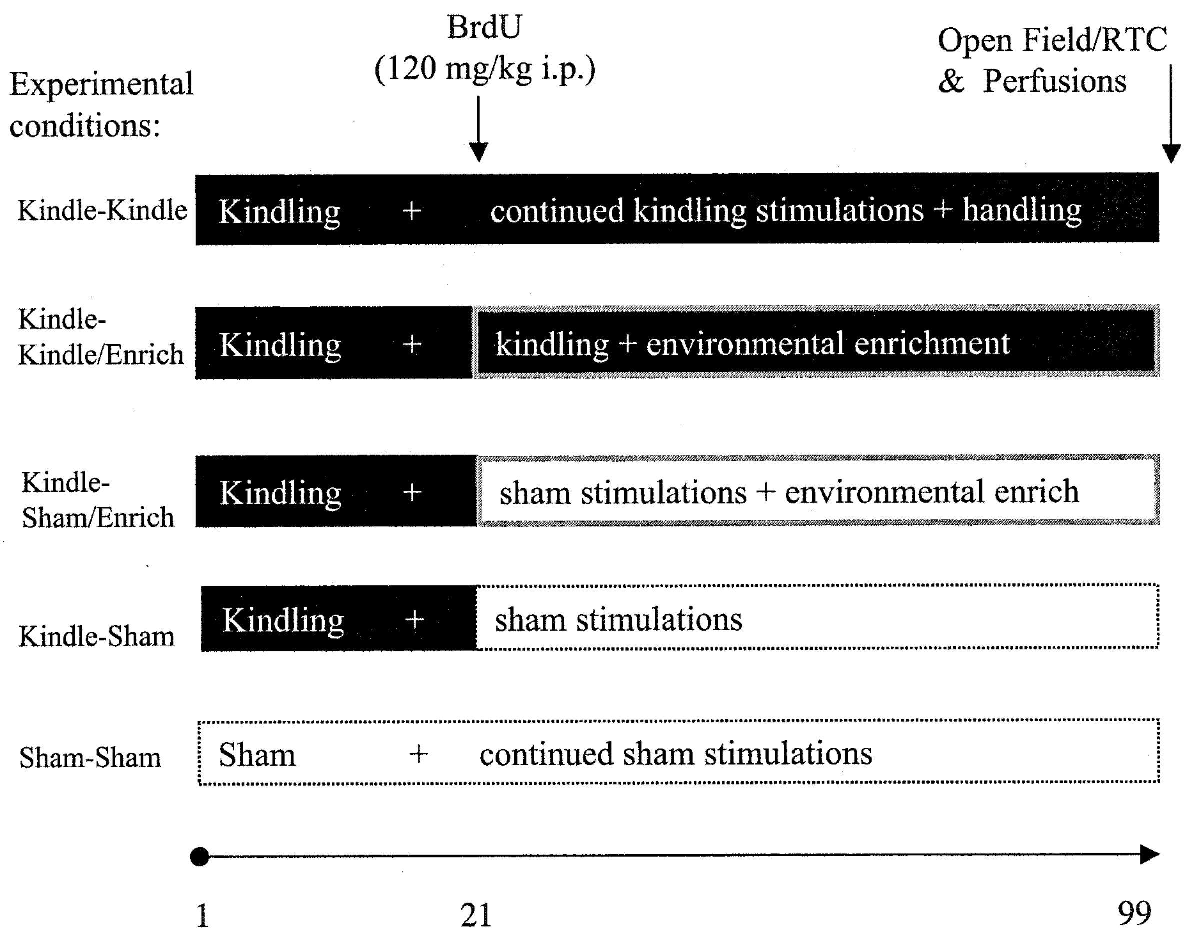


*Figure 4.1.*A schematic representation of the experimental groups and schedule. Arrow indicates a timeline for the kindling stimulations from the first stimulation to the final stimulation.

served as sham-stimulated controls. After the 21st kindling stimulation, the kindled rats were divided into one of four groups: One group of male and female rats received an additional 78 stimulations, for a total of 99 kindling stimulations (Kindle-Kindle males, n = 10; Kindle-Kindle females, n = 10), one group of male and female rats received an additional 78 stimulations plus enrichment as described below (Kindle-Kindle/Enrich males, n = 10; Kindle-Kindle/Enrich females, n = 10), one group of male and female rats received 78 sham stimulations and enrichment (Kindle-Sham/Enrich males, n = 10; Kindle-Sham/Enrich females, n = 10), and one group of male and female rats received 78 sham stimulations without enrichment (Kindle-Sham males, n = 10; Kindle-Sham females, n = 10). The sham-stimulated rats continued to receive sham stimulations following the initial 21 stimulations for a total of 99 sham stimulations and received no enrichment (Sham-Sham males, n = 10; Sham-Sham females, n = 10). These conditions are depicted in Figure 4.1. The measure of seizure severity was the convulsion class elicited by each kindling stimulation, scored as per the criteria used in the three previous experiments.

Enrichment Exposure

Exposure to the first enrichment session began 1 hour and 30 minutes after the 21st stimulation and continued until 99 kindling or sham stimulations had been delivered to all rats. Each enrichment session involved placing each rat into one of three different environments for 30-60 minutes, 6 days a week. On kindling days, the enrichment session followed the three kindling/sham stimulations by 30 min to 2 hr and 30 min. On non-kindling days, the enrichment occurred some time during the light cycle. The rats received a total of 31 enrichment sessions. The environments used for each session are described separately below. All of the environments had a range of objects, textures, sugared cereal treats (Fruit Loops), and climbing apparatus. These environments were static throughout the entire experiment and contained soiled wood shaving retrieved from the kindling bins used for the kindling stimulations. Some of the environments were visited multiple times and some of the environments were visited only once.

Large Complex Environments: Four different large complex environments were used (see Figure 4.2 for photographs and dimensions of each environment). The rats were exposed to the large complex environments a total of 20 times (5 times per environment). For the first four days of enrichment, the rats spent 1 hour in each environment and then revisited the environments four additional times (30 min each subsequent time) throughout the remainder of the experiment. Within each environment, sugar treats were strategically placed prior to each rat's visit to promote spatial learning and memory.

Small Enclosures: The small enclosures were made of wire mesh walls and top filled with soiled wood shavings from the kindling bins as was done with the large environments (see Figure 4.3 for photographs and dimensions). There were six enclosures that were identical in shape and size but the objects and spatial arrangement differed inside. The objects inside the enclosures varied among wooden blocks, paper balls, straws, foam pieces, ladders, tubing, milk containers, and toilet paper rolls. Each enclosure contained two sugar treats among the objects for each rat to find. Each rat visited each enclosure once for 30 min each.

Single Exposure Environments: The single exposure environments differed in layout, objects, size, and shape (see Figure 4.4 for photographs and dimensions). There

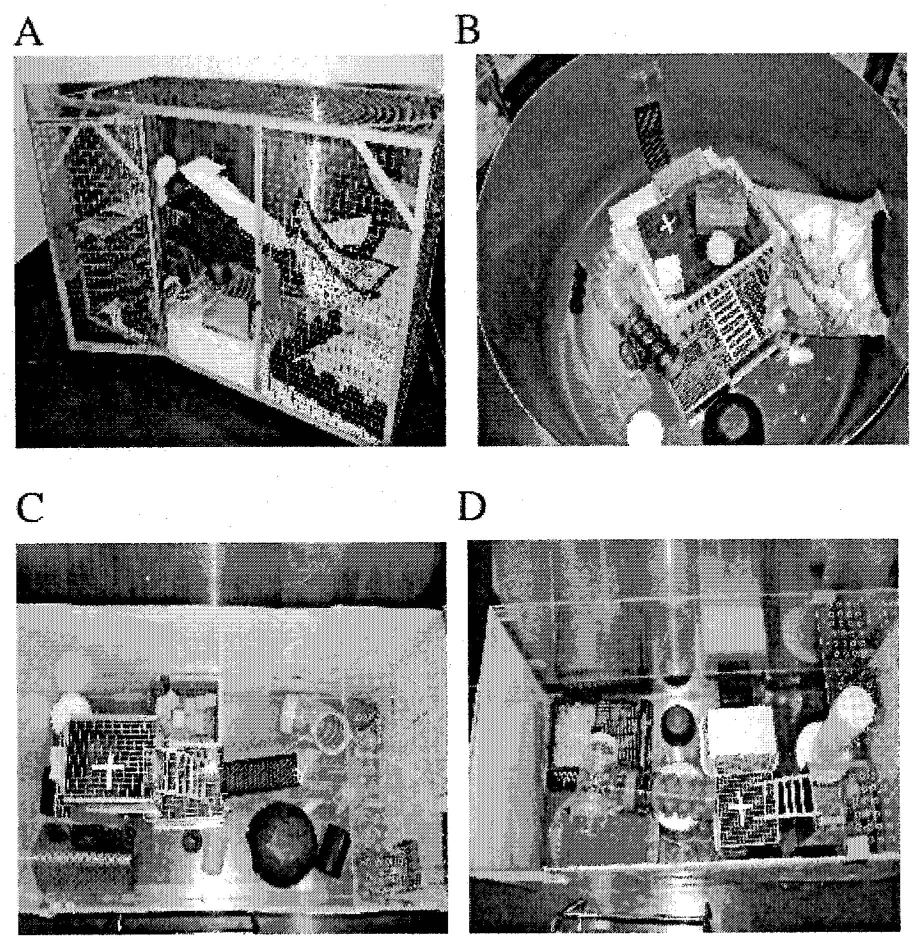


Figure 4.2. Photographs of the large complex environments. A) A former cat cage measuring 1.5 m (L) X 1 m (W) X 60 cm (H). B) A circular fiber glass enclosure measuring 1.5 m in diameter. C & D) Plexiglass enclosure covered with paper to make opaque measuring 1.2 m (L) X 65 cm (W) X 60 cm (H).

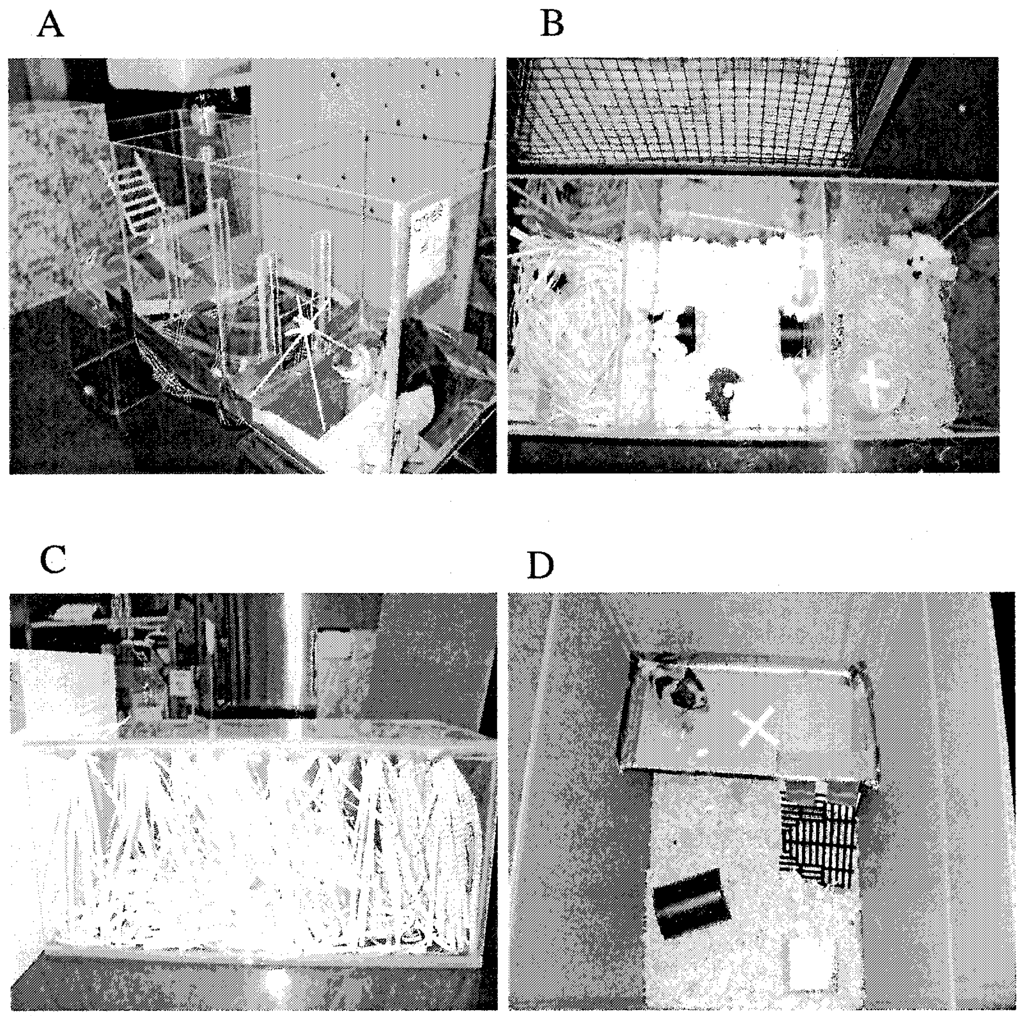
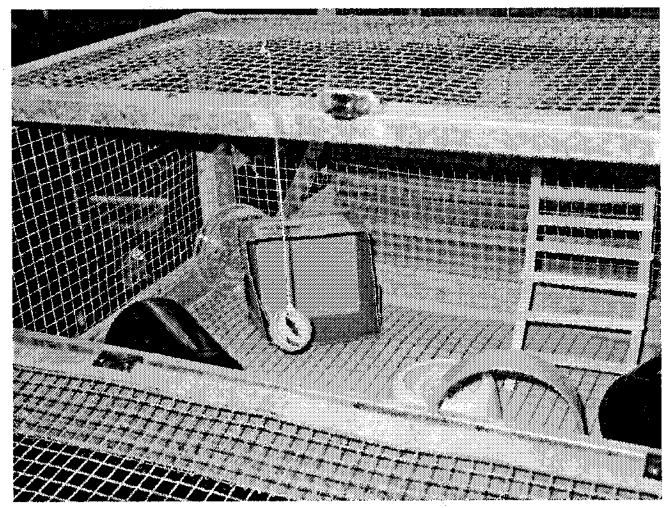


Figure 4.3. Photographs of the small enclosures. A& B) 1.05 m (L)X 40 cm (W) X 50 cm (H) with equal chambers. Each chamber contained a different flooring and different objects inside for exploration. C) 70 cm (L) X 40 cm (W) X 40 cm (H) with shreaded paper hanging from the top. All four sides were enclosed. D) Cardboard box measuring 60 cm (L) X 60 cm (W) X 90 cm (H) with a platform visible in the top portion of the photo and a ramp leading up to the platform.

A



B

Figure 4.4. Photographs of the wire mesh enclosure (A) and a few of the items used in the twice-weekly home cage objects (B).

were five environments in which the rats visited once only for 30 min each. These environments did not contain any sugar treats.

Open-Field and Resistance-to-Capture Tests

The open field and resistance-to-capture testing occurred in the same manner as described in the methods for Experiments 1 and 2. The testing was done on the day following the final kindling/sham stimulation and enrichment session.

BrdU Immunohistochemistry and Tissue Preparation

A single dose of BrdU (120 mg/kg, i.p. dissolved in 0.9% warm saline) was administered one hour following the 21st kindling or sham stimulation (i.e., the third stimulation on the 7th kindling day) to label cells born around that time. The rats were transcardially perfused 4 hours after the open field session (i.e., approximately 24 h after the last kindling stimulation). The rats were anaesthetized with a lethal dose of sodium pentobarbital (100 mg/kg, i.p.) and perfused. The brains were stored in 4% paraformaldehyde for 48 hours and then stored in PBS/azide. Approximately five days prior to sectioning, the brains were cryoprotected by immersion in a 30% sucrose solution (0.1M PB). The brains were then frozen and then sectioned at 40 μm through the entire hippocampus. The sections were divided into 12 series and stored in 0.1M PBS, which contained 0.05% sodium azide to prevent bacterial growth.

The procedure for BrdU determination of cell proliferation differed slightly from Experiment 3. The tissue was denatured (formamide and SSC steps) and blocked with normal horse serum in the same manner; however, the tissue was incubated in a lower concentration of primary antibody (mouse anti-BrdU, 1:1000, Roche Diagnostics) for 48 hr at 4°C. It was incubated in the same concentration of the secondary antibody (biotinylated horse, anti-mouse, 1:200, Vector Laboratories) overnight; however, it was incubated in a higher concentration of ABC (1:100, Vector Laboratories) for 3 hr. The same DAB procedures from Experiment 3 were used. The tissue was mounted, dried, counter stained, and cover-slipped as per the methods of Experiment 3.

Cell Counting & Sampling

BrdU-labelled cells were counted in brain sections –3.14 mm from bregma by an observer who was blind to the experimental condition of the animals using a Nikon E800 light microscope under 40X and 100X magnification. Pyknotic cells were identified based on examples depicted in Cameron & McKay (2001). These cells were not included in the count. The total number of BrdU-labelled (non-pyknotic) and total granule-like cells (non-pyknotic) cells were each counted separately in the left and right hemisphere of the following hippocampal regions: the inner granule cell layer (iGCL), the subgranule layer (subGCL: defined by cells that border the GCL and the hilus), the supragranule layer (supGCL: defined by cells that border the granule layer and the molecular layer), the polymorphic layer (plm), the molecular layer (mol), pyramidal cell layers (CA3, CA2/1), and the strata of the pyramidal cell layers (strata; see Figure 4.5 for a diagram and Figure 4.6 for examples from histological tissue).

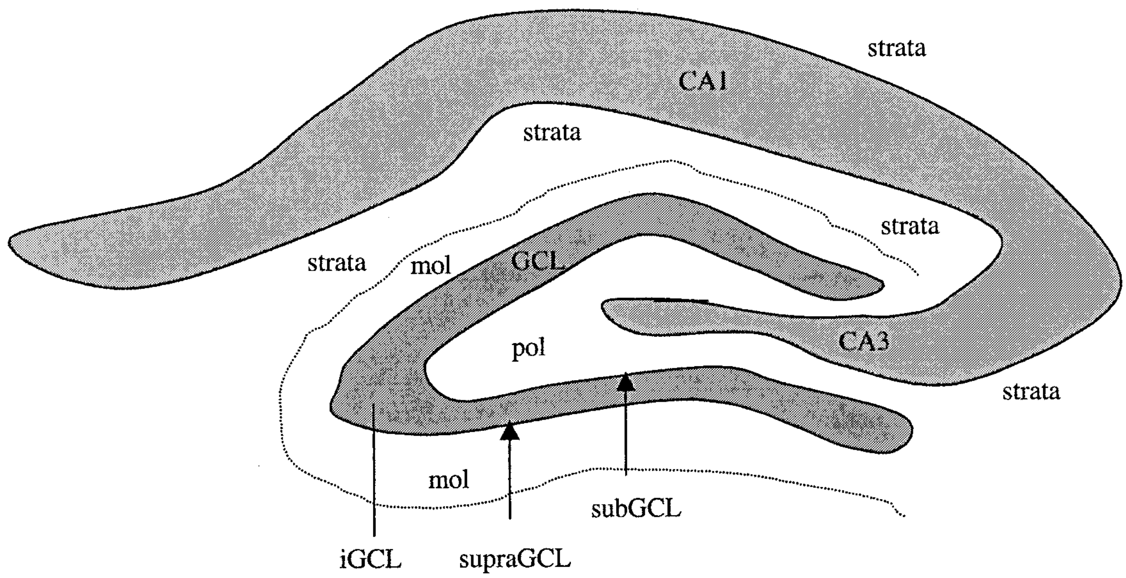


Figure 4.5. Diagram of the areas within the hippocampal formation where BrdU-labeled cells were counted. Abbreviations: GCL - granule cell layer, iGCL - inner GCL, mol - molecular layer of the dentate, pol - polymorphic layber of the dentate.

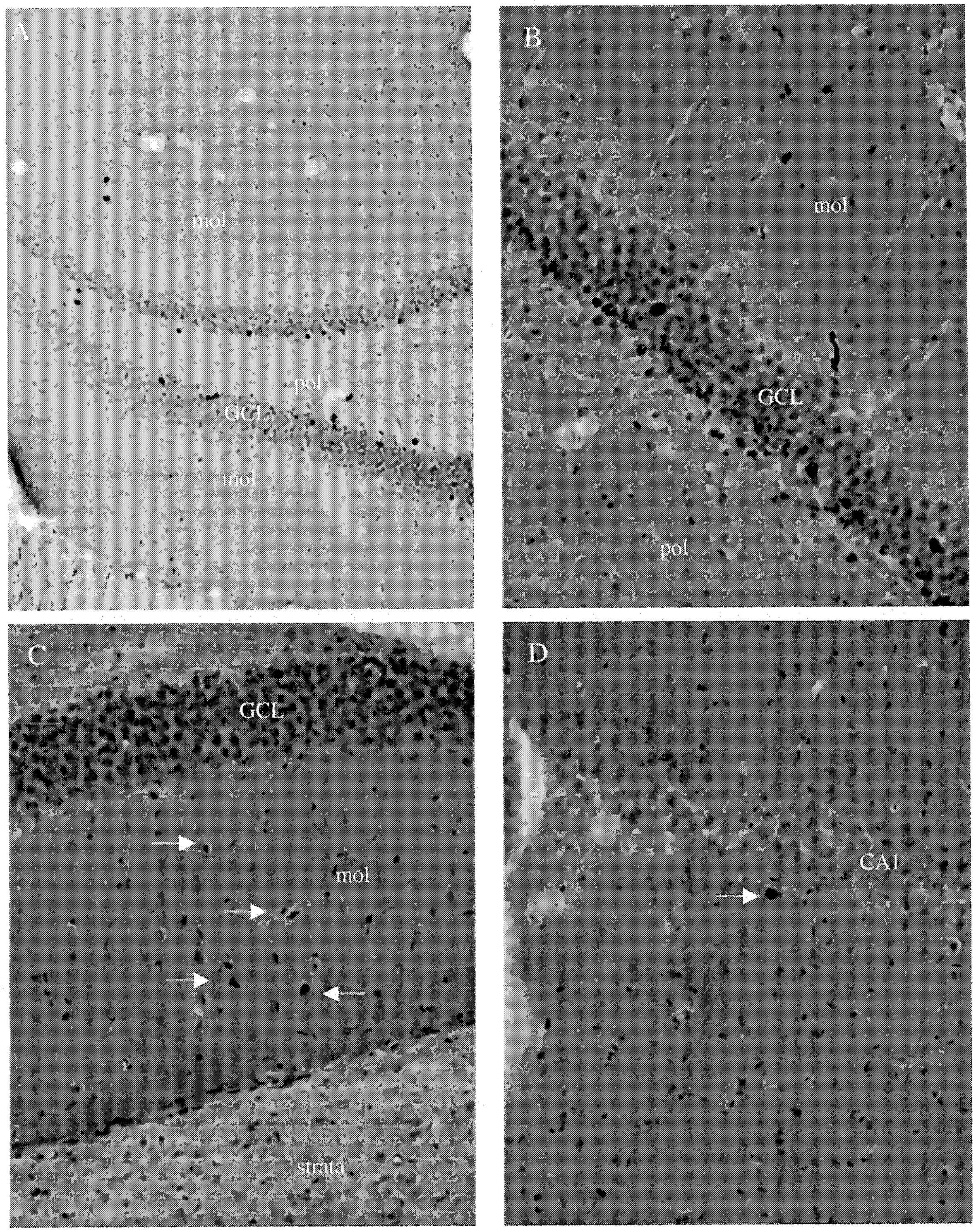


Figure 4.6. Photographs of BrdU-labeled cells. A) labeled cells throughout the granule cell layer at 10X magnification. B) labeled cells at 20X magnification. C) non-granule-like cells in the molecular layger of the DG (at 20X magnification). D) granule-like cells in the strata of CA3 (at 20X magnification). Abbreviations: GCL - granule cell layer, mol - molcular layger, pol - polymorphic layger. Arrows indicate BrdU-labelled cells outside the GCL.

Data Analysis

Simple effects ANOVA was used to assess the mean number of lines crossed per minute in the open field for each condition separately for males and females. Polynomial trend analysis was performed to examine the linear, cubic, and quadratic patterns for each male and female group of rats across the five minutes of the open-field session. Resistance-to-capture scores were compared across all five groups separately for males and females using the Kruskal-Wallis test and Mann-Whitney post hoc tests (alpha adjusted for the number of comparisons). Mann-Whitney tests were used to assess sex differences in resistance-to-capture scores within each condition. Separate one-way ANOVAs were used to assess the mean number of BrdU-labelled cells in each region of the hippocampus (collapsing across hemisphere), separately for males and females. Statistical significance was set at $p \leq .050$

Results

Histology

As in the previous experiments, rats that lost their electrode head apparatus at any point during the experiment or had electrode placements that terminated outside the amygdala were removed from the experiment. Therefore, the data analyses were based on a total of 48 male and 46 female rats: Kindle-Kindle (males, $n = 10$; females, $n = 9$); Kindle-Kindle/Enrich (males, $n = 9$; females, $n = 10$); Kindle-Sham/Enrich (males, $n = 10$; females, $n = 9$); Kindle-Sham (males, $n = 10$; females, $n = 9$); Sham-Sham (males = 9, females = 9).

Open Field Line Crosses and Resistance to Capture

Open Field. Figure 4.7 illustrates the mean number of lines crossed in each minute in the open-field session for the male (Figure 4.6a) and female (Figure 4.6b) rats in each group. The males and females displayed similar patterns per group; however, the females displayed an overall increase in mean number of line crosses. The Kindle-Kindled males and females displayed a clear difference across the 5 minutes compared to the Sham-Sham rats: The Kindle-Kindled rats exhibited few line crosses in the first minute and an increase in line crosses in the subsequent minutes whereas the Sham-Sham rats showed similar levels of line crosses across minutes. The Kindle-Sham and the Kindle-Sham/Enrich rats displayed similar activity to the Sham-Sham rats. In contrast, the Kindle-Kindle/Enrich rats appeared somewhat distinct from all the other groups with an overall increased mean number of line crosses across each of the five minutes.

Statistically, this pattern was assessed using simple effects repeated measures ANOVA separately for males and females of each group. The analysis revealed a statistically significant change across minute for the Kindle-Kindle males, $F(4, 336) = 26.16, p < .001$, the Kindle-Kindle females, $F(4,336) = 19.82, p < .001$, the Kindle-Kindle/Enrich males, $F(4,336) = 3.68, p = .006$, the Kindle-Kindle/Enrich females, $F(4,336) = 3.34, p = .011$, the Kindle-Sham/Enrich females, $F(4,336) = 4.73, p = .001$, and the Kindle-Sham males, $F(4,336) = 4.49, p = .002$. The Kindle-Sham/Enrich males, the Kindle-Sham females, and the Sham-Sham males, showed no statistically significant difference across minute, $F(4,336) = 1.56, p = .185$, $F(4,336) = 1.70$, and $p = .148$, $F(4,336) < 1.0$, respectively. The Sham-Sham females showed a nearly significant difference across minutes, $F(4,336) = 2.06, p = .086$.

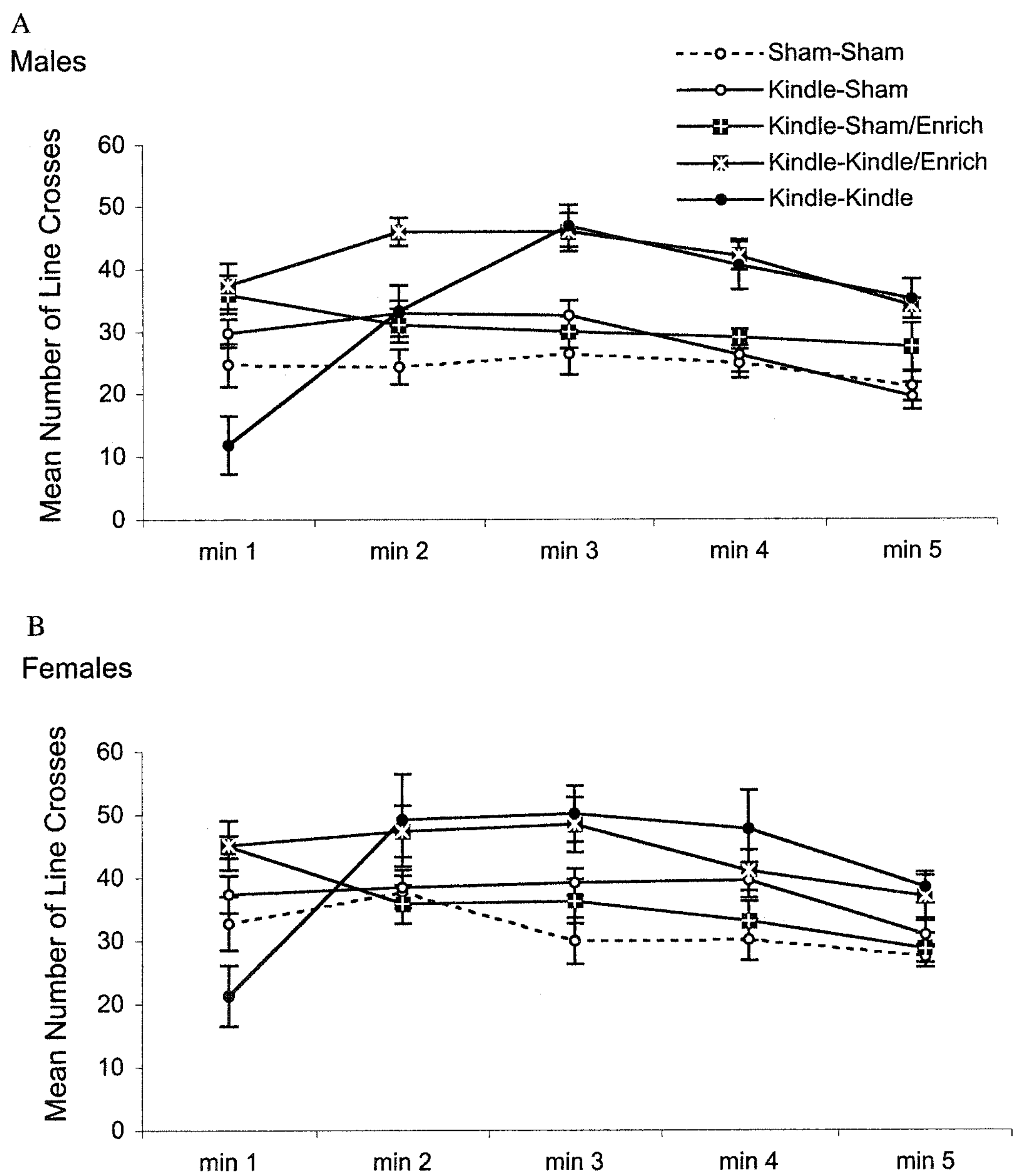


Figure 4.7. Mean (± S.E.M.) number of line crosses across each minute of the open field for males (A) and females (B) of each kindling/enrichment condition. See text for statistically significant differences. See text for statistically significant change across minute for each condition.

Polynomial trend analysis was done for the statistically significant effects listed above in order to examine the pattern of activity across the five minutes of the open-field session. This results for males showed that the Kindle-Sham and the Kindle-Kindle rats displayed a significant linear trend, $F(1,8) = 32.258, p < .000$, $F(1,8) = 8.872, p = .015$, respectively, and significant quadratic trend, $F(1,8) = 15.562, p = .003$, $F(1,8) = 32.525$, $p < .001$, respectively. In contrast, the Kindle-Kindle/Enrich rats showed a significant quadratic trend only, $F(1,8) = 15.734, p = .004$. The results for the female conditions indicated that the Kindle-Kindle rats displayed a significant linear, quadratic, and cubic trend, $F(1,8) = 5.854, p = .042$, $F(1,8) = 19.696, p = .002$, and $F(1,8) = 7.113, p = .028$, respectively. The Kindle-Sham/Enrich rats displayed a significant linear trend, $F(1,8) = 16.798, p = .003$. The Kindle-Kindle/Enrich rats displayed significant linear, $F(1,8) = 5.349, p = .046$, and quadratic trends, $F(1,8) = 5.351, p = .018$.

Resistance to Capture. The median and mean resistance-to-capture scores displayed by the rats in each group are presented in Figure 4.8. In general, enrichment reduced the resistance-to-capture scores. The difference in resistance to capture among the conditions was assessed with the Kruskal-Wallis nonparametric test for males and females separately. The results indicated that for both males and females there was a statistically significant effect of treatment condition on resistance-to-capture scores, $H(4) = 24.210, p < .001$ and $H(4) = 26.339, p < .001$, respectively.

Post hoc tests (Mann-Whitney, adjusted alpha = .05/9 comparisons = .006) for males revealed that the Kindle-Kindle rats had higher resistance-to-capture scores than all other male conditions, all $U(9)$'s $\geq 4.5, p \leq .006$. The Kindle-Kindle/Enrich males had statistically significantly higher resistance-to-capture scores than the Kindle-Sham/Enrich

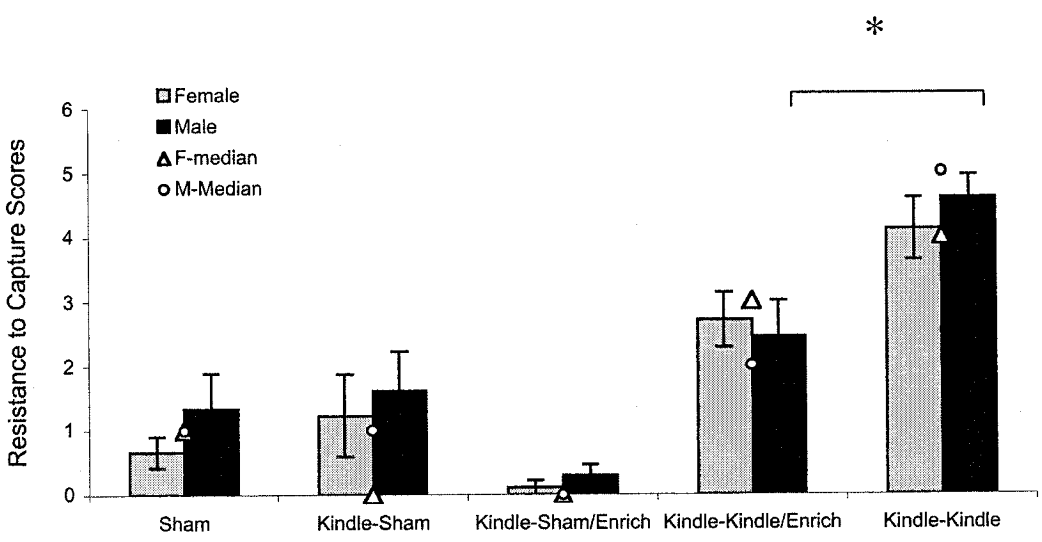


Figure 4.8. Mean (bars + S.E.M.) and median (points) open field resistance-to-capture scores for male and female rats across kindling conditions. * significant difference between the Kindle-Kindle males and the Kindle-Kindle/Enrich males. The difference did not reach statistical significance for the females. All other significant differences are listed in the text of the results section.

rats only, $U(9) = 9.5, p = .002$. The resistance-to-capture scores of the Kindle-Kindle/Enrich rats were not significantly different from the Kindle-Sham, $U(9) = 31.0, p = .278$, or the Sham-Sham rats, $U(9) = 24.0, p = .161$.

Post hoc tests for females revealed that the Kindle-Kindle rats had statistically significantly higher resistance-to-capture scores than the Kindle-Sham/Enrich, $U(9) = 0$, $p < .001$, the Kindle-Sham rats, $U(9) = 10.5, p = .006$, and Sham-Sham rats, $U(9) = 1.0$, $p < .001$, but they did not differ from the Kindle-Kindle/Enrich, $U(9) = 21.0, p = .053$. The Kindle-Kindle/Enrich females had statistically significantly higher resistance-to-capture scores than the Kindle-Sham/Enrich females, $U(9) = 5.5, p < .001$, and the Sham-Sham females, $U(9) = 10.5, p = .003$. The difference between the Kindle-Kindle/Enrich females and the Kindle-Sham females was not statistically significant, $U(9) = 24.0, p = .095$

Sex differences in resistance-to-capture scores were compared using the Mann-Whitney test in each group. There was no statistically significant sex difference in resistance-to-capture scores in any group, $U = 34.50, p = .571$ (Sham-Sham), $U = 37.00$, $p = .487$ (Kindle-Sham), $U = 36.50, p = .326$ (Kindle-Sham/Enrich), $U = 40.50, p = .707$ (Kindle-Kindle/Enrich), $U = 34.00, p = .347$ (Kindle-Kindle).

BrdU Immunohistochemistry

In general, there was no difference among the groups in the expression of BrdU-labelled cells (see Figures 4.9 – 4.14). A series of one-way ANOVA tests conducted separately for males and females confirmed this observation. First the total number of BrdU-labelled cells and the total number of granule-like BrdU-labelled cells

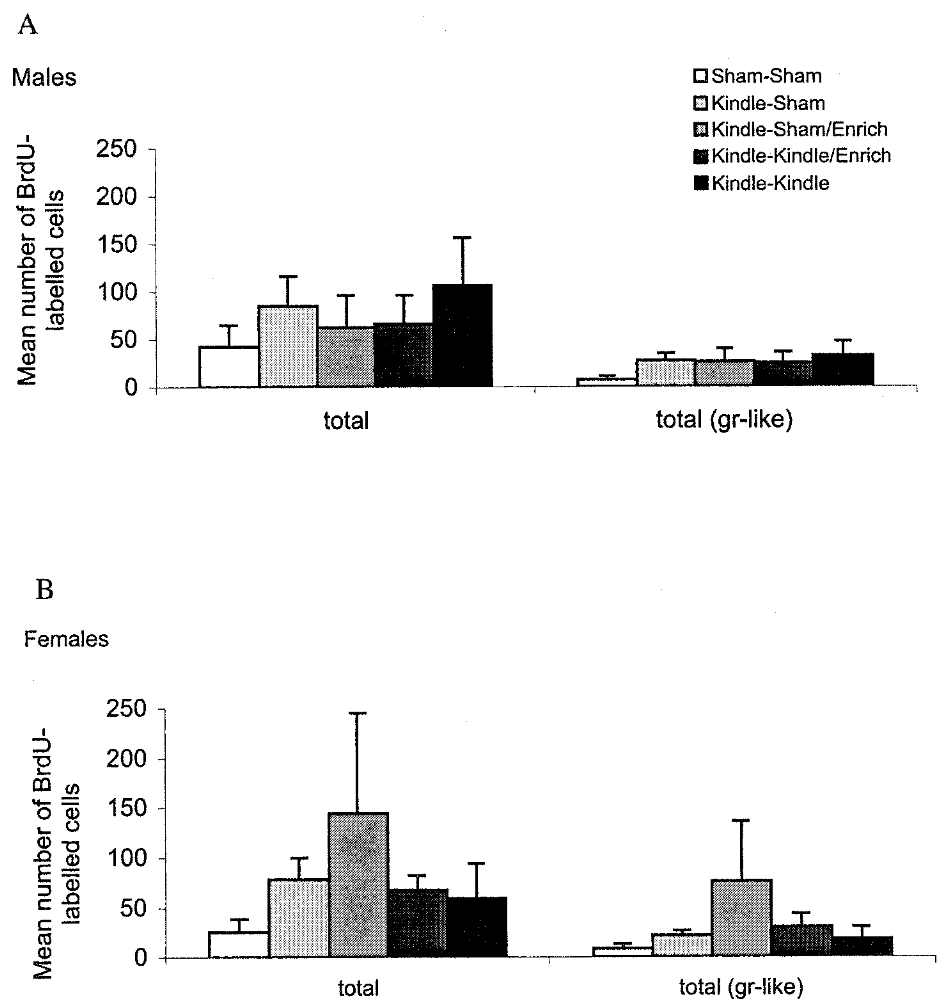
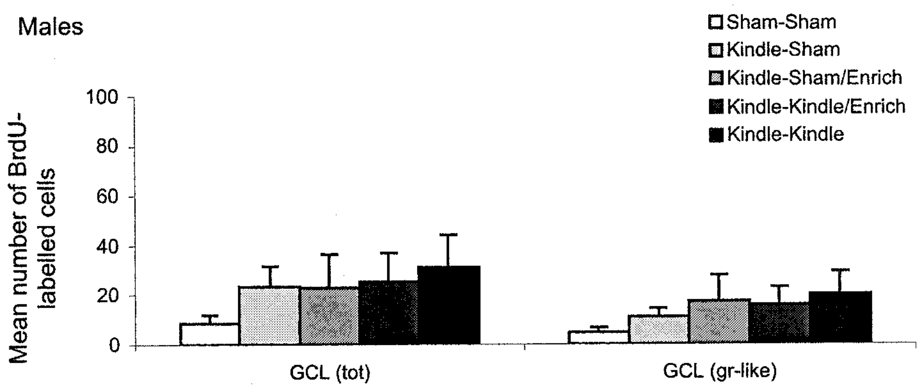


Figure 4.9. Mean (± SEM) number of total and granule-like (gr-like) BrdU-labelled cells for males (A) and females (B).

A



B

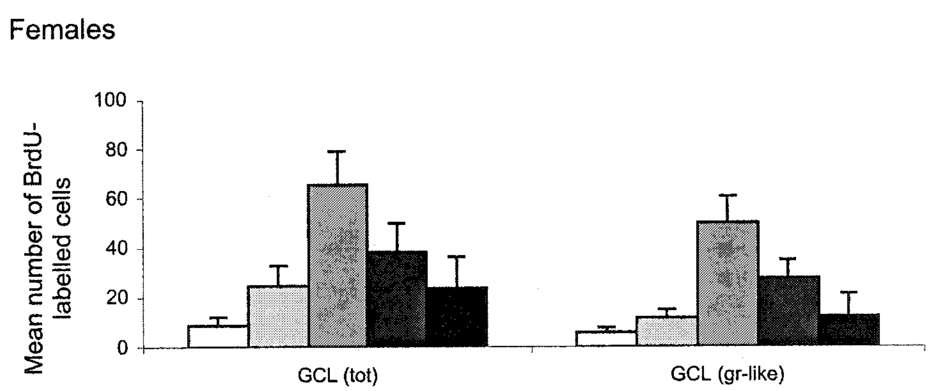
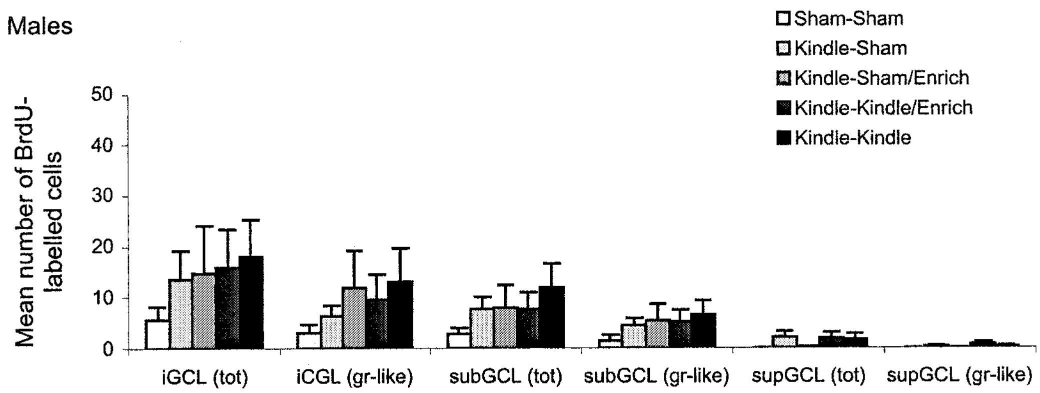


Figure 4.10. Mean (± SEM) number of total (tot) and granule-like (gr-like) BrdU-labelled cells in the total granule cell layer (CGL) for males (A) and females (B).

A



B

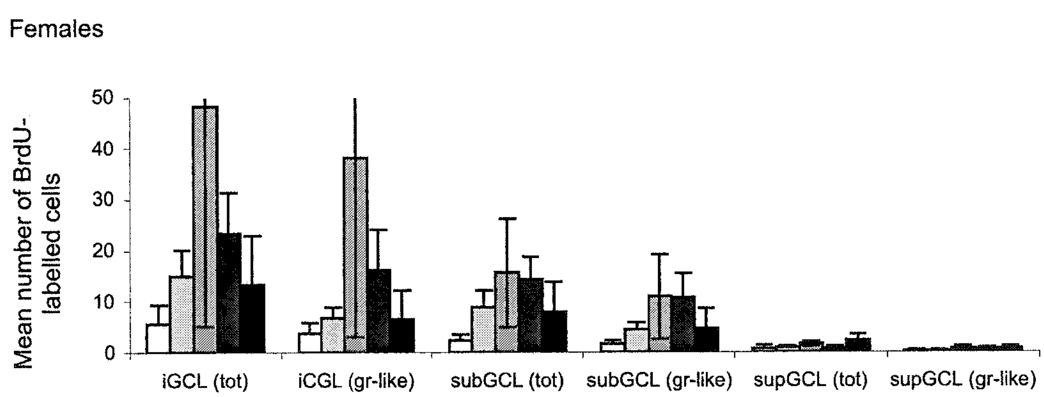


Figure 4.11. Mean (± SEM) number of total (tot) and granule-like (gr-like) cells in the inner granule cell layer (iGCL), the subGCL, and the supra GCL (supGCL) for males (A) and females (B). *NB* only minus S.E.M. is shown for the Kindle-Sham/Enrich females in order to maintain the same scale as the males.

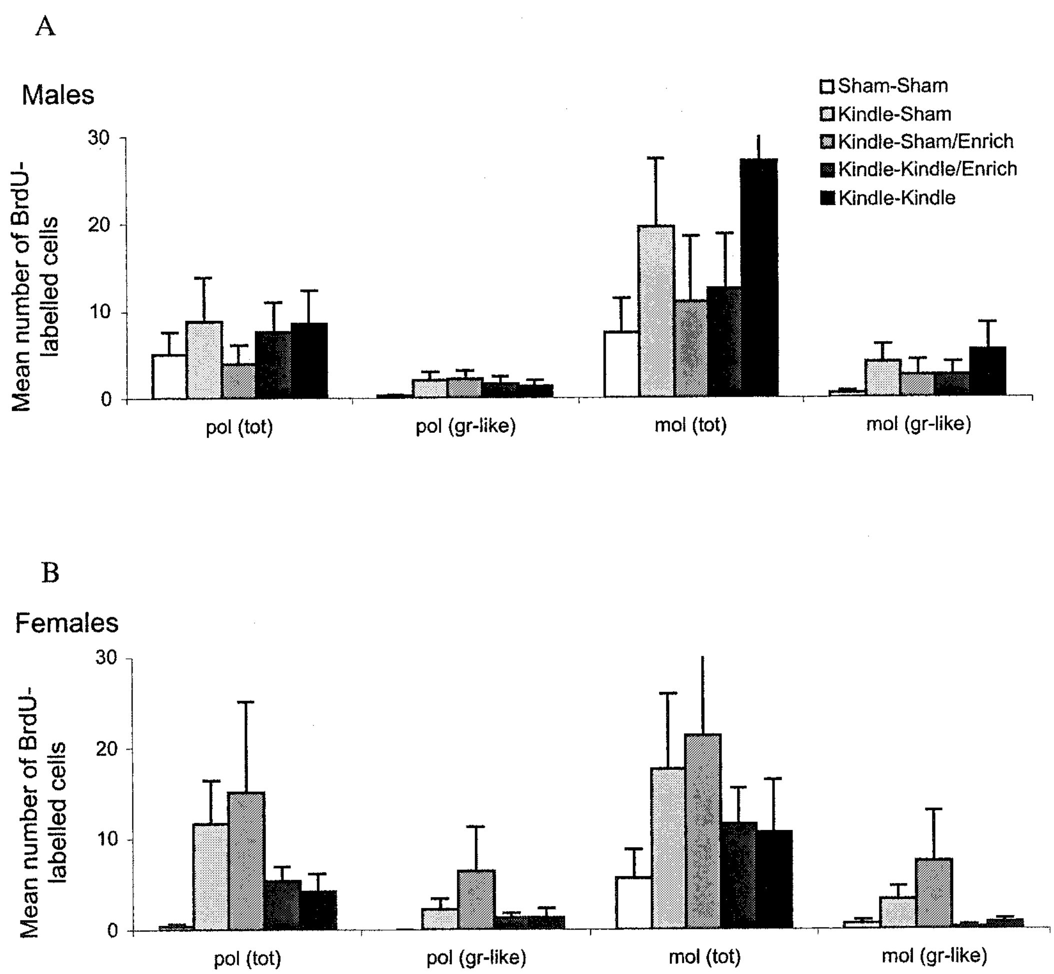
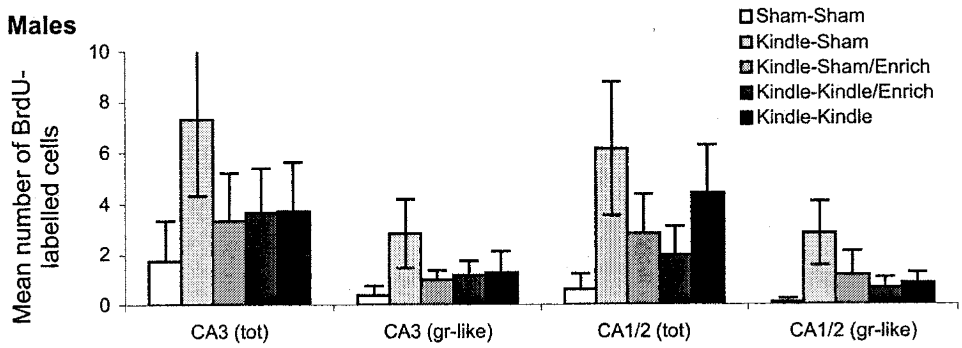


Figure 4.12. Mean (± SEM) number of total (tot) and granule-like (gr-like) cells in the polymorphic (pol) and molecular (mol) regions of the dentate gyrus for males (A) and females (B).

A



B

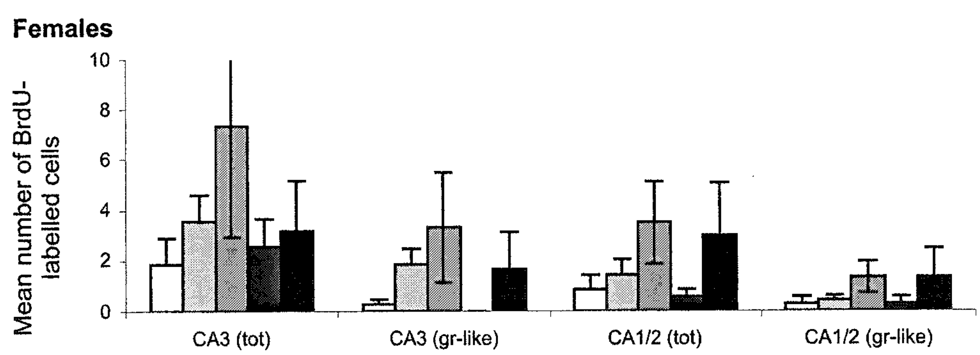
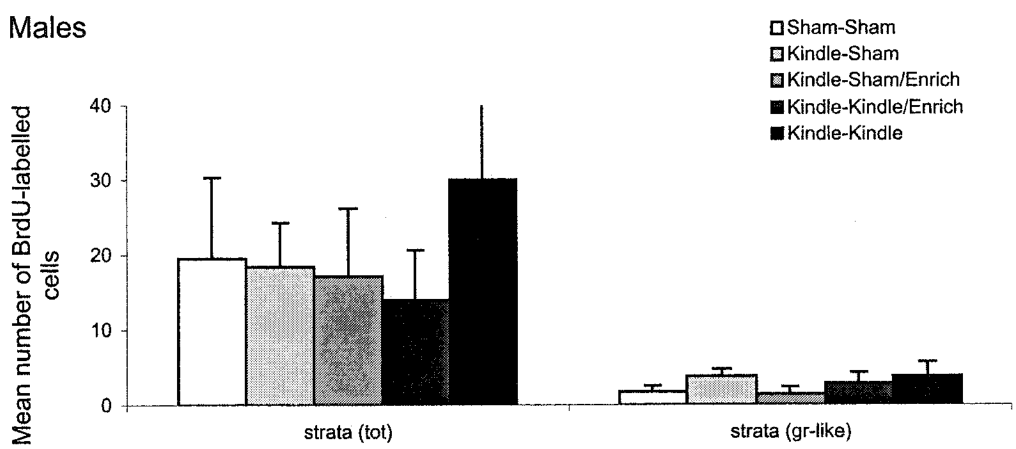


Figure 4.13. Mean (± SEM) number of total (tot) and granule-like (gr-like) cells in the CA3 and the CA1/2 region of the hippocampus for males (A) and females (B).

A



B

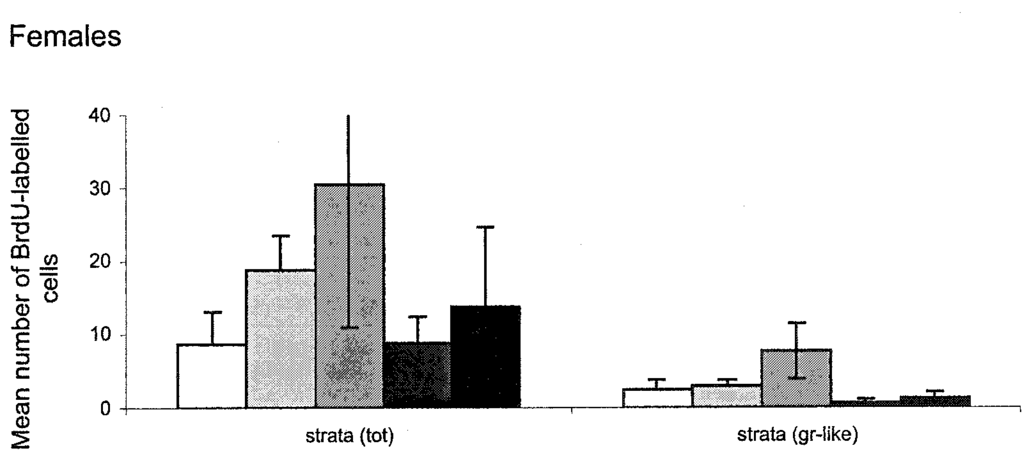


Figure 4.14. Mean (± SEM) number of total (tot) and granule-like (gr-like) cells in the strata of the CA region of the hippocampus for males (A) and females (B).

both showed no significant difference across the groups, all F's < 1.0, n.s (Figure 4.8). Second, the total number of BrdU-labelled cells in the granule cell layer of the dentate gyrus (DG) and the total number of granule cell-like BrdU-labelled cells in the DG were examined (Figure 4.9); however, no statistically significant differences emerged, all F's < 1.0, n.s. Third, the total number of BrdU-labelled cells and the total number of granule cell-like BrdU-labelled cells were analyzed for three areas of the granule cell layer: subgranule layer, supragranule layer, and granule cell layer proper (Figure 4.10). There were no statistically significant effects for any of these regions, $F(4,28) \leq 1.477, p \geq .236$, except for a statistically significant effect in the total cells of the supragranule layer, $F(4,28) = 2.719, p = .050$. Fourth, the total number of BrdU-labelled cells and the total number of granule-like BrdU-labelled cells were examined in the polymorphic and molecular regions of the DG (Figure 4.11); however, there was no statistically significant effect across treatment conditions, $F(4,28) \leq 1.564, p \geq .211$. Finally, the total number of BrdU-labelled cells and the total number of granule-like BrdU-labelled cells were examined in CA3 and CA1/2 (Figure 4.12) and in the strata of the CA regions (Figure 4.13). No statistically significant effect emerged, $F(4,28) = 2.094, p \leq .108$, except for a nearly significant effect for granule-like cells in the strata of the CA regions for females, $F(4,28) = 2.360, p = .078$. No post hoc tests were conducted.

These tests were repeated with the exclusion of the sham-sham condition in order to assess differences among the kindled groups irrespective of kindling-induced baseline differences at the time of the BrdU injection. These tests resulted in no statistically significant effects across treatment conditions for any hippocampal region, $F(3,21) \leq 1.995, p \geq .146$, except for a nearly significant difference in granule-like cells in the strata of the CA region for females, $F(4,28) = 2.842$, $p = .061$.No post hoc tests were conducted.

Discussion

The purpose of this experiment was to examine the effect of a hippocampal-dependent behavioural treatment on kindled fear and hippocampal cell proliferation. The results showed that exposure to enriched environments significantly reduced the magnitude of fear behaviour in kindled rats; however, this same treatment did not affect the survival and dispersal of proliferating hippocampal cells. The behavioural results support the hypothesis that exposure to hippocampal-dependent behavioural treatments during kindling can reduce the magnitude of kindled fear. In contrast, the number of surviving cells and the number of cells dispersed in particular hippocampal regions was not related to the development of kindled fear behaviour, at least with respect to the time of labelling and the time of assessment used in this experiment.

Open Field and Resistance to Capture

One purpose of this experiment was to examine whether challenging kindled rats with frequent exposure to stimuli that activate the hippocampus reduces the magnitude of kindled fear. This hypothesis arose from the results of Experiment 2, in which limited exposure to a hippocampal-dependent task significantly reduced fear behaviour in kindled females. In the present experiment, the rats had the opportunity to engage in more extensive hippocampal-dependent behaviours: They were exposed to novel and complex environments for 30-60 minutes six days a week and received novel objects twice weekly in their home cages. In Experiment 2, the rats were exposed to a hippocampal-dependent version of the water maze only once per week for a maximum of 1 hour and 6 minutes (including the 1-hour delay between trials). The treatment in the present experiment reduced the magnitude of fear in the kindled males and females, as measured by lower resistance-to-capture scores (particularly in the kindled males) and increased line crosses within the first minute of open-field exposure (both males and females). Although the enrichment paradigm used in this experiment did not lower kindled fear to the level shown by the sham-stimulated rats (i.e., Sham-Sham or Kindle-Sham) it did lower fear in comparison to the Kindle-Kindled rats by a significant amount. This confirms that amygdala-kindled rats can benefit from environmental enrichment by experiencing a reduced magnitude of kindled fear.

Hippocampal Cell Proliferation

The second purpose of this experiment was to examine whether the survival and dispersal of kindling-induced proliferating hippocampal cells was related to kindled-fear behaviour. Several different cell counts were taken, including the total number of BrdU-labelled cells and the total number of granule-like BrdU-labelled cells in several hippocampal regions, and yet no statistically significant effect emerged. A few effects nearly reached statistical significance; however, these effects were not robust and should be replicated before further consideration is justified. Accordingly, the results of this experiment indicate that neither the number of surviving cells nor the number of labelled cells dispersed in various hippocampal regions are related to kindled fear.

Environmental enrichment has previously been shown to increase the survival of proliferating hippocampal cells and promote neurogenesis (Brown et al., 2003; Nilsson, Perfilieva, Johansson, et al., 1999). Given that kindling was also hypothesized to increase the survival of proliferating cells, the location of surviving cells was also taken into account. There has been evidence from a few non-kindling seizure models that newly born cells aberrantly locate within the hippocampus (i.e., in the hilar and CA3 regions: Scharfman, Coogman, & Sollas, 2000). However, neither treatment (i.e., kindling or enrichment) appeared to increase the survival or dispersal of new cells in the present experiment.

The fact that there were no significant effects of kindling or enrichment on cell survival and dispersal is an important observation. For example, it is the only study that examined the effects of continued stimulations on cell proliferation and showed that kindling does not facilitate the survival of new cells born around the 21-stimulation point. However, it is of further interest to know whether this is a phenomenon that is stable throughout kindling or if there are times at which the survival rate is facilitated by kindling. Whether this is stable or not could have consequences for the development of behavioural co-morbidities or it may be related to mechanisms of epileptogenesis that are not immediately obvious when gross measures of cell number are taken.

Furthermore, although the results of this experiment were negative, they have not ruled out a range of alternative hypotheses concerning the role of hippocampal cell proliferation in kindled fear. For example, it is possible that the cells contributing to fear behaviour were not captured with a single BrdU injection after 21 stimulations. Also, the net number of cells surviving may be the same yet the types of cells that develop may be related to kindled fear. These ideas are discussed in more detail in the general discussion along with a discussion of the logistics of cell proliferation analysis.

Sex Differences

A few sex differences emerged particularly in terms of the pattern of open-field line crosses. However, these sex differences appeared to be due to non-specific effects that were not produced by the kindling or enrichment conditions. These sex differences were described in detail in Experiment 1 and are discussed again in the general discussion. There was also a sex difference in resistance-to-capture scores in terms of the magnitude of the fear reduction. The Kindle-Kindle/Enrich males were significantly less resistant to capture than the Kindle-Kindle males; however, there was no statistically significant difference between these two groups of females. This is true when the median values are compared; however, the mean values also depicted on in the figure (but not statistically assessed) display a similar pattern between all groups of males and females. Accordingly, it is difficult to say whether this effect represents a true sex difference or an effect that did not reach statistical significance.

If the sex difference is a reliable effect, then it is possible that the enrichment paradigm did not activate the hippocampus to the same degree in females as it did in males. This same suggestion as was made for the reverse effect observed in Experiment 2 in which the kindled males appeared to benefit less from the spatial task than the kindled females. One reason why this paradigm might have not affected the females as much may have to do with sex differences in navigation strategy. For example, females tend to be as proficient or more proficient than males when using proximal cues for navigation, i.e., swimming to a raised or probed platform in the Morris water maze (Astur, Tropp, Sava, et al., 2004; Berger-Sweeney, Arnold, Gabeau, & Mills, 1995). However, when the use of distal spatial cues or landmarks to configure the destination (i.e., cognitive mapping), females tend to perform worse (Gwinn, Fernando, James, & Wilson, 2002). The use of these strategies may have differed between Experiments 2 and 4, particularly for the females. The Morris water maze task in Experiment 2 required the use of distal cues or a cognitive map to navigate to the hidden platform with little potential for other navigational strategies, and as a result may have created a more challenging task for the females who normally do not adopt such strategy.

In comparison within the present experiment, the females may have naturally adopted a proximal-cue navigational strategy rather than the distal cue/cognitive mapping strategy, making the task less challenging for the females. The fact that the kindled males did show a benefit from the paradigm may have been because the design was appropriate for a more challenging and extensive hippocampal activation, involving more frequent and lengthier sessions than the ones used in Experiment 2.These speculations are interesting and testing them would likely be fruitful for our understanding of how hippocampal activation can reduce the magnitude of kindled fear. As was mentioned in the discussion of Experiment 2, a direct measure of the neural activation incurred as a result of the behavioural treatments would be necessary.

There were also a few effects of cell proliferation that appeared in the female rats; however, these results only nearly reached statistical significance and were not followed-up statistically. In general, the behavioural pattern between males and females was similar; however, in conjunction with the results of the previous three experiments, they do suggest that something different is occurring with males and females particularly with respect to those factors that reduce the magnitude of kindled fear. Given that there are so few data using female rats in kindling experiments, it is difficult to speculate further. These data promote the further use of both males and females during follow-up studies.

Environmental Enrichment

Environmental enrichment is generally considered to be beneficial for a laboratory animal. For example, environmental enrichment facilitates memory on spatial tasks (Dahlqvist, Ronnback, Bergstrom, et al., 2004; Frick & Fernandez, 2001) and helps reduce the cognitive decline associated with old age (Frick, Stearns, Pan, & Berger-Sweeney, 2003). These behavioural benefits are thought to occur because a period of enrichment stimulates changes within the hippocampus and cortex that promotes learning, for example by increasing neurogenesis through promoting cell survival (Brown, et al., 2003), increasing NGF (Torasdotter, Metsis, Henriksson, et al, 1996), and increasing cortical dendritic arborization (Turner, Lewis, & King, 2003).

In the present experiment, the enrichment paradigm deviated slightly from convention in a manner that attempted to capitalize upon hippocampal activation. This was done in order to approximate the paradigm used in Experiment 2, in which exposure to a hippocampal-dependent task reduced the magnitude of fear in kindled females. Accordingly, in the present experiment, each individual rat was presented with opportunities to navigate through the environments to find a dry-cereal sugared treat. Each of the four large complex environments contained one food cache that was identical among the environments and was baited with food for each exposure and also contained a second food cache that was environment specific and always baited with food prior to each exposure. In addition, each of the small environments contained two hidden sugared treats. The small environments were identical in size and outer structure; however, the inner objects were distinct from one environment to another. In these environments, the rats could navigate through each small environment with the certainty that two sugared treats were to be found somewhere.

The rats also received novel objects in their home cages twice per week. The objects consisted of novel materials such as tin foil, pipe cleaners, popsicle sticks, paper balls, toilet paper roles, wood blocks for climbing and chewing, pipe tubing for sleeping and hiding, and various paper or similarly packaged sugared treat for the rats to discover. In general, the rats in this experiment were given the opportunity to informally learn their environments and to explore novel objects and their surroundings. However, none of the environments contained any structured opportunity for exercise other than what the rats would normally engage in while navigating through the environment. Voluntary exercise (i.e., free access to running wheels) has been previously reported as contributing to the neurogenesis seen after environmental enrichment (van Praag, Kempermann & Gage, 1999). Currently, there is evidence to suggest that both voluntary exercise and environmental enrichment each contribute to increased neurogenesis (Brown et al., 2003).

As a result of this paradigm, the magnitude of kindled fear was reduced; however, it was not reduced to the level of sham-stimulated control rats. The enrichment paradigm used was thought to be a more potent activator than the water maze exposure used in Experiment 2. Although the magnitude of fear was reduced substantially, especially for kindled males, it is possible that it could still be further reduced with a refined protocol and more extensive enrichment exposure. Furthermore, housing the animals in full conventional enrichment environments was not done in the present experiment because most of those paradigms include housing animals in groups. Housing animals together with other kindled or non-kindled animals present potential confounds with respect to social interaction among cage-mates. Such effects, although highly interesting, could complicate the understanding of the role of the hippocampus in kindled fear during these initial studies. The results thus far, suggest that more comprehensive methods to activate the hippocampus should be investigated.

Conclusion

The results of this experiment showed that exposure to environmental enrichment during the course of kindling reduces the magnitude of fear behaviour in amygdala-kindled male and female rats, although the effects were more pronounced in males. Hippocampal cell proliferation, as measured here, did not appear to be related to kindled fear; however, these behavioural results are an important step toward a cognitive treatment of kindling-induced fear and the anxiety associated with epileptogenesis.

Chapter 6: General Discussion

The purpose of this thesis was to investigate sex differences in the behavioural consequences of amygdala kindling and to evaluate the hypothesis that kindling-induced fear is mediated by neural changes within hippocampal circuits. The results of these experiments have provided some important new information about kindling-induced fear – information that might also be important for the development, expression, and treatment of pathological anxiety in general. These results are as follows. First, the results from Experiment 1 showed for the first time that fear behaviour does develop in female amygdala-kindled rats. Second, the results from Experiment 2 demonstrated that amygdala-kindled male rats are impaired on a hippocampal-dependent task beginning around the time when kindled fear becomes evident. Third, Experiment 3 showed that hippocampal cell proliferation is elevated at a time that could contribute to the development of kindled fear; however, Experiment 4 showed that the survival and dispersal of hippocampal cell proliferation around this same time does not appear to be related to the magnitude of kindled fear. Finally, Experiment 2 and 4 showed that exposure to hippocampal-dependent behavioural treatments can reduce the magnitude of kindled fear. These results emphasize the relationship between hippocampal-dependent behaviour and amygdala-kindled fear. They also identify the potential for behavioural and cognitive therapies as possible treatment strategies for anxiety-like disorders.

Kindled Fear

Amygdala kindling produces highly fearful behaviour in rats (Kalynchuk, 2000). This has been well documented in male amygdala-kindled rats. Experiment 1 of this thesis confirmed that kindling also increases fearful behaviour in female rats, as measured in the open-field test, the resistance-to-capture test, and the forced-swim test. The behavioural observations from each of these tests are discussed below.

Open-field. Open-field presents itself as reliable indicator of fear behaviour in kindled rats. This was originally seen as low exploratory activity in kindled rats during the first 30s of an open-field session, representing freezing behaviour, followed by increased exploratory activity over the entire five-minute session, representing hyperactivity (Kalynchuk et al., 2001). The results of this thesis provide much more detail about open-field activity in kindled rats. In the three behavioural experiments described here, open-field activity was examined during each minute of the session. The analysis revealed an intriguing pattern of exploratory activity, in that fully kindled rats were less active during the first minute in the open field with a dramatic increase in activity during the second minute that persisted through minutes three, four, and five. This pattern of activity was distinct from the sham-stimulated rats that remained fairly consistent across the 5-min session. This activity difference between kindled and sham-stimulated rats has become very interesting particularly because it has been replicated in several other experiments in addition to the three experiments presented here (unpublished laboratory observations). This activity probably further exemplifies the inability of amygdala-kindled rats to familiarize themselves with their environment, resulting in hyperactivity (i.e., increased line crosses) and panic-like mannerisms (i.e., jumping behavior and high resistance-to-capture scores; Wintink, personal communication). These results warrant more specific investigations of hippocampal-mediated exploratory behaviour in an open field, such as the spatial acquisition of a homebase and exploratory trajectories (Golani et al., 1993; Eilam & Golani, 1989; Tchernichowski & Golani, 1995). Interestingly, rats with hippocampal lesions also show hyperactivity in an open field (Whishaw, Cassel, Majchrzak, et al., 1994).

Interestingly, open-field activity during the first minute of the open-field session seemed to correspond quite closely with the resistance-to-capture scores at the end of the open-field session. For example, the manipulations that reduced resistance-to-capture levels also reduced freezing during minute 1 of the open field. This was evident in the group of rats that received 30 stimulations from Experiment 1 (i.e., the 30-stim condition), the kindled female rats exposed to the water maze during kindling from Experiment 2 (i.e., the kindle-WM condition), and all the conditions in Experiment 4 except the non-enriched fully kindled rats (i.e., the Kindle-Kindle). Thus, activity during the first minute of the open-field session is probably a reliable indicator of fearful behaviour, as is the pattern over the entire 5-minute session.

Resistance-to-capture. As mentioned, the resistance-to-capture test is a very sensitive test for measuring the magnitude of fearfulness in kindled rats. High levels of resistance to capture in kindled rats include attempting to bite the hand of the experimenter and launching jump attacks at the hand, whereas moderate levels include running from the experimenter's hand. The experiments of this thesis with males and females support previous work in males showing that kindled fear intensifies with the number of stimulations, as measured by resistance-to-capture behaviour. Interestingly, some of the experimental manipulations showed that these high levels of resistance to capture can be reduced under certain circumstances. For example, the group of kindled females and, to a lesser extent, males that were exposed to the water maze had reduced resistance-to-capture levels compared to the group of kindled rats that were not exposed to the water maze (Experiment 2). A similar reduced effect was observed in both males and, to a lesser extent, females that received enrichment during kindling (Experiment 4). These sex differences are described below.

Forced-swim test. The forced-swim test was only used in Experiment 1. This test provided additional corroborative data that kindled rats experience high levels of fear in novel situations. In this test, kindled rats displayed a longer latency for immobility and less time spent immobile. If this behaviour is indicative of panic-like behaviour, as was speculated in Experiment 1, then the mechanism by which the kindled rats respond to this stressor may have serious implications for a mechanism of kindled fear. For example, the kindled rats may be responding to dysregulated activity of the hypothalamic-pituitary-adrenal (HPA) axis. Under stressful conditions, the HPA axis is normally activated in a positive-feedback manner and the hippocampus is responsible for shutting off the response through negative feedback (see Brown, Rush, & McEwen, 1999; Jacobson & Sapolsky, 1991). Should the hippocampus be disrupted, HPA axis activity could persist in a hyperactive manner in response to stressful stimuli like environmental novelty or the forced swimming. For example, without the hippocampal negative feedback the kindled rats would react with a greater and greater stress response. The kindled rats would not be able to habituate to their environment and would consequently arrive at a panic-like state. This could explain activity in the open field and the hyperactivity in the forced-swim test.

Elucidating the nature of kindled rats' stress response, for example, by examining the peak and recovery of stress hormone elevation following these behavioural tests may be an important step for understanding how kindled fear manifests itself physiologically. In any case, the behaviour observed in the forced-swim test has provided an additional dimension of kindled fear and may help in considering the role of the hippocampus in mediated kindled fear.

Sex differences: There were some interesting sex differences that emerged in the experiments of this thesis, particularly when kindled rats were challenged with hippocampal-dependent tasks. In Experiment 1, no sex differences in resistance-to-capture levels were observed in any condition. Both groups of kindled rats (i.e., 99-stim and 30-stim) and the sham-stimulated rats had similar levels of resistance to capture between males and females. The sex differences that were observed were predominantly in activity within the open field. For example, females (kindled or sham) were generally more active: They displayed more line crosses and more wall jumping. However, when kindled females were exposed to the hippocampal-dependent water maze task in Experiment 2, their resistance-to-capture scores were lowered. A similar effect was found in Experiment 4 when kindled males were enriched. Furthermore, in Experiment 2, the impaired spatial memory of the males approached the performance of the females.

These sex differences are very intriguing, especially given that they surfaced under some conditions and not others. However, these sex differences are difficult to fully interpret at this time given that a very limited amount of data concerning female kindled rats exists. In the kindling field, the vast majority of research is conducted using male rats. For example, Experiment 1 is the first to consider and compare the behavioural consequences of kindling in both male and female rats. In contrast, many previous experiments have compared male and female rats' behaviour on hippocampal-mediated tasks. Consequently, we know that females typically do not perform as well as males on tasks that require the use of distal spatial (configuration) cues to navigate to a location (Daniel, & Lee, 2004; Roof & Stein, 1999). This difference in performance has been attributed to sex hormones (estrogen and testosterone) by specifically affecting the hippocampus (Luine, 1994; McEwen, 1996). Others have argued that male brains are more lateralized and that the lateralization contributes to their better performance (Vogel, Bowers, & Vogel, 2003). The difficulty is that these and many other studies imply that the hippocampus of males and females are differentially organized. This idea creates the possibility that manipulations that target the hippocampus, such as those used in the present experiments, may have different effects on males and females because of different neural starting points. Accordingly, the idea was raised in Experiment 2 that the intensity of the hippocampal activation might be different for males and females.

The possibility that the intensity of hippocampal activation might be different for males and females is not easily measurable. What is needed is a way in which the hippocampal activation can be measured according to reception rather than delivery. To start, one possibility might be to assess the response of both male and female kindled rats using immediate early genes. For example, the response of kindled and sham males and females to novel objects or novel environments such as those used in Experiment 4 could be assessed by examining FOS protein, which was described earlier as a marker of neuronal activity (Dragunow, Peterson, & Robertson, 1987). If a certain task more intensely activates the hippocampus between males and females then an increase in FOS protein immunoreactivity would also be seen. A second possibility would be to examine hippocampal activation electrophysiologically. Hippocampal excitatory post-synaptic potentials or evoked potentials could also be measured in response to novel objects and novel environments. Assessing hippocampal activity could also be done during the process of neural spatial map representation (i.e., place cell formation as described in by O'Keefe & Nadel, 1978; O'Keefe, 1999). These measurements of hippocampal activation would also necessarily provide information regarding hippocampal functioning.

Unfortunately, the data from which many theories are built are often derived from male data and thus, not necessarily analogous to the behaviour of females. This is one reason why both males and females were included in this thesis. Another reason was that the majority of clinical anxiety diagnoses are in women. Therefore, as a potential model for anxiety, the kindled fear model should incorporate females. There were several interesting sex differences that emerged that can now help refine this model for use in both sexes, particularly as the role of the hippocampus becomes more obvious.

Rate of Kindling

The manipulations used in the experiments of this thesis had some interesting effects on the rate of amygdala kindling. That is, the rate of kindling was delayed in two conditions: The 99-stim males were slower to kindle than the 30-stim males (Experiment 1 & 3) and the water-maze exposed rats were slower to kindle than those not exposed to the water maze (Experiment 2). The first effect was seen in two experiments suggesting that the effect is reliable. However, this effect was only statistically evident in the kindled males. There was a trend for the kindled females to also show this effect, but it failed to reach statistical significance twice. It does appear that the lack of difference was a delay in the 30-stim females compared to the 30-stim males; however, this was not statistically supported either. In the discussion of Experiment 1, the argument was made that the 69 sham stimulations prior to the 30 kindling stimulations in the 30-stim rats made the kindling box very familiar to those rats prior to the first kindling stimulation. As a result, the kindling box may have been less stressful for the 30-stim rats than it was for the 99-stim rats, which may have facilitated the development of kindling specifically in the 30-stim rats. This result is now more intriguing given that it occurred to the same degree in two separate experiments.

The reason why this was observed in males and not females is not known. One possibility is that familiarity with the kindling box might have been more important in facilitating kindling for males than for females. Although speculative, the possibility that contextual information is more important for male kindling behaviour compared to female kindling behaviour may be mediated by the same mechanism by which the kindled males' spatial memory is more impaired than that of the kindled females.

In Experiment 2, the kindled rats exposed to the water maze kindled slower than the rats not exposed to the water maze. Originally, it was thought that this might occur because the learning paradigm recruited new cells into beneficial locations (i.e., the granule cell layer) rather than aberrant locations for epileptogenesis (i.e., cells in the hilus and molecular layers). However, given that the dispersal of cells was not affected by kindling or environmental enrichment paradigms, this hypothesis has been abandoned. In fact, the relationship between kindling rates and enrichment is interesting given that in two previous experiments, environmental enrichment has been shown to both delay

(Auvergne, Lere, El Bahh, et al., 2002) and facilitate (Young, Wintink, & Kalynchuk, 2004) kindling. It appears that different environmental manipulations can have opposite effects in a manner that is not well understood. In any case, the rate of kindling, as mentioned in Experiment 1, has been previously dissociated from the expression of kindling-induced fear (Adamec, 1990b). However, at this point it would be too speculative to consider the issue any further, as curious as both these results are.

The Role of the Hippocampus in Kindled Fear

Hippocampal Dysfunction

One of the major questions of this thesis was whether the hippocampus is functioning properly in rats subjected to long-term kindling. Collectively, several prior studies suggested that the hippocampus is altered in some manner that was related to kindled fear. For example, receptor-binding levels in the hippocampus (i.e., $5HT_{1A}$, $BZ/GABA_A$, NMDA, AMPA) were correlated with resistance-to-capture scores in kindled males (Kalynchuk, Pearson, et al, 1999; McEachern et al., 1996). In addition, as mentioned above, animals with hippocampal lesions seem to behave in a similar manner to amygdala-kindled rats in the open field with greater hyperactivity and less habituation (Bannerman et al., 2001; Coutureau et al., 2000). Furthermore, the comprehensive kindling literature also suggests that the hippocampus is the structure most affected by the development of kindling.

From a behavioural perspective, kindled fear seems rooted in hippocampal-mediated behaviour. For example, kindled fear appears to manifest itself specifically in novel environments (Kalynchuk et al. 1998). As described earlier, the hippocampus is known for its role in processing contextual and spatial information specifically related to novel environments (for e.g., Jenkins, Amin, Pearce et al., 2004; Kemp & Manahan-Vaughan, 2004; Frank, Stanley, & Brown, 2004). It was speculated that kindled fear resulted from a sensitivity to environmental novelty. To begin to test this, amygdala-kindled rats were tested on a hippocampal-dependent version of the Morris water maze spatial task at the end of each week of kindling. Under these circumstances, male rats were shown to be impaired on the task. The results of Experiment 2 suggest that the ability to spatially re-configure landmarks for navigational purposes was impaired. Along with the results of Cammisuli et al. (1997) in which the acquisition of novel spatial information was impaired in male rats subjected to 300 stimulations, amygdala-kindled rats appear to have some sort of spatial dysfunction that emerges after a large number of stimulations. Consequently, hippocampal dysfunction is suspected.

Hippocampal Cell Proliferation as a Mechanism of Kindled Fear

Amygdala kindling stimulates an increase in hippocampal cell proliferation (Parent et al., 1998; Scott et al., 1998). This phenomenon was investigated as a potential mechanism of kindled fear because it was hypothesized that the new cells might be surviving and aberrantly dispersing within the hippocampus, possibly to impose secondary inhibition. Experiment 3 of this thesis revealed that cell proliferation was only increased after 30 stimulations and returned to baseline levels after 99 stimulations. This suggested that cell proliferation could contribute to the development of kindled fear, as the time course in which cells born after 30 stimulations could differentiate into neurons and migrate to form pathological connections with other hippocampal cells matched the time course of subjecting rats to 99 kindling stimulations. However, the results of

Experiment 4 revealed that the survival and dispersal of cells did not depend on kindling or on the kindling-plus-enrichment treatment, and consequently did not correspond to the level of fear behaviour observed in this experiment. Although negative, these results do not eliminate the possibility that cell proliferation is still involved in the development of kindled fear.

Examining hippocampal cell proliferation with the purpose of determining a mechanistic link is particularly difficult. One major problem concerns the timing of the cell proliferation sample. Cell proliferation is now known to occur continuously in the adult mammalian brain. Consequently, cell sampling requires the knowledge of when a particular phenomenon develops in order to time lock cell proliferation to the particular phenomenon. These efforts have been relatively fruitful in the learning and memory field. For example, the marker of cell proliferation (i.e., BrdU) can be administered prior to a learning task and the learning task promotes the survival of new cells and destines them to a neuronal fate (Shors et al., 2002). In contrast, the destruction of proliferating cells with methyloxymethanol acetate (a pharmaceutical agent that inhibits cell proliferation) prior to a learning task impedes learning (Shors et al., 2001). Together, these results suggest that hippocampal cell proliferation is important for learning and memory. However, with kindling, sampling is more difficult because kindled fear likely develops over a long time frame compared to a trace memory. Consequently, capturing the cells that are potentially involved in the development of kindled fear is difficult when done in this snapshot manner, particularly because the phenomenon could be a result of compounded snapshots. To fully examine the role of hippocampal cell proliferation in kindled fear efforts should include samples at multiple time points and compounded time points.

The results of Experiment 4 are important when considered in conjunction with the fact that kindling does increase the rate of cell proliferation around the 30-stimulation point. This begs the question of what happens to the cells that proliferate around this time? If these cells are not surviving at a higher rate, then why does cell proliferation increase and what becomes of these proliferating cells? These cells were suspected to integrate aberrantly within the hippocampal network, possibly as a secondary inhibitory mechanism for seizure suppression. An alternative possibility is that these cells are surviving but in replacement of cells that are lost through kindled-induced apoptosis. This is an interesting suggestion because, as mentioned in the introduction, kindling does not appear to produce much cell loss. Accordingly, an increase in the survival of proliferating hippocampal cells and a complementary loss of cells in kindled rats (Cavazos & Sutula, 1990; Spiller & Racine, 1994) could result in a similar net number of cells so that kindled and non-kindled rats appear to have similar hippocampal cell density. Furthermore, the same argument could be made regarding the issues of time sampling in both cell counting and immunohistochemical techniques, which are the techniques used to assess cell loss and cell damage (i.e., these techniques may be just as sensitive to the snapshot error discussed above with cell proliferation sampling).

An additional way to assess the role of hippocampal cell proliferation in the development of kindled fear would be to inhibit proliferation during kindling and assess fear behaviour. Cell proliferation could be inhibited from the very first stimulation and then the development of kindled fear could be assessed as in Experiment 1 with rats that received 30 and 99 stimulations. In fact, this type of experiment has recently been done in our laboratory. Inhibition of cell proliferation with a 14-day pre-treatment of methyloxymethanol acetate (MAM) prior to the onset of amygdala kindling actually increased the rate of amygdala kindling (Fournier et al., 2004). Unfortunately, these animals did not undergo any formal behavioural testing but personal observation of their behaviour suggested that they were much less fearful than the kindled rats that did not receive the MAM pre-treatment. The results of this experiment support a previous proposal that the function of the increased rate of cell proliferation is to provide a secondary inhibitory suppression on seizures: When hippocampal cell proliferation is removed, kindling develops more quickly. By extension, these results are therefore interesting the context of hippocampal dysfunction or inhibition contributing to kindled fear.

Hippocampal Inhibition-Excitation

A major component of kindling is the breakdown of $GABA_A$-mediated inhibition and the glutamate-mediated overexcitation in the hippocampus. The purpose of examining cell proliferation was to begin to identify a neural mechanism of kindled fear, in which aberrant cell growth in the hippocampus was suspected. The data have been somewhat inconclusive and suggest that cell typing is required. This may be especially important for assessing whether inhibition and/or excitation contributes to fearfulness in kindled rats. Assessing inhibition within the hippocampus is clearly necessary in the context of cell proliferation and the general integrity of the hippocampus. Thus, in addition to examining whether the cells born as a result of kindling are inhibitory or excitatory within the regions sampled in Experiment 4, general electrophysiological properties of the hippocampus during exposure to a novel open field is necessary. Such experiments could also assist in deciphering the sex differences that emerged in resistance-to-capture scores after hippocampal treatments: Not only would it be useful to assess the response of males and females to the hippocampal activation, it would also be useful to assess the response of both sexes to a novel environment after kindling.

Hippocampal-Dependent Cognitive Treatments for Kindled Fear

Perhaps the most interesting and potentially important finding from this thesis was the identification of a strategy for reducing the magnitude of kindled fear. When kindled females were exposed to the water maze during the course of kindling, they displayed significantly lower resistance to capture at the end of the experiment. This prompted the investigation of whether a behavioural treatment that activates the hippocampus, or more generally, facilitates plasticity within the hippocampus, could reduce the magnitude of kindled fear. Using an environmental enrichment paradigm, this was confirmed in Experiment 4 for both males and females.

Environmental enrichment of laboratory animals is currently an attractive topic in neuroscience research because it enhances of a variety of neuroplastic changes within the hippocampus and cortical brain regions and promotes many behavioural benefits. It is interesting to speculate on whether environmental enrichment reduces fearfulness in kindled rats by promoting the survival of new hippocampal cells into functionally and appropriately active cells. Enrichment is known to promote the survival of proliferating hippocampal cells as neurons (Kempermann et al., 1998); however, seizures are known to promote aberrant organization of new cells into the polymorphic and molecular layers of the DG, and into the CA3 region (Parent, et al. 1997; Scharfman et al., 2000) Furthermore, these cells become functional and are activated by spontaneous seizures (Scharfman, Sollas, & Goodman, 2002). The idea that enrichment might promote a beneficial functional state could also explain why the number and dispersal of surviving cells did not differ according to fear and offers the possibility that specific cell functioning may be related. Accordingly, rather than number, the difference in cells among the treatment conditions of Experiment 4 might be confirmed using more comprehensive immunohistochemical (such as GAD immunoreactivity for GABA cells identification) or electrophysiological techniques that examine functional neurogenesis by examining passive membrane properties, action potentials, and functional synaptic inputs, as was done previously in newly generated cells (van Praag, Schnider, Christie, et al., 2002).

Conclusion

The purpose of this thesis was to examine the role of the hippocampus in fearful behaviour in both male and female amygdala-kindled rats. The main result was that long-term kindling does produce a dysfunctional hippocampus: Kindled males were impaired on the hippocampal-dependent task and both kindled males and females showed abnormal amounts of hippocampal cell proliferation, although the extent and nature of this abnormal cell proliferation is not yet known. The most interesting finding was that treatments that activated the hippocampus reduced the magnitude of kindled fear. The possibility of exploiting this finding to eliminate kindled fear with more intensive hippocampal-dependent behavioural treatments is very enticing. Not only could this treatment serve to alleviate the fear and anxiety associated with epilepsy, it could also serve to alleviate some anxiety disorders. If one considers the proposal that kindling is an example of normal processes gone awry as described by Goddard (cited in McNaughton, 2003), then it is not surprising that exploiting normal function can result in pathologies such as fear sensitization and by extension, anxiety-like behaviour. Consequently, the kindled fear model may be generalizable to a wide range of hippocampal-dependent disorders. The fact that the process of kindling itself is still somewhat of a mystery, makes the finding that hippocampal-dependent behaviour can reduce the magnitude of kindling-induced fear even more enticing in relation to the mechanisms of kindling and anxiety-like behaviours.

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