BEHAVIOURAL CONSEQUENCES OF KINDLING IN THE ANTERIOR CLAUSTRUM

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Abstract

The anterior claustrum (CLA) has been implicated in epileptogenesis and epileptiform activity due to its abundant and widespread bilateral connections to some of the structures believed to play an important role in seizure generalization: the motor cortex, entorhinal cortex, limbic structures, and brainstem sites. Kindling in the CLA has been characterized as comprising two distinct phases: an early phase and a late phase. Early phase seizures progress quickly into generalized seizures, are short in duration, and resemble cortical seizures. Late phase seizures are characterized as being more severe in intensity, having longer durations, and resembling limbic-type seizures.

It is unknown whether kindling in the CLA will lead to changes in behaviour as seen after kindling of limbic sites. Thus, I measured the behavioural effects of kindling in the anterior CLA to investigate potential changes in learning, memory, and anxiety-related behaviours. I hypothesized that changes in behaviour would occur after kindling of late phase seizures, because of their close resemblance to limbic-type seizures, but not after kindling of early phase seizures. Anxiety-like behaviour was assessed in the elevated plus maze and open field. Object memory was assessed in an object recognition test, and spatial learning and memory were assessed in the water maze.

I found no significant changes in behaviour in the late phase group in comparison to the early phase and control groups. Thus, contrary to my hypothesis, late phase kindling of the CLA does not produce changes in learning and memory or alterations in anxiety-related behaviours.

Acknowledgements

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LIST OF ABBREVIATIONS

AD ..... afterdischarge

ADT ..... afterdischarge threshold

ANOVA ..... analysis of variance

Cg1 ..... cingulated cortex

CLA ..... claustrum

dHPC ..... dorsal hippocampus

EPM ..... elevated plus maze

fmi ..... anterior forceps of the corpus callosum

M1 ..... primary motor cortex

M2 ..... secondary motor cortex

PrL ..... prelimbic cortex

S1J ..... somatosensory cortex (jaw)

SEM ..... standard error of the mean

T ..... trial

WM ..... water maze

Behavioural consequences of kindling in the anterior claustrum

It is estimated that 10% of people will experience at least one seizure during their lifetime. The disease of epilepsy is, however, distinct from simply having a seizure. Epilepsy is defined as a chronic neurological disorder that currently affects approximately 1 to 3% of the population (Shneker & Fountain, 2003). There is currently considerable interest in understanding the neurobiological basis of epilepsy. Unfortunately, due to numerous uncontrollable factors such as age of onset, medical history, seizure focus, frequency, duration and severity, studying epilepsy within a clinical setting introduces many confounds. However, animal models of epilepsy are extremely useful, because they permit control of such factors.

Kindling

First described by Goddard (Goddard, 1967), kindling in rats as well as other animals has become a commonly used model for complex partial epilepsy, the most common type of temporal lobe epilepsy. Complex partial epilepsy involves focal seizures that, due to propagation to other parts of the brain, result in loss of consciousness. When generalized behavioural convulsions are triggered, complex partial seizures are considered to include secondary generalization.

Kindling at limbic sites involves development of a variety of seizure stages, from simple partial seizures, to hemiconvulsions, and eventually secondarily generalized seizures. Kindling also provides experimental control of seizure frequency, severity, and focus, in that it involves the development of robust electrographic seizures with repeated electrical stimulation to cortical or subcortical sites through chronically implanted electrodes. Like the clinical disorder, kindling involves an increase in seizure susceptibility, duration, severity, behavioural convulsions and secondarily generalized seizures, leading, in some studies to the development of spontaneous

seizures. Furthermore, kindling has been shown to be a robust phenomenon across species (Coulter, McIntyre, & Loscher, 2002).

Kindling is provoked by short trains of electrical stimulation that trigger epileptiform afterdischarge (AD) in the EEG. With stimulation of limbic sites, repeated ADs result in progressive development of behavioural convulsions. Racine's classification scheme (Racine, 1972b) is universally used to categorize the increase in severity of seizures into 5 different behavioural stages: stage 1 includes chewing, stage 2 includes head nodding, stage 3 includes unilateral forelimb clonus, stage 4 includes bilateral forelimb clonus, and stage 5 includes rearing and falling over, associated with hindlimb clonus.

The increased susceptibility to seizures associated with kindling is, as far as has been determined, permanent. Given the profound and permanent physiological changes associated with kindling, including a very significant increase in the magnitude and duration of AD propagated to distant sites, one might expect to see evidence of associated functional changes. In particular, it might be reasonable to expect to observe behavioural changes after kindling, given that forebrain areas susceptible to kindling are also thought to play a role in important behavioural functions such as motivation, learning and memory.

Behavioural testing

Before a brief discussion on how kindling can affect behaviour, I will review the kind of behavioural tests that have been used in our laboratory to investigate potential alterations in behaviour after kindling: the Elevated Plus Maze, the Open Field test, Object Recognition task, and the Water Maze.

Elevated Plus Maze. The Elevated Plus Maze (EPM) is used to test for anxiety-like behaviours as well as activity and exploratory behaviours. It consists of two perpendicular arms,

alternating between open and closed, interlocked together in the center (see Materials and Methods Section; Figure 1). During a single 5 min trial, measures are taken of the total time spent in the open/exposed arms versus the closed arms, or the number of entries from the closed arms into the open arms. For example, lower values in the time spent in the open arms would indicate higher levels of anxiety. Likewise, higher values in the total distance travelled within the EPM or higher values in entering the four arms indicate higher levels of activity/exploratory behaviour. In addition to simply looking at total distance or duration spent in each arm, one can also measure grooming behaviour, rearing, locomotor activity, and immobility.

Grooming is characterized as forepaw licking, face washing and/or licking body fur. It functions as self-cleaning and maintenance, but it has also been used as an indication of behavioural adaptation to stressors (Ladurelle, Sebret, Garbay, Roques, & Dauge, 1998; Moody, Merali, & Crawley, 1988). That is, placing a rat in a novel environment can cause enough stress to increase grooming (Moody et al., 1988). Intense and chronic stress, however, will decrease the incidence of grooming (Ladurelle et al., 1998; Moody et al., 1988). Rearing, related to exploratory behaviour, is defined as standing on both hind legs while having both front paws off the ground. Locomotor activity is defined as the rat's moving about freely, exploring its environment. High levels of activity indicate high levels of exploratory behaviour as well as lower levels of anxiety. Immobility is characterized as an episode in which all four limbs are on the ground and the rat does not display any movement other than necessary for respiration. High levels of immobility would suggest higher levels of anxiety.

Open Field. The Open Field also tests for anxiety and exploratory behaviours. It consists of a circular arena (see Material and Methods Section; Figure 2) in which the rat can freely move. Testing was conducted over 3 consecutive days following measurements in the

EPM. Testing for 3 consecutive days is to allow rats to habituate to the arena (Hannesson, Howland, Pollock, Mohapel, Wallace, & Corcoran, 2005). The total distance travelled within the arena is a measure of exploratory activity. Furthermore, greater time spent in the outer region than in the central region of the arena would suggest higher levels of anxiety. It is also possible to quantify time spent grooming, rearing, and in locomotor activity or immobility.

Object Recognition Test. This task tests for object recognition memory. The same test arena used in the Open Field task was used for the Object Recognition task. However, Object Recognition consisted of 2 separate trials. On the first trial, rats were presented with 2 identical toys. On the second trial, one of the familiar toys was switched for a novel toy the rat had never been exposed to. Thus, if rats recall the familiar object they were previously exposed to, they should spend more time investigating the novel object in the second trial due to their preference for novel objects (Ennaceur & Delacour, 1988).

Water Maze. In the Water Maze (WM), rats were individually placed in a pool of water and required to learn the location of a submerged platform. After acquisition was complete, memory was assessed in retention tests at various intervals. The WM is a test of spatial cognition because the pool itself is featureless and contains no proximal cues to guide learning; hence the rats must rely on distal spatial cues (e.g., a poster hung on one wall and a paper maché parrot hung from the ceiling in another corner of the WM room) to learn the location of the submerged platform.

Because my thesis research was concerned with the behavioural consequences of kindling the claustrum, I shall now turn to a selective review of the anatomy of the claustrum and its susceptibility to epileptiform activity.

Anatomy of the Claustrum

The claustrum (CLA) is a column of gray matter located between the anterior half of the basal ganglia and cortex of the forebrain (Kowianski, Dziewiatkowski, Kowianska, & Morys, 1999; Sheerin, Nylen, Zhang, Saucier, & Corcoran, 2004). More specifically, it is found beneath the insular and piriform cortices. In the rodent, it is described as elliptical in shape, decreasing in size from the anterior to posterior end (Zhang, Hannesson, Saucier, Wallace, Howland, & Corcoran, 2001). It is reciprocally connected to the visual cortex, motor cortex, olfactory areas, limbic structure, the thalamus, and brainstem structures (Zhang et al., 2001). In addition, studies in felines, monkey, and humans show that the CLA cortical connections are topographically organized (Macchi, Bentivoglio, Minciacchi, & Molinari, 1981, 1983; Morys, Narkiewicz, & Wisniewski, 1993; Sadowski, Morys, Jakubowska-Sadowska, & Narkiewicz, 1997). That is, the anterior CLA is mainly linked to the motor and prefrontal cortical regions, the central CLA to somatosensory regions, and the posterior CLA to the visual cortex (Sadowski et al., 1997). Ventral to the visual and somatosensory areas of the CLA are connections to the auditory cortex (Sadowski et al., 1997). All these connections have varying degrees of overlap.

For rats in particular, Sadowski et al (1997) showed that injections of retrograde tracers Fast Blue and Diamidino Yellow into the motor, somatosensory, visual, and auditory cortices resulted in prominent projections between the motor cortex and the anterior CLA, somatosensory cortex with the central CLA, as well as the visual and auditory cortex with the ventroposterior CLA. Thus, with minor overlap in the rat CLA, a sensorimotor region and a visuoauditory region can be distinguished (Sadowski et al., 1997).

Zhang et al. (2001) examined in detail the connections of the anterior CLA in rats using the anterograde tract-tracer Phaseolus vulgaris leucoagglutinin and retrograde tract-tracer Fluoro-Gold. They found that the anterior CLA is extensively connected to structures thought to be

important in epileptogenesis: the motor cortex, orbital cortex, and insular cortex (Zhang et al., 2001). Projections were also found in the perirhinal cortex, entorhinal cortex, and piriform cortex, structures highly susceptible to epileptiform activity. Furthermore, the anterior CLA projects to various regions of the thalamus, the amygdala, and brainstem structures including the substantia nigra, the periaquiductal gray and dorsal raphe nucleus, which have been implicated in seizure susceptibility (Zhang et al., 2001).

Although the anatomy and circuitry of the CLA is well characterized, the functional role of the CLA nevertheless remains unresolved. Research has suggested that the CLA may be involved in coordinating motor and sensorimotor control (Cortimiglia, Crescimanno, Salerno, & Amato, 1991; Olson & Graybiel, 1980; Sheerin et al., 2004; Shima, Hoshi, & Tanji, 1996), and voluntary swallowing (Zald & Pardo, 1999). It may be involved in nociception (Sloniewski, Morys, & Pilgrim, 1995), and it may become pathological in Alzheimer's disease (Morys, Bobinski, Wegiel, Wisniewski, & Narkiewicz, 1996; Morys, Narkiewicz, Maciejewska, Wegiel, & Wisniewski, 1994), Parkinson's disease (Yoshimura, Yoshimura, Asada, Hayashi, Fukushima, Sato, & Kudo, 1988), and ulegyria or gyral scaring; (Morys et al., 1993). Furthermore, due to the abundant reciprocal associations between the CLA and the frontal cortex, motor cortex, perirhinal cortex, entorhinal cortex, and the amygdala as just some of the many examples, it has been proposed that the CLA may be part of the network of structures involved in the generalization of seizures (Zhang et al., 2001).

Several studies have investigated the potential role of the CLA in the generalization of seizures in different species. For instance, Kudo & Wada (1990) found that anterior CLA electrolytic lesions in felines ipsilateral to the kindled amygdala can delay kindling. In Senegalese baboons, electrolytic lesions in the CLA produce similar effects (Wada &

Tsuchimochi, 1997). Mohapel, Hannesson, Armitage, Gillespie, and Corcoran (2000) found that radiofrequency lesions destroying approximately 13% of the rat posterior CLA were able to delay amygdaloid kindling, but not block it completely. Additional studies investigating the potential role the CLA in epileptogenesis involves directly kindling the CLA.

Early Phase versus Late Phase

Zhang et al. (2001) kindled seizures in rats with stimulation of the anterior CLA and found that it was extremely susceptible to kindling. They also discovered that repeated CLA stimulation resulted in the progression of seizures from one distinct phase to another. The early phase was characterized as by rapid development into generalized seizures, and the late phase was characterized as involving a more gradual increase in the AD duration and resembled limbic-type seizures (Zhang et al., 2001). The criterion used to define the transition from early phase kindling into late phase kindling was when at least a twofold increase in AD duration from the immediately preceding generalized seizure was observed (Zhang et al., 2001).

Behavioural effects of kindling

It has been known for decades that epileptic patients often experience changes in behaviour and personality, including alterations in learning and memory (Hermann, Black, & Chhabria, 1981). Evidence from kindling research in animals demonstrates similar effects, indicating that the behavioural changes in patients with epilepsy are not secondary to drug treatment or other considerations. For instance, many researchers have shown that hippocampal kindling produces deficits in spatial cognition (Gilbert, Hannesson, & Corcoran, 2000; Gilbert, McNamara, & Corcoran, 1996; Hannesson & Corcoran, 2000; Hannesson, Howland, Pollock, Mohapel, Wallace, & Corcoran, 2001a; Hannesson, Mohapel, & Corcoran, 2001b; Leung & Shen, 1991, 2006a; Leung, Zhao, & Shen, 1994; Lopes da Silva, Gorter, & Wadman, 1986).

Kindling of the nucleus accumbens can produce changes in motor behaviour (Ehlers & Koob, 1985) and alterations in anxiety-related behaviours can occur after amygdaloid kindling (Adamec, 1990; Adamec & Morgan, 1994; Adamec & Shallow, 2000a, 2000b; Kalynchuk, Pinel, & Treit, 1998a; Kalynchuk, Pinel, Treit, Barnes, McEachern, & Kippin, 1998b).

The behavioural effects of kindling in the CLA have yet to be investigated. Thus, even though the fact remains that the CLA has close connections with structures implicated in epileptogenesis, kindling research has been directed mainly to sites in the limbic system or neocortex, and virtually nothing is known about the behavioural effects of kindling elsewhere. Therefore, kindling in the CLA will provide further insight into analyses into the consequences of kindling an atypical site. In my thesis research I have measured the behavioural effects of kindling in the CLA. Because late phase kindling in the CLA involves development of limbic-type seizures, and limbic kindling produces distinct and reliable behavioural effects, I hypothesize the late phase kindling of the CLA, but not early phase kindling, will produce changes in at least some of the behaviours affected by limbic kindling.

Method

Subjects

A total of 37 male Long-Evans hooded rats (Charles River Canada, St. Constant, PQ, Canada) weighing 250g-350g at the time of surgery were used in this experiment. Rats were individually housed in standard shoebox cages on a 12:12h light:dark cycle; experiments were conducted during the light portion of this cycle; food and water was available *ad libitum*. All procedures were in accordance with the guidelines of the Canadian Council on Animal Care as approved by the University of Saskatchewan Animal Care Committee.

Surgery

Rats were anaesthetized with isofluorane and received bilateral implantation of bipolar stimulating/recording electrodes constructed of two twisted strands of nichrome wire, 127 μm dia., into the anterior claustrum (CLA; Anterior-Posterior +2.8; Medial-Lateral +2.1; Dorsal-Ventral -4.5 from skull surface). A reference wire and four additional stainless steel dental screws were secured to the skull with dental acrylic.

Kindling

The rats were given 7 days for postsurgical recovery as well as daily handling before their afterdischarge threshold (ADT) was determined, arbitrarily defined as the minimum intensity of stimulation sufficient to trigger an afterdischarge (AD) of at least 7 sec in one of the 2 electrodes. Electrical stimulation consisted of a 1 sec train of biphasic square-wave pulses; 1 msec in duration, delivered at 60pps from a Grass S8800 stimulator. Stimulation began at 150 μA and was increased by 100uA every 1 min until at least 7 sec of AD was elicited. Stimulation was then administered once daily at ADT until rats developed 5 consecutive stage 5 generalized seizures, at which point kindling stopped. Behavioural testing began one week later. Of the 37 rats, 10 were randomly chosen as yoked controls, 7 were chosen for the early phase kindling group, and six were chosen for the late phase kindling group. The remaining 14 rats were not included in the analysis due to electrode misplacement, the early loss of their electrode pedestal, or their failure to meet the kindling criterion. The increase in the severity of seizures during kindling was categorized according to (Racine, 1972b).

Behavioural tests

Behavioural testing began 1 week after kindling. Testing in the EPM started on Day 7; testing in the Open Field was on Day 8, 9 and 10; behavioural testing was suspended on Day 11;

testing in Object Recognition was on Day 12; Day 13 was another day off; and WM acquisition occurred on Days 14 and 15, with retention being measured 28 days later (Figure 1).

Tracking and recording behaviours

All behavioural tests were recorded with a video camera mounted directly above each test arena, which was connected to a computer using a program called EthoVision®, Version 3.1.16 (1993), Nodulus. EthoVision® tracked a rat's position by comparing and contrasting its movement and colour to its surroundings.

Elevated Plus Maze

Anxiety-like behaviour was measured in the EPM. The EPM consisted of two perpendicular arms interlocked together (length = 110 cm; width = 10 cm) with alternating open and closed arms (length = 110 cm; width = 10 cm, height = 45 cm; Figure 2). Rats were placed in the center of the EPM maze facing the East open arm, and the time spent in each of the arms was recorded for 5 min. Their behaviour (total time spent in rearing, grooming, activity, or immobility) in each of the arms was also tracked and recorded during the 5 min. In order to normalize for differences in activity levels, the data were converted into percentage time in the open and closed arms. In the interval between testing different rats, the EPM was cleaned with a 70% ethanol solution.

Open Field

Anxiety-like behaviour and locomotor activity/exploration were measured in the Open Field. A circular arena measuring 150 cm in diameter and 50 cm in height was used. Two identical 400 ml glass beakers, with alternating rings of black and white tape, were placed 51.25 cm apart from each other and 25 cm from the side wall of the arena (Figure 3). For the purposes of analysis, EthoVision® divided the arena into three concentric rings: the outer ring (0 to 15 cm

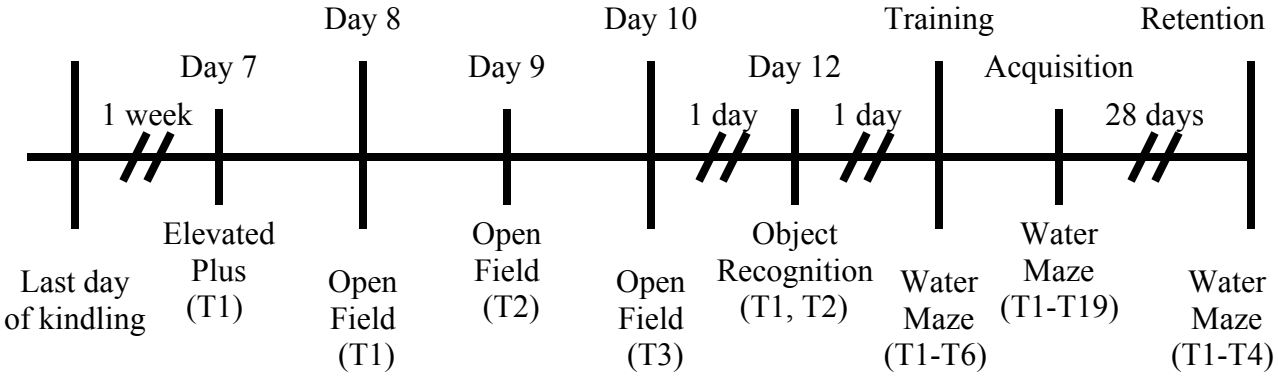

Figure 1. Timeline of behavioural testing. All rats were tested individually one week after they met the kindling criterion of 5 consecutive stage 5 generalized seizures (T = trial).

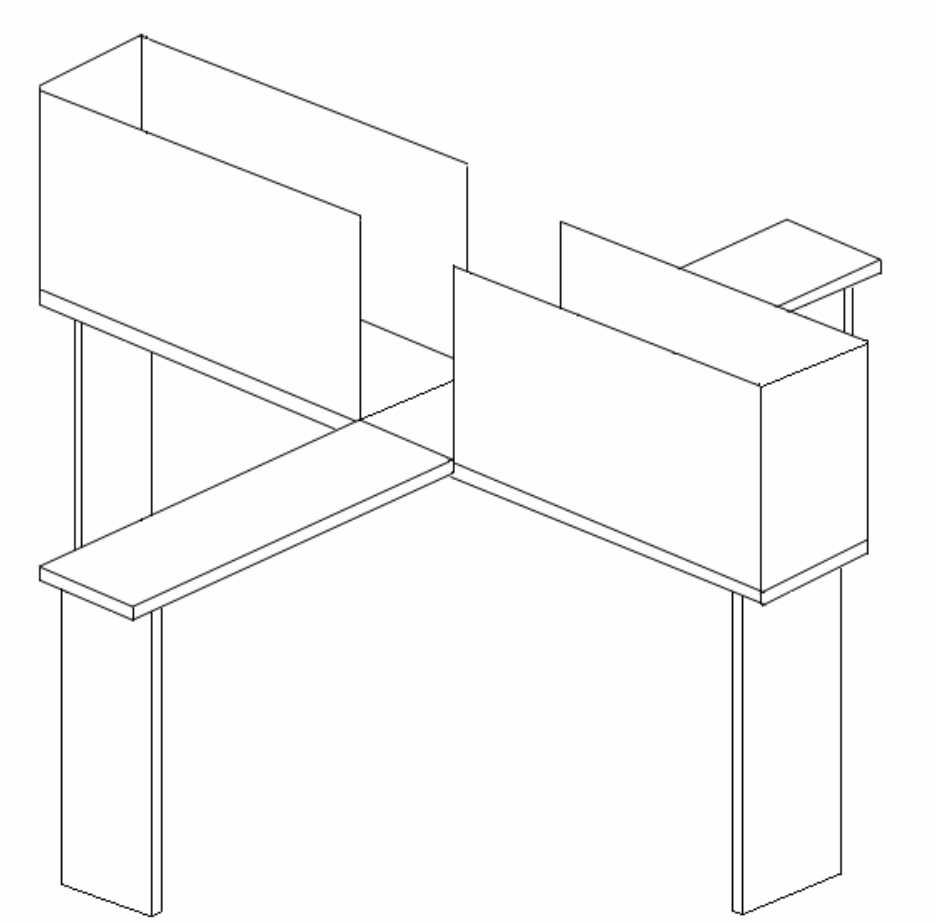

Figure 2. The elevated plus maze. At the start of each trial, each rats was placed at the center of the alternating open and closed arms facing East (facing the left open arm in the diagram).

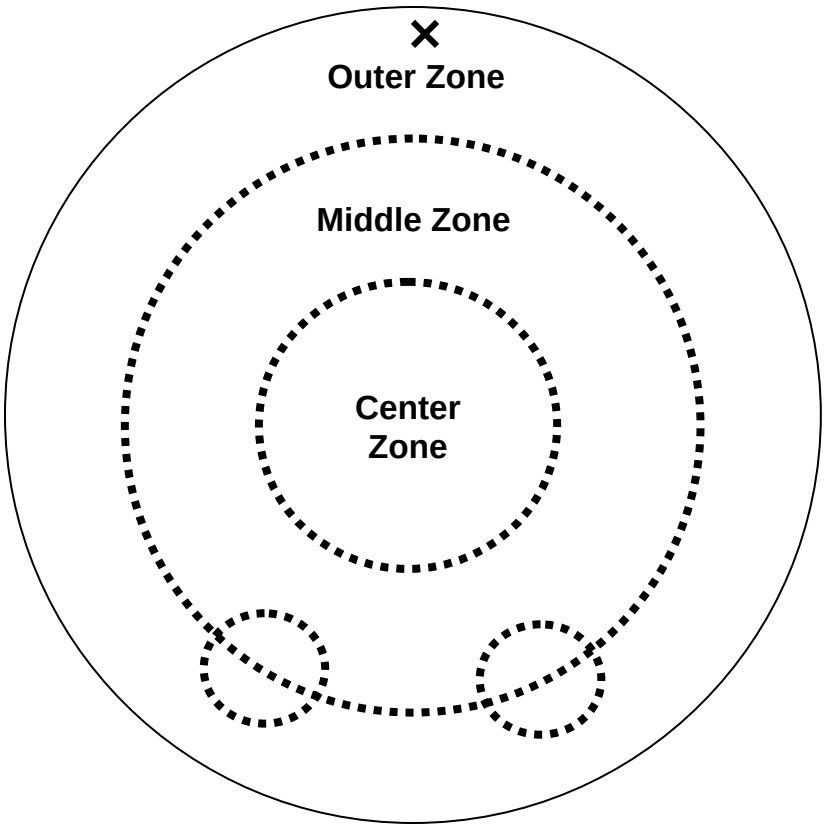

Figure 3. Top view of the open field arena. The computer program (EthoVision®) divides the arena into three concentric rings, to permit quantification of time spent in different zones. The 2 smallest rings represent the 2 object zones, and **X** represents the spot where each rat was placed at the start of the testing period.

from the wall), the middle ring (15 to 40 cm from the wall), and the inner ring (40 to 75 cm from the wall). Two object zones (30 cm in diameter) were also designated for the glass beakers to be placed in the center. Rats were individually placed into the arena for 5 min in one trial daily, for 3 consecutive testing days, and the time spent in each ring as well as the percentage of time spent performing various behaviours (rearing, grooming, locomotor activity, immobility) were tracked and recorded.

Object Recognition Test

This test measures memory for familiar objects. The circular arena for the test was the same arena used for the Open Field. The same behaviours as in the Open Field task were tracked and recorded. This test consisted of 2 separate trials: the first trial lasted 5 min in duration in the presence of 2 identical objects. Ten min later, a second trial that lasted 3 min was conducted in which one of the previously identical objects was switched for a novel object.

The 2 objects were a toy car or a toy jester of a smooth, non-porous plastic surface, served as either the novel or familiar object. Approximately 8 cm x 6 cm x 6 cm, the toy car was orange, black, blue, grey, and white in colour. The toy jester figurine was yellow, blue, green, and purple in colour standing inside a purple circular ring measuring approximately 9.5 cm in diameter and 3.5 cm in width. The toy jester was taped in place with masking tape so that it stood perpendicular to the floor. Each object was cleaned with 70% ethanol solution between every exposure.

Water Maze

Learning and retention of spatial information was examined in the WM. Testing was conducted in a circular pool, made of industrial plastic and painted white, measuring 200 cm in diameter and 45 cm in height. The water temperature was maintained at 25 $\pm$ 2ºC and the water

surface was rendered completely opaque with floating white plastic beads. Rats were individually tested in the WM on 3 separate days: Day 1 involved training with a visible platform, and Day 2 involved acquisition (learning the location of a submerged platform). For the next 27 days, rats were only handled. On Day 28 they were given a test of retention or long-term memory. A Plexiglass® structure measuring 23 cm in height with a 10 by 12 cm upper face was used throughout WM testing as the platform rats had to locate

For analytical purposes, EthoVision® divided the WM into 4 quadrants corresponding to Northeast (NE), Northwest (NW), Southeast (SE) and Southwest (SW). Each trial was terminated whenever the rat was able to locate and remain on the platform for 5 sec, or at the end of the 60 sec trial, whichever came first. Upon locating the platform and remaining on it for 5 sec, the rat was removed from the WM and placed into an empty cage with a 250W red heat lamp in one corner of the room. If the rat had not located the platform at the end of the 60 sec, it was gently guided to the location of the platform and left there for 5 sec before it was removed. There was approximately a 1min interval between each trial throughout WM testing.

Training day. The first day of WM testing familiarized rats with the WM. Rats were required to locate a black, square platform measuring 10 by 12 cm upper face, and 5 cm in height. This black platform was attached on top of the Plexiglass® structure. The WM pool was filled so that the black platform was protruding 3 cm above water surface. This was therefore referred to as a 'visible platform.' The platform was randomly placed in 1 of the 4 different quadrants for each trial, for a total of 6 trials. Rats were then randomly released from different points from the side of the pool corresponding to the NE, NW, SE, and SW quadrants.

Acquisition day. On Day 2 of the WM test, 19 individual acquisition trials were conducted in which rats were required to learn the location of the submerged or hidden platform

using extra-maze cues. The hidden platform was the same Plexiglass® platform but without the visible platform attached on top. The platform was submerged 2 cm beneath water surface, hence 'hidden/submerged platform.' A total of 19 trials were conducted during the acquisition trial with the submerged platform consistently located in the middle of the NE quadrant while rats were randomly released from different points.

Retention day. The rats' long-term memory for the location of the submerged platform was measured 28 days after acquisition day. Rats were once again individually placed into the WM for a total of 3 trials in order to locate the presence of the submerged platform in the middle of the NE quadrant.

Probe trials. A single probe trial was conducted on the 16th trial during acquisition, and another probe trial was conducted on the final trial during retention testing. The probe trial involved removing the hidden platform from the WM and measuring the time rats spent in each quadrant of the WM.

Histology

After behavioural testing, rats were euthanized with an overdose of sodium pentobarbital (euthanyl) at a concentration of 240mg/kg and perfused through the heart with 250 ml of Phosphate Buffered Saline followed by 250ml of 4% paraformaldehyde. Brains were extracted and placed overnight in PFA before immersion in a 30% sucrose solution for approximately 2 - 3 days at 4^{o}C. A sliding microtome was used to section each brain in frozen sections 45µm-thick. Sections were then mounted on glass microscope slides and stained with cresyl violet in order to confirm electrode placement. Electrode placements were visualized (Figure 4) using *The Rat Brain In Stereotaxic Coordinates* (Paxinos, 1995; Paxinos & Watson, 1997), figures 7 – 9.

Data analysis

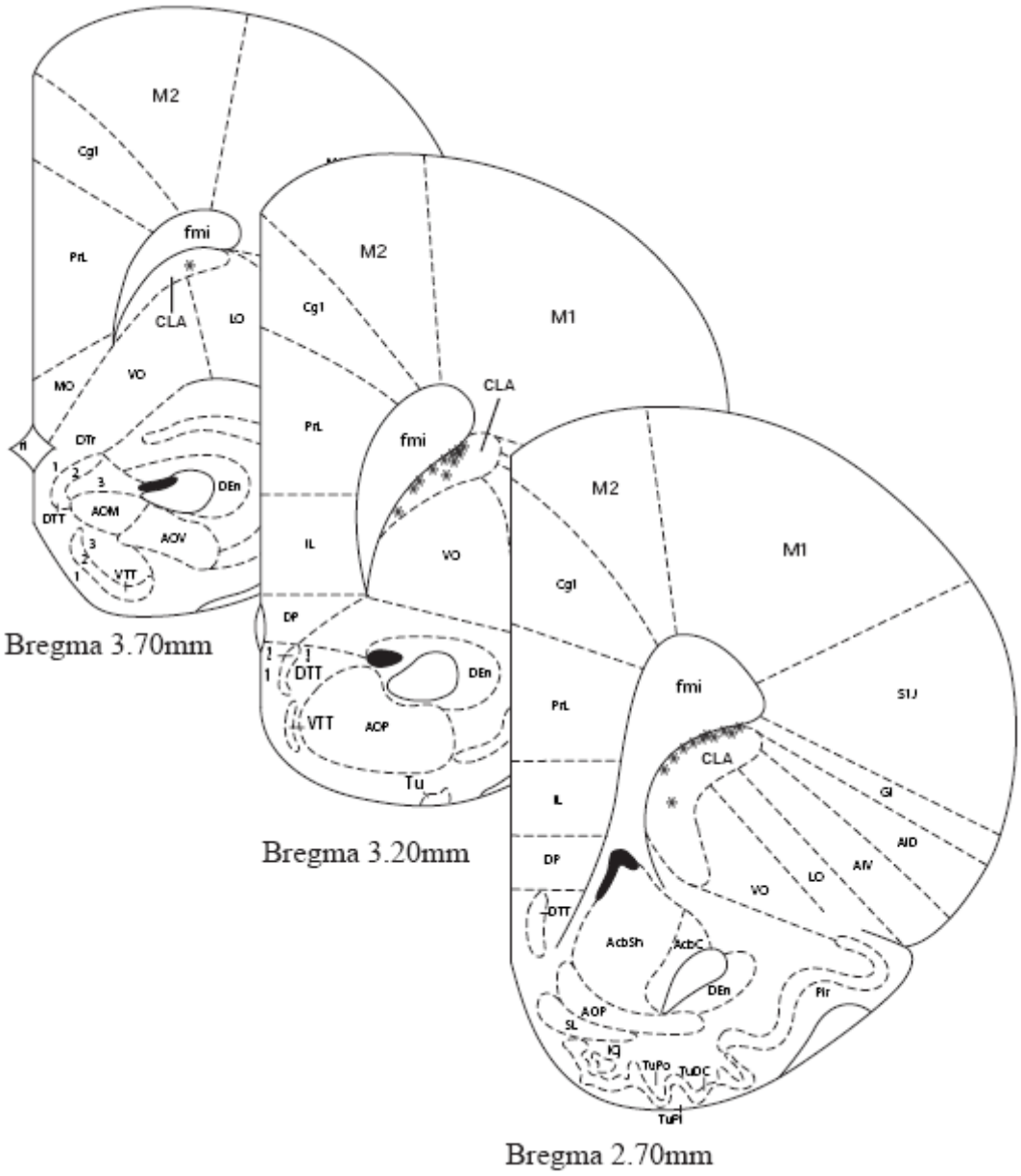

Figure 4. Electrode placements. Images were adapted from Paxinos and Watson (1997); used with permission. The electrode placements for all 23 rats that met the criterion for kindling are represented by the solid black dots (*). Structures surrounding the claustrum include the anterior forceps of the corpus callosum (fmi), prelimbic cortex (PrL), cingulated cortex (Cg1), secondary motor cortex (M2), primary motor cortex (M1), somatosensory cortex (S1).

Data were analysed with the statistical software package SPSS® 11.5 for Windows. Dependent measures were analyzed with one-way Analysis of Variances (ANOVA) or mixed-model ANOVAs where appropriate. Paired samples t-tests, independent samples t-tests, or Fisher's LSD were used for post-hoc tests when applicable. When multiple comparisons were required, the Bonferroni correction was used to adjust the alpha level of $p < 0.05$, to guard against Type 1 errors.

Results

Kindling

Initial kindling of the anterior CLA resulted in very short durations of AD (9 to 15 sec) and cortical-like AD in which brief stage 3 or 4 seizures would be elicited within the first several days of stimulation. A quick progression into stage 5 seizures would occur, which is characteristic of early phase kindling and was seen consistently. When seizure development progressed to the late phase, stage 5 limbic-like generalized seizures became evident. In addition, there was at least a 2 fold increase in AD duration compared to the immediately preceding ADs.

The mean ADT ($\pm$ SEM) of the early phase group and the late phase group did not differ significantly: $500.00 \pm 65.47\mu A$ versus $658.33 \pm 114.32\mu A$; $t(11) = -1.248$, $p = 0.238$. However, as intended, the early phase group required significantly fewer ADs to reach the 5^{th} consecutive generalized stage 5 seizure (14.86 ± 0.60) than the late phase group (25.33 ± 0.96), $t(11) = -9.615$, $p < 0.0005$.

Ictal measures included latency to clonus, clonus duration, AD duration, log roll, wet dog shakes, and secondary AD. Log rolls are characterized as the rat rolling or twisting along its entire longitudinal axis. Wet dog shakes are defined as rotational shaking of both the head and

body, not unlike what one would see when a dog shakes water off its body (Barnes & Mitchell, 1990; Bedard & Pycock, 1977; Leung & Shen, 2006b). Six 2 (group: early phase and late phase) X 5 (trial) mixed-model ANOVAs were used to analyze the dependent variables. The Bonferroni correction for the increased likelihood of a Type I error associated with multiple tests resulted in the alpha (α) level $\alpha/6 = 0.05/6 = 0.008$.

Latency to clonus. There was no main effect of trial for latency to clonus, $F(4, 44) = 0.511$, $p = 0.728$, but there was a main effect of group, $F(1, 11) = 20.502$, $p = 0.001$. This indicates that there was a difference between the late phase group and the early phase group. The interaction between trial X group for latency to clonus did not reach significance, $F(4, 44) = 0.666$, $p = 0.619$. Three independent samples t-tests were performed to further investigate the main effect of group on the 1st, 3rd and 5th generalized seizure. A Bonferroni correction of $\alpha/3 = 0.017$ was used. The difference in the latencies of the 2 groups in the 1st generalized seizure was not significant. However, the early phase group displayed a significantly longer latency to clonus than the late phase group in the 3rd generalized seizure (early phase group, 2.71 ± 0.36 sec; late phase group, 1.00 ± 0.00 sec; $t(11) = 4.386$, $p = 0.001$). A similar difference occurred in the 5th generalized seizure (early phase group, 2.43 ± 0.20 sec; late phase group, 1.33 ± 0.21 sec; $t(11) = 3.740$, $p = 0.003$).

Clonus duration. A main effect of trial for clonus duration was found, $F(4, 44) = 5.515$, $p = 0.001$. A main effect of group was found as well, $F(1, 11) = 72.759$, $p = 0.001$, suggesting again that there was a difference between the early phase group versus the late phase group in clonus duration. In addition, the interaction between trial X group for clonus duration was statistically significant, $F(4, 44) = 4.136$, $p = 0.006$. Three independent samples t-tests (between groups) were used to compare the clonus duration means of the 1st, 3rd, and last generalized

seizure, with a Bonferroni corrected alpha level of $\alpha/3 = 0.0167$. The value for clonus duration between the early phase group (8.71 ± 0.99 sec) and late phase group (34.33 ± 5.02 sec) in the 1st generalized seizure was statistically significant ($t(11) = -5.404$, $p < 0.0005$). The difference between the early phase group (9.57 ± 1.19 sec) and the late phase group (44.67 ± 3.68 sec) in 3rd generalized seizure was significant ($t(11) = -9.702$, $p < 0.0005$). The difference between the groups in the 5th generalized seizure was also significant, in that the early phase group had a shorter clonus duration (13.14 ± 2.00 sec) than the late phase group (40.83 ± 3.32 sec), $t(11) = -7.385$, $p < 0.0005$ (see Figure 5).

AD duration. There was a main effect of trial, $F(4, 44) = 9.312$, $p < 0.001$, indicating that there was at least one significant difference among the 5 trials. There was also a main effect of group for the AD duration between the early and late phase groups, $F(1, 11) = 104.206$, $p < 0.001$. The interaction of trials X group was significant, $F(4, 44) = 7.388$, $p < 0.0005$. Thus 3 independent samples t-tests were used to examine the 1st, 3rd, and 5th generalized seizure (Figure 6), with a Bonferroni correction of the p value of $\alpha/3 = 0.05/3 = 0.0167$. The independent samples t-tests showed significant differences in the 1st generalized seizure (early phase: 11.57 ± 0.48 sec; late phase: 47.17 ± 5.62 sec), $t(11) = -6.854$, $p < 0.0005$. The difference in AD duration between the early phase group (12.57 ± 1.11 sec) and the late phase group (57.83 ± 4.95 sec) in the 3rd generalized seizure was also significant, $t(11) = -9.621$, $p < 0.0005$. Furthermore, the difference between groups in the last generalized seizure was significant as well (early phase: 15.71 ± 2.09 sec; late phase: 54.33 ± 2.75 sec), $t(11) = -11.359$, $p < 0.001$. Thus, as expected, the late phase group displayed consistently longer AD duration than the early phase group.

S*econdary AD duration*. No significant main effect of trial was found on secondary AD duration, $F < 1$, suggesting that none of the 5 trials differed from each other. The trials X group

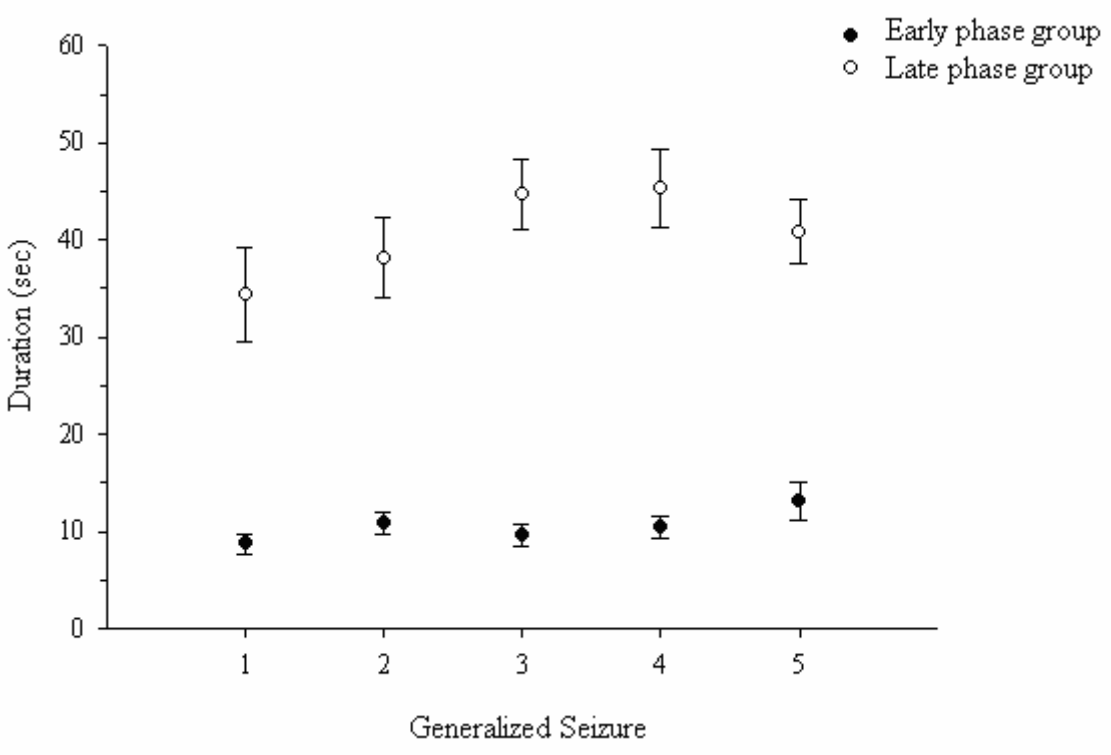

Figure 5. Clonus duration. A main effect of group was present for clonus duration. The 1st, 3rd and 5th generalized seizures were chosen for post-hoc analysis in order to investigate whether significant differences were present between the 2 groups. The difference between the early phase and late phase groups in duration of clonus in the 1st generalized seizure was significant, $p = 0.003$. The difference between the early phase and late phase groups in clonus duration in the 3rd and 5th generalized seizure was also significant, $p < 0.001$.

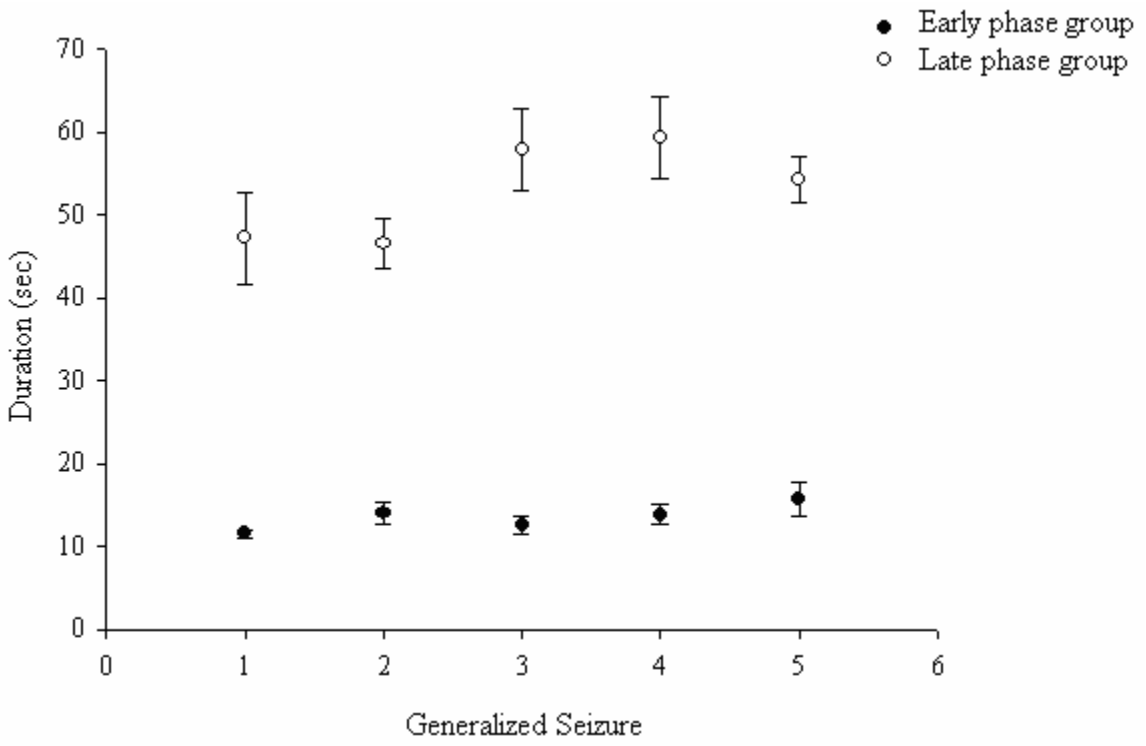

Figure 6. AD duration. A main effect of group was present for AD duration. The 1st, 3rd and 5th generalized seizures were chosen for further analysis in order to investigate whether significant differences were present between the 2 groups. The difference between the early phase and late phase groups in duration of AD in the1st generalized seizure was significant, $p = 0.001$. The difference between the early phase and late phase groups in AD duration in the 3rd and 5th generalized seizure was also significant, $p < 0.001$.

interaction was not significant, F < 1, and the main effect of group did not differ significantly, $F(1, 11) = 7.027$, $p = 0.023$, suggesting that the presence of secondary AD did not vary between the groups.

Log roll. The main effect of trials, group, and trials X group interaction did not vary significantly, $F < 1$.

Wet dog shakes. The main effect of trial was not significant, $F(4, 44) = 2.708$, $p = 0.042$. However, the main effect of group missed statistical significance, $F(1, 11) = 8.236$, $p = 0.015$. The trial X group interaction was not significant, $F(4, 44) = 2.708$, p = 0.042, indicating that during the 5 generalized seizures, the incidence of wet dog shakes did not differ between the groups.

Behavioural tests

Elevated Plus Maze. A one-way ANOVA was used to analyze the percentage of time and total distance travelled within the closed and open arms during the 5 min EPM trial. Thus, a Bonferroni correction of $\alpha/2 = 0.05/2 = 0.025$ was used. However, because the percentage of time and distance travelled are highly correlated, only the percentage of time spent in the EPM will be reported.

Duration. The percentage of time spent between the closed arms and open arms was not significant, $F < 1$, suggesting the early phase group, late phase group, and control group did not differ. Paired samples t-tests indicated that each group spent a higher percentage of time in the closed arms (early phase group: 64.49 $\pm$ 5.31%; late phase group: 72.86 $\pm$ 6.72%; control group: 68.85 $\pm$ 5.84%) compared to the open arms (early phase group: 16.37 $\pm$ 4.52%; late phase group: 11.09 $\pm$ 3.75%; control group: 15.31 $\pm$ 3.43%; Figure 7), $p < 0.005$.

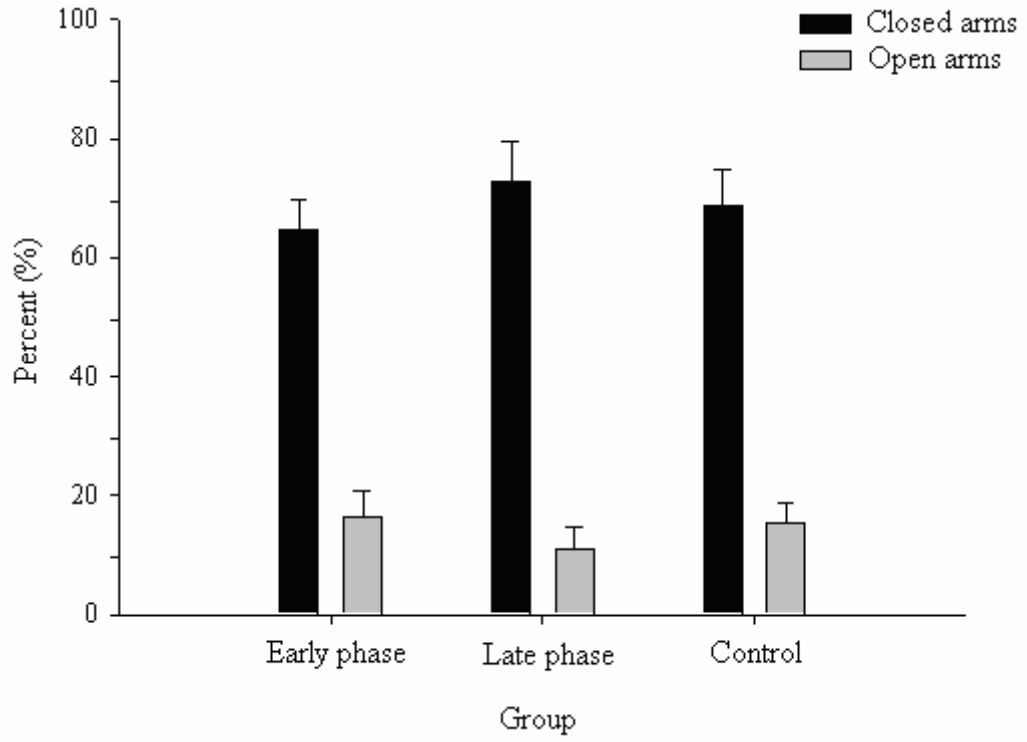

Figure 7. Percent of time spent in the closed versus open arms in the EPM. All groups spent a significantly higher percentage of time in the closed arms compared to the open arms, $p < 0.005$. Differences between the 3 groups were not significant.

Four specific behaviours in the EPM were quantified separately: grooming, locomotor activity, rearing, and immobility. Four one-way ANOVAs were used to analyze the proportion of time spent in the closed versus open arm performing these behaviours, with an alpha correction of $\alpha/4 = 0.05/4 = 0.0125$.

Grooming. A between-subjects analysis showed no difference in the percentage of time spent grooming in the closed arms, $F(2, 20) = 1.125$, $p = 0.344$. No grooming was ever performed in the open arms. Three paired samples t-tests for each group, with a Bonferroni correction of $\alpha/3 = 0.05/3 = 0.017$, indicated that the early phase group did not spend a higher proportion of time grooming in the open ($0.00 \pm 0.00\%$) versus closed arms ($2.16 \pm 0.94\%$), $t(6) = -2.286$, $p = 0.062$. The difference in the percentage of time grooming by the late phase group in the open ($0.00 \pm 0.00\%$) versus closed arms ($2.11 \pm 0.73\%$) was not significant, $t(5) = -2.895$, $p = 0.034$. In the control group the difference in the percentage of time grooming in the open versus the closed arms was significant, ($4.28 \pm 1.40\%$ versus $0.00 \pm 0.00\%$), $t(9) = -3.053$, $p = 0.014$.

Locomotor activity. The percentage of time spent locomoting in the open arms and the closed arms between the groups was not significant, $F < 1$. Three paired samples t-tests for locomotor activity between each group, with an alpha correction of $\alpha/3 = 0.05/3 = 0.017$, were conducted. There was a significant difference in the percentage of time spent locomoting in the open ($13.48 \pm 4.17\%$) versus closed arms ($46.00 \pm 3.31\%$) by the early phase group, $t(6) = -4.417$, $p = 0.004$. Differences were also revealed between the open ($7.95 \pm 2.46\%$) and closed arms ($56.28 \pm 6.39\%$) for the late phase group, $t(5) = -6.080$, $p = 0.002$. The controls also spent a significantly greater percentage of time in the open arms ($11.34 \pm 2.93\%$) compared to the closed arms ($48.33 \pm 4.82\%$), $t(9) = -5.065$, $p = 0.001$.

Rearing. The differences amongst the groups in the percentage of time spent rearing in the closed arms were not significant, $F < 1$. Nor were differences in the percentage of time spent rearing in the open arms significant, $F(2, 20) = 1.421$, $p = 0.265$. Three paired samples t-tests, with a Bonferroni correction of $\alpha/3 = 0.05/3 = 0.017$, revealed significant differences in the percentage of rearing between all groups. That is, the early phase group spent significantly less time rearing in the open arms ($0.47 \pm 0.47\%$) versus the closed arms ($14.14 \pm 1.72\%$), $t(6) = -8.149$, $p < 0.0005$. This was that case for the late phase group (open arms: $0.00 \pm 0.00\%$ versus closed arms: $11.91 \pm 1.62\%$), $t(5) = -7.333$, $p = 0.001$, and controls as well (open arms: $0.11 \pm 0.05\%$ and closed arms: $13.88 \pm 1.15\%$), $t(9) = -12.007$, $p < 0.0005$.

Immobility. Differences in the percentage of time spent immobile in the open arms were not significant between the 3 groups, $F < 1$; nor were the differences in the closed arms, $F(2, 20) = 1.695$, $p = 0.209$. Three paired samples t-tests, with an alpha correction of $\alpha/3 = 0.05/3 = 0.017$, were conducted. No significant differences were found in the percentage of time spent immobile between the open ($0.00 \pm 0.00\%$) and closed arms ($0.27 \pm 0.27\%$) for the early phase group, $t(6) = -1.00$, $p = 0.356$; the late phase group (open arms: $0.00 \pm 0.00\%$ versus closed arms: $0.07 \pm 0.07\%$), $t(5) = -1.00$, $p = 0.363$; and control group (open arms: $0.11 \pm 0.11\%$ versus closed arms: $0.78 \pm 0.32\%$), $t(9) = -1.88$, $p = 0.093$.

Open field

Two 3 (zones: outer, middle and center zone) X 3 (days: testing days 8, 9 and 10) X 3 (group) mixed-model ANOVAs were used to analyze the dependent variables of total duration and total distance travelled in the open field. A Bonferroni correction of $\alpha/2 = 0.025$ was used. Because duration and total distance travelled were highly correlated, only durations are reported.

Duration. The main effect of zones was significant, suggesting that the time spent in at least one of the 3 zones differed significantly from the others, $F(2, 40) = 490.317$, $p < 0.0005$. The main effect of days failed to reach significance, $F(2, 40) = 3.954$, $p = 0.027$, as did the main effect of group, $F(2, 20) = 3.155$, $p = 0.064$, indicating that the differences between the early phase, late phase, and control groups were not significant. The days X group interaction was not significant, $F < 1$. The zones X days interaction was statistically significant, $F(4, 80) = 32.655$, $p < 0.0005$, indicating that differences in duration associated with at least 1 of the 3 zones was influenced by days or vice versa. The zones X group interaction was also evident in the duration spent in the open field, $F(4, 40) = 3.164$, $p = 0.024$. That is, at least one of the groups differed from the others in time spent in at least one of the zones. The 3-way interaction of zones X days X group was not significant, $F(8, 80) = 2.101$, $p = 0.045$, indicating that the groups performed similarly throughout the open field test.

Three repeated measures ANOVAs of the mean duration spent in the outer, middle, and center zone for each day were used to analyze further the significant zones X days interaction, employing a Bonferroni correction of $\alpha/3 = 0.0167$. The main effect of days was significant for the mean duration spent in the outer zone, $F(2, 44) = 37.378$, $p < 0.0005$; the middle zone, $F(2, 44) = 12.863$, $p < 0.0005$; and the center zone, $F(2, 44) = 10.524$, $p < 0.0005$. Three paired t-tests for each zone were computed to follow up the main effect of days, with the alpha level adjusted to $\alpha/9 = 0.006$. The average duration spent in the outer zone on day 8 (227.01 ± 5.14 sec) was significantly higher than on day 9 (185.09 ± 7.51 sec), $t(22) = 6.070$, $p < 0.0005$. Likewise, duration of time in the outer zone on day 8 versus day 10 (171.00 ± 7.20 sec) was significantly different, $t(22) = 9.736$, $p < 0.0005$. No differences were found in the mean duration spent in the outer zone between day 9 and 10, $t(22) = 1.892$, $p = 0.072$. The average

duration spent in the middle zone on day 8 (34.38 ± 2.56 sec) and day 9 (52.31 ± 5.23 sec) was significantly different, $t(22) = -4.419$, $p < 0.0005$. Comparing day 8 and day 10 (56.66 ± 5.87 sec), the time spent in the middle zone on day 10 was significantly higher, $t(22) = -4.608$, $p < 0.0005$. The difference between the time spent on day 9 and 10 in the middle zone was not significant, $t(22) = -0.866$, $p = 0.396$. With regard to the center zone, the difference in the time spent on day 8 (8.07 ± 1.69 sec) and day 9 (23.31 ± 4.40 sec) was significant, $t(22) = -3.472$, $p = 0.002$. As well, the times spent in the center zone on day 8 and 10 (26.94 ± 4.80 sec) were different from each other, $t(22) = -3.995$, $p = 0.001$. No differences were found between day 9 and 10, $t(22) = -0.921$, $p = 0.367$. Considering that only zone comparisons between day 8 and day 10 were significantly different, this indicates that habituation of locomotor activity occurred in the open field.

The significant zones X group interaction was also further analyzed with 3 one-way ANOVAs: one for the average duration spent in the outer zone, one for the middle zone, and one for the center zone. A corrected alpha of $\alpha/3 = 0.0167$ was used. The average duration of time spent in the center zone differed significantly between groups, $p = 0.001$. Moreover, the mean duration spent in the middle zone differed significantly between groups, $p = 0.007$. These results indicate that at least one of the groups differed significantly in the average time spent in the center and middle zone. The mean duration spent in the outer zone did not differ significantly, $p = 0.428$. Fisher's LSD ($\alpha = 0.05$) was used for the post-hoc test. For the middle zone, the late phase group (67.07 ± 6.67 sec) spent significantly more time there than the early phase group (38.31 ± 6.68 sec), $p = 0.003$. The difference between the late phase group and the control group was also significant (42.84 ± 6.29 sec), $p = 0.006$, in that the control group spent less time in the middle zone. For the average duration spent in the center zone, the late phase group (35.80 ±

6.39 sec) spent significantly more time the early phase group (11.28 ± 3.91sec), $p < 0.0005$. Likewise, the late phase group differed from the control group (15.34 ± 4.92 sec), which spent less time in the center zone, $p = 0.001$.

Grooming, rearing, locomotor activity, and immobility were also recorded in the open field. Four zones X day X group mixed-model ANOVAs were used to analyze the proportion of time groups performed each behaviour in the zones across the 3 days, with a corrected alpha of $\alpha/4 = 0.0125$. Any t-tests conducted for grooming behaviour, rearing, and immobility employed a Bonferroni correction of $\alpha/9 = 0.006$, because 3 paired samples t-tests were conducted for each behaviour.

Grooming. The main effect of days, days X group, zones X days and zones X days X group was not significant, $F < 1$. The main effect of group in the proportion of time spent grooming was also not significant, $F(2, 20) = 2.879$, $p = 0.080$, nor was the zones X group interaction, $F(4, 40) = 3.105$, $p = 0.024$. Only a main effect of zones was significant, $F(2, 40) = 48.966$, $p < 0.0005$. In other words, the proportion of grooming was more or less in at least one of the zones. Three paired samples t-tests were used to investigate where this significance lay. The proportion of time spent grooming in the outer zone (0.00 ± 0.00%) and middle zone (0.16 ± 0.05%) was significantly different, $t(22) = -3.346$, $p = 0.003$. Likewise, significant differences were found in the proportion of grooming time between the middle and center zone (0.00 ± 0.00%), $t(22) = -3.3456$, $p = 0.003$, due to the fact that there was no grooming exhibited in the center zone. No differences were found in the proportion of grooming between the outer and center zone.

Rearing. The main effect of group, zones X group, and days X group were not significant, $F < 1$. The zones X days interaction was not significant, $F(4, 80) = 2.257$, $p = 0.070$,

nor was the 3-way interaction of zones X day X group, $F(8, 80) = 1.029$, $p = 0.422$, implying that all groups reared a similar amount in the open field. There was, however, a main effect of zones, $F(2, 40) = 234.340$, $p < 0.0005$, indicating that the proportion of time spent rearing in at least one of the zones differed from another. The significant main effect of days suggests that the percentage spent rearing was different in at least one of the 3 days of open field, $F(2, 40) = 18.666$, $p < 0.0005$. Six paired samples t-tests were then employed to identify the source of the difference, with a Bonferroni correction of $\alpha/9 = 0.006$. For the main effect of zones, the difference in proportion of time spent in the outer zone ($8.04 \pm 0.45\%$) versus the middle zone ($1.04 \pm 0.25\%$) was highly significant, $t(22) = 16.344$, $p < 0.0005$. The difference in proportion of time spent in the middle and center zones ($0.42 \pm 0.11\%$) was significant, $t(22) = 3.339$, $p = 0.003$. In addition, the proportion spent rearing in the outer versus center zone was significant, $t(22) = 17.759$, $p < 0.0005$. With regard to the main effect of days, the difference in the proportion of time spent rearing on day 10 ($4.05 \pm 0.33\%$) relative to day 9 ($2.98 \pm 0.24\%$) was significant, $t(22) = -3.984$, $p = 0.001$. The difference in rearing on day 10 and day 8 ($2.48 \pm 0.17\%$) was also significant, $t(22) = -6.080$, $p < 0.0005$, because groups also spent a higher proportion of their time rearing on day 10. The difference in the proportion of time spent rearing between day 8 and 9 was not significant, $t(22) = -2.548$, $p = 0.018$.

Locomotor activity. The main effect of group was not significant, $F(2, 20) = 1.313$, $p = 0.291$, nor was the days X group interaction, $F < 1$, or the zones X group interaction, $F(4, 40) = 1.669$, $p = 0.176$. The main effect of zones was significant, $F(2, 40) = 515.370$, $p < 0.0005$, implying that a significant difference in the proportion of locomotor activity was present in at least one of the zones. The main effect of days, $F(2, 40) = 35.598$, $p < 0.0005$, the zones X day interaction, $F(4, 80) = 59.984$, $p < 0.0005$, and the 3 way interaction of zones X days X group

were all statistically significant, $F(8, 80) = 2.763$, $p = 0.010$, indicating that groups performed differently in regard to locomotor activity in the open field.

Three zones X group mixed model ANOVAs were performed on the percentage of locomotor activity during day 8, 9 and 10, with an adjusted alpha level of $\alpha/3 = 0.0167$. On day 8, the group effect, $F(2, 20) = 2.163$, $p = 0.141$, and the zones X group interaction, $F < 1$, were not significant. The main effect of zones was significant, $F(2, 40) = 829.739$, $p < 0.0005$. Paired samples t-tests showed that on day 8, the percent of time spent on locomotor activity in the outer zone ($64.33 \pm 1.71\%$) was significantly higher than that of the middle zone ($9.05 \pm 0.62\%$), $t(22) = 27.769$, $p < 0.0005$. The difference in activity in the outer zone versus the center zone ($2.03 \pm 0.44\%$) was statistically significant, $t(22) = 31.163$, $p < 0.0005$. In addition, the difference in activity in the middle zone versus the center zone was significant as well, $t(22) = 15.609$, $p < 0.0005$.

The percentage of locomotor activity during day 9 did not show a main effect of group, $F < 1$. However, there was a significant main effect of zone, $F(2, 40) = 231.426$, $p < 0.0005$, and the zones X group interaction was statistically significant, $F(4, 40) = 3.736$, $p = 0.011$. Three one way ANOVAs of the percentage of locomotor activity in each zone during day 9 were conducted to further evaluate the significant interaction, employing a Bonferroni correction of $\alpha/3 = 0.0167$. Percent time spent in locomotor activity in the outer zone on day 9 did not differ significantly between the 3 groups, $p = 0.165$. On the other hand, the groups differed in activity in the middle zone, $p = 0.003$. The differences in the groups' activity in the center zone was not significant as well, $p = 0.023$. Fisher's LSD ($\alpha = 0.05$) was used for the post hoc tests to investigate where the group differences lie in the middle and center zone for locomotor activity. The percentage of locomotor activity in the late phase group ($18.80 \pm 1.41\%$) was significantly

greater than that of the early phase group (9.40 ± 1.97%) in the middle zone, $p = 0.001$. Moreover, the late phase group also displayed a greater percentage in locomotion than the control group (12.29 ± 1.30%), $p = 0.009$. In the center zone, the late phase group (8.78 ± 1.72%) displayed a greater percentage in locomotion than the early phase group (3.25 ± 1.08%), $p = 0.011$, and the control group (4.05 ± 1.12%), $p = 0.018$.

Finally, the analyses revealed a nonsignificant main effect of group of the percent of locomotor activity on day 10, $F(2, 20) = 1.066$, $p = 0.363$. The main effect of zones was significant, $F(2, 40) = 181.887$, $p < 0.0005$, but the zones X group interaction was not significant, $F < 1$. Paired samples t-tests comparing the percentage of locomotor activity in the different zones on day 10 revealed a significant difference between the outer zone (43.84 ± 2.10%) and middle zone (13.65 ± 0.98%), $t(22) = 12.509$, $p < 0.0005$. Percentage scores in the outer zone were significantly higher than scores in the center zone (5.39 ± 0.74%), $t(22) = 15.850$, $p < 0.0005$, and scores in the middle zone were significantly higher than in the center zone, $t(22) = 8.701$, $p < 0.0005$.

Immobility. The main effect of group, the days X group interaction, and the 3-way interaction of zones X day X group were not significant, $F < 1$. Nor were the main effect of days, $F(2, 40) = 2.033$, $p = 0.144$, the zones X group interaction, $F(4, 40) = 1.695$, $p = 0.176$, and the zones X days interaction, $F(4, 80) = 2.283$, $p = 0.0.68$, significant. The main effect of zones was significant, $F(2, 40) = 13.92$, $p = 0.0005$, which means that the proportion of time recorded for immobility in the open field differed between at least one of the zones. To analyze this further, 2 paired samples t-tests were completed. Across groups, the percentage of time spent immobile in the outer zone (1.52 ± 0.33%) was significantly higher than in the middle zone (0.14

± 0.04%), $t(22) = -3.976$, $p = 0.001$. The difference in immobility in the middle zone in comparison to the center zone (0.05 ± 0.04%) was not significant, $t(22) = -1.423$, $p = 0.172$.

Differences between the groups emerged in the total distance travelled in the open field. As mentioned above, the duration and total distance travelled were analyzed together, although previously only the duration data were reported. Employing a Bonferroni correction of $\alpha/2 = 0.025$, a 3 way zones X days X group mixed model ANOVA revealed a significant main effect of group in the total distance travelled, $F(2, 20) = 5.681$, $p = 0.011$. Note that the duration data did not reveal a significant main effect of group. The zones X days X group interaction was also significant, $F(8, 80) = 3.247$, $p = 0.003$, indicating that the total distance travelled differed among groups within the open field. Three separate zones X group mixed models ANOVAs were therefore used to further investigate distance data from days 8, 9, and 10, with the alpha adjusted to $\alpha /3 = 0.017$.

The main effect of group for total distance travelled on day 8 was significant, $F(2, 20) = 5.582$, $p = 0.012$, suggesting that at least one of the groups differed from the others. The zones X group interaction was significant, $F(4, 40) = 5.896$, $p = 0.001$. Three one way ANOVAs of distance travelled in the outer, middle, and center zones further analyzed the significant zones X group interaction, with a Bonferroni correction of $\alpha/3 = 0.017$. Differences in the total distance travelled in the outer zone on day 8 was statistically significant, $p = 0.005$, whereas differences in the middle zone, $p = 0.323$, and the center zone, $p = 0.637$, were not significant. Fisher's LSD ($\alpha = 0.05$) was used to analyze further where the group differences were. The distance travelled by the early phase kindled group (3798.59 ± 278.78 cm) was significantly greater than the distance travelled by the control group (3010.99 ± 94.96 cm), $p = 0.010$. Furthermore, the

distance travelled by the late phase group (3983.04 ± 277.66 cm) was significantly greater than the control group, $p = 0.003$.

Of total duration of activity on day 9, analyses revealed no significant differences other than a significant main effect of zone, which was found on all days of the open field for the duration and the total distance travelled. On day 10, however, the main effect of group was significant, $F(2, 20) = 5.240$, $p = 0.015$, which was not reflected in the analyses of duration. Further analysis with Fisher's LSD ($\alpha = 0.05$) indicated that the late phase group (1497.57 ± 126.09 cm) and control group (1158.16 ± 86.40 cm) differed significantly from each other, $p = 0.004$. The total distance travelled on day 10 for the early phase group (1264.60 ± 98.17 cm) versus the late phase did not reach significance, $p = 0.053$ (Figure 8).

Object Recognition Test

A one-way ANOVA, with an alpha level = 0.05, was used to investigate the proportion of time spent between the 2 object zones during trial 2 of the object recognition test. No significant differences were found between groups in the percentage of time spent in the zone containing the familiar object as well as in the zone with the novel object, $F < 1$. This suggests that the early phase group, late phase group, and control groups did not perform differently in the percentage of time spent exploring the familiar or the novel object. Between the 2 object zones, however, a paired samples t-tests revealed that all groups spent a higher proportion of time in the object zone with the novel object (early phase: 19.93 ± 2.13%; late phase: 18.98 ± 7.75%; controls: 16.99 ± 5.37%) compared to the object zone with the familiar toy (early phase: 11.20 ± 1.34%; late phase: 13.52 ± 5.52%; controls: 11.64 ± 3.68%; Figure 9), $p = 0.001$.

Water Maze

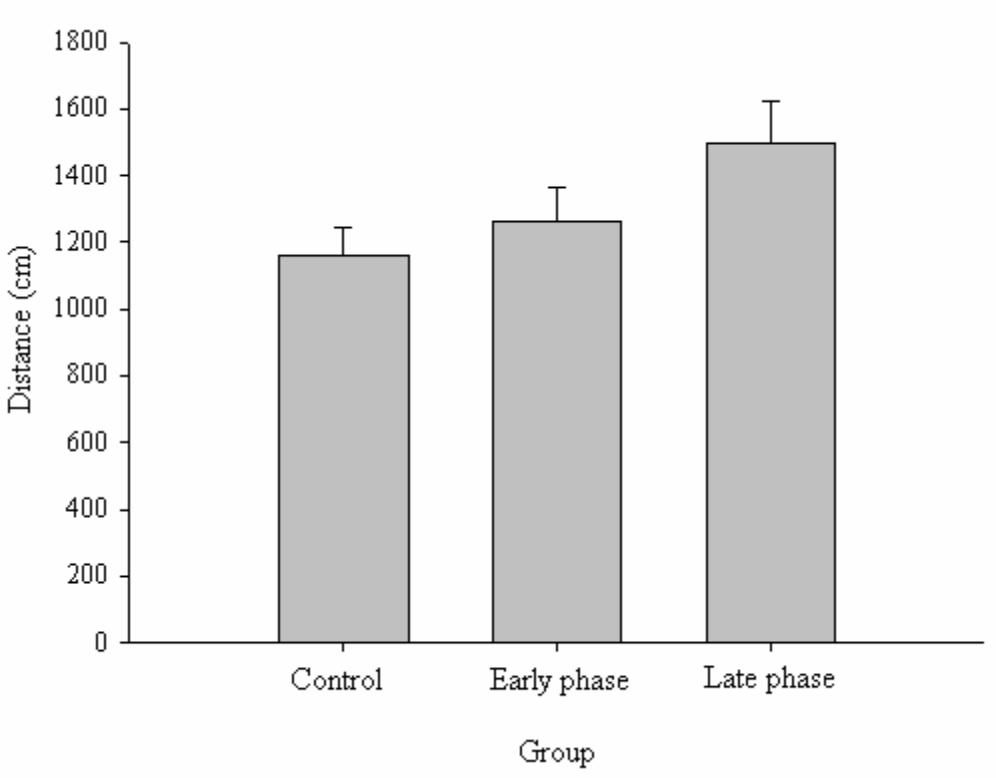

Figure 8. Mean distance travelled on day 10 between groups. A significant main effect was found between the late phase group compared to the control group only ($p = 0.004$). That is, the late phase group on average travelled a significantly greater distance in the open field on day 10 compared to the control group ($p = 0.004$).

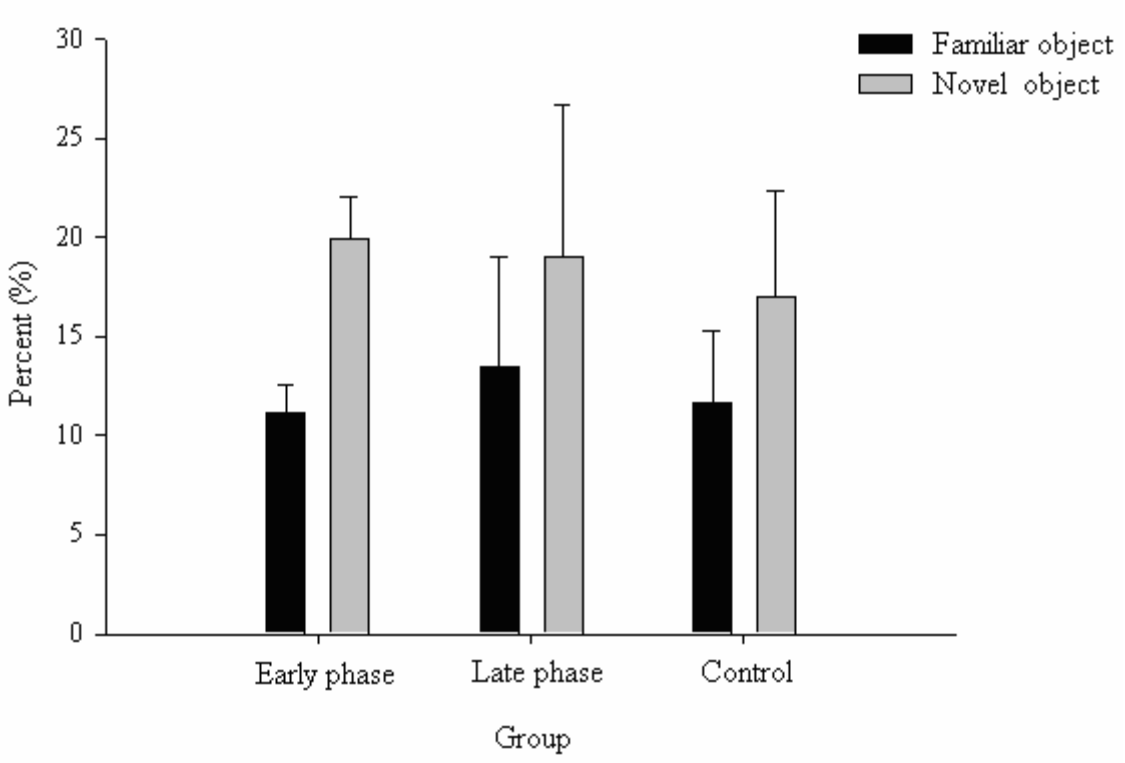

Figure 9. Percent of time spent with the familiar and novel objects in the object recognition task. Trial 2 of the object recognition task lasted 3 min. The location of the novel object was counterbalanced across animals. There was a significant difference between the percent of time spent exploring the familiar object compared to the novel object ($p = 0.002$). The main effect of group and interaction were not significant.

The latency to escape to the submerged platform and total distance swum to reach the submerged platform during acquisition day were recorded. An alpha correction of $\alpha/2 = 0.025$ was employed. However, because the 2 dependent variables were highly correlated, only the analyses for latency to escape will be reported.

Acquisition in the WM comprised 15 trials that terminated when rats located the submerged platform or at 60 sec. Data from every 3 trials were grouped into Blocks. Therefore, Block 1 contained the data for trials 1 to 3, Block 2 for trials 4 to 6, and so on. The main effect of group was not significant, $F(2, 20) = 0.492$, $p = 0.619$, which indicates that no difference existed between the groups in the speed of acquisition of spatial information. The main effect of Block was significant, $F(4, 80) = 33.410$, $p < 0.0005$, suggesting that at least one of the Blocks differed significantly from another Block. The Block X group interaction was not statistically significant, $F(8, 80) = 2.343$, $p = 0.026$.

Paired samples t-tests were conducted between Block 1 and 2, and Block 2 and 5 with a Bonferroni correction of $\alpha/3 = 0.017$. As illustrated in Figure 10, the latency to escape from Block 1 (33.78 ± 2.73 sec) was greater than the latency in Block 2 (16.34 ± 1.26 sec), $t(22) = 5.477$, $p < 0.0005$. In addition, latencies to escape in Blocks 1 and 5 (14.03 ± 1.09 sec) were also significantly different, $t(22) = 6.317$, $p < 0.0005$. The comparison between Block 2 and 5, however, was not significant, $t(22) = 1.346$, $p = 0.192$.

Trial 16 was a probe trial in which the submerged platform was removed and the percentage time spent in each quadrant was measured. The main effect of group and the quadrant X group interaction were not significant, $F < 1$. However, the main effect of quadrant was significant, $F(3, 60) = 50.113$, $p < 0.0005$. Post hoc analysis was performed with five paired samples t-tests, with a Bonferroni correction of $\alpha/5 = 0.01$. Figure 11 reveals that groups spent

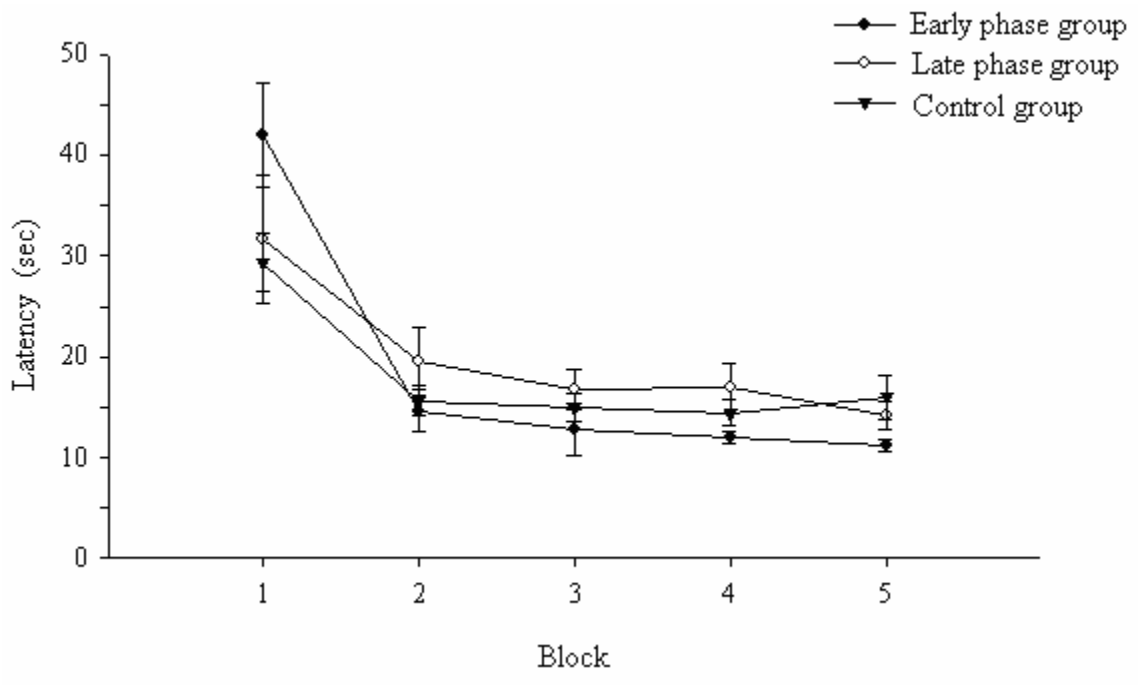

Figure 10. Latency to escape to the platform on acquisition day in the WM. Each Block represents data from 3 trials collapsed. Data from Blocks 1, 2 and 5 were chosen for t-tests to investigate where significant differences in latency to escape could be found between the 1st and 2nd Block, as well as the 2nd and 5th Block. There was a significant reduction in the latency to escape to the submerged platform from Block 1 to 2 ($p < 0.0005$), but the difference between Block 2 to 5 was not statistically significant ($p = 0.192$). There were no differences in performance found between the early phase, late phase, and control groups.

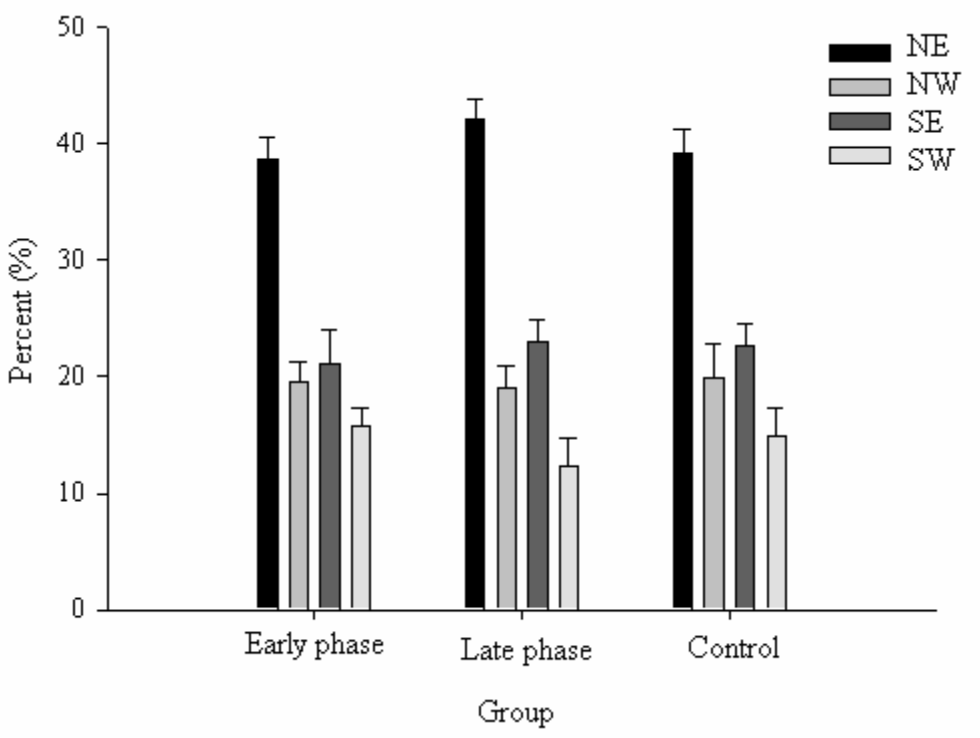

Figure 11. Percent of time spent in each quadrant during the probe trial on acquisition day. The percent of time spent in the NE quadrant was significantly higher compared to the mean duration spent in the NW, SE and SW quadrants ($p < 0.0005$). The difference in amount of time spent in the SE quadrant compared to the SW quadrant was also significant ($p < 0.005$). Differences between groups were not significant.

a much higher percentage of time in the NE quadrant (39.78 $\pm$ 1.18%) compared to the NW quadrant (19.59 $\pm$ 1.43%), $t(22) = 9.442$, $p < 0.0005$; the SE quadrant (22.29 $\pm$ 1.26%), $t(22) = 9.183$, $p < 0.0005$; and the SW quadrant (14.49 $\pm$ 1.28%), $t(22) = 11.873$, $p < 0.0005$. The difference between the SE and SW quadrant was also statistically significant, $t(22) = 4.046$, $p = 0.001$. On the other hand, the proportion of time spent in the SE versus NW quadrant was not significant, $t(22) = -1.164$, $p = 0.257$.

Following the probe trial, 3 more trials (trials 17 to 19) in the presence of the submerged platform continued. A trial X group mixed model ANOVA was performed on the latency to escape on trials 17 to 19, with a Bonferroni correction of $\alpha/2 = 0.025$. The main effect of group and the trial X group interaction for trials 17 to 19 were not significant, $F < 1$. The insignificant interaction of trial X group indicated that regardless of which trial, groups did not show performance differences on trials 17 to 19 or vice versa. The main effect of trial was also not significant, $F(2, 40) = 2.444$, $p = 0.100$ (Figure 12).

After a 28 day break, the retention day involved a total of 3 trials with the submerged platform and a final probe trial. A trial X group mixed model ANOVA was carried out for the latency to escape and distance swum, with a Bonferroni correction of $\alpha/2 = 0.025$. Only the latency to escape will be reported. Figure 13 illustrates the latency to escape times for trials 1 to 3 on retention day. No group effects were found, $F < 1$. The trial X group interaction was also not significant, $F(4, 40) = 1.286$, $p = 0.292$. The main effect of trial was significant, $F(2, 40) = 13.320$, $p < 0.0005$; therefore a paired samples t-test was performed on data from the 1st (25.25 $\pm$ 3.20 sec) and 2nd trial (13.21 $\pm$ 0.94 sec). The difference was significant, $t(22) = 3.872$, $p = 0.001$, suggesting that the 1st trial back after the 28 day break did result in a transient deterioration in performance, presumably due to decay of memory.

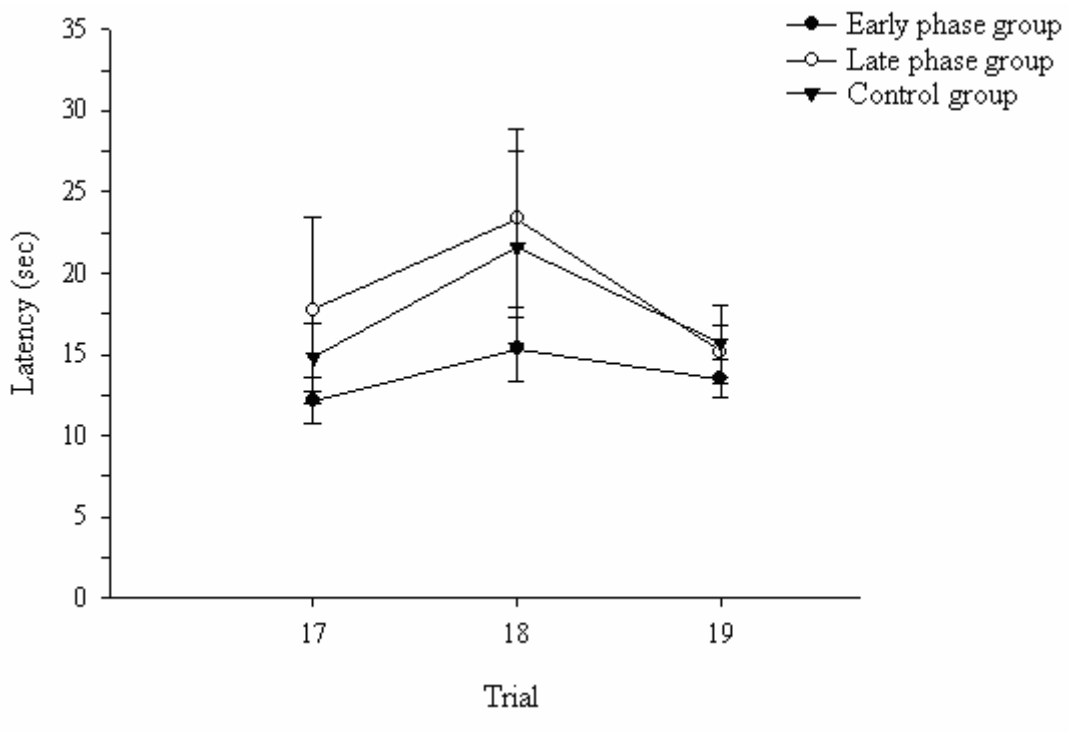

Figure 12. Latency to escape on acquisition day trials 17 to 19. No significant differences in performance were found between experimental groups. In addition, differences between the 3 trials were not statistically significant.

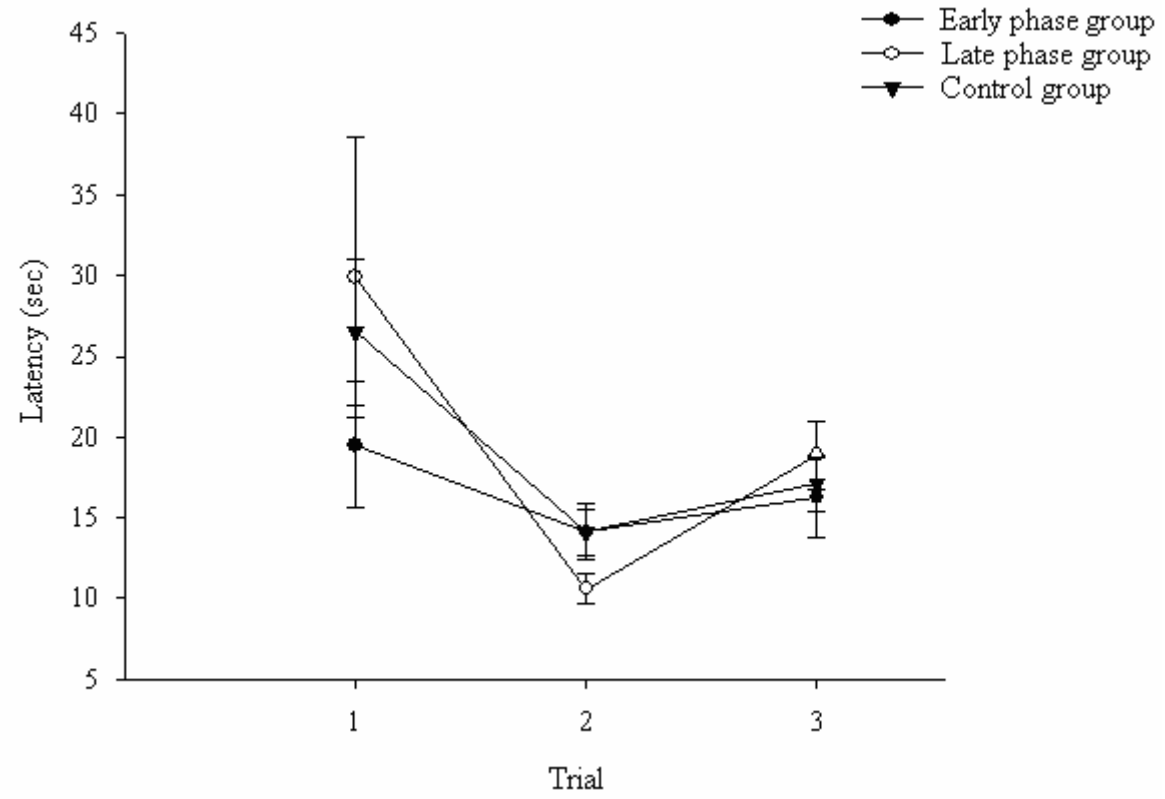

Figure 13. Latency to escape to the platform on retention day. Performance on the first retention trial after the 28 day break shows a significantly higher latency to escape to the submerged platform compared to trials 2 and 3. Differences between the groups were not significant.

Analysis of the latency to escape on the last trial of acquisition day compared to the first trial on retention day also further demonstrated evidence of minor memory deficits; although the main effect of group and interaction were not significant, $F < 1$, the main effect of trial was significant, $F(1, 20) = 15.044$, $p = 0.001$. That is, a comparable memory deficit was found in the early phase, late phase, and control groups between the last trial of acquisition day (early phase: 13.52 $\pm$ 1.16 sec; late phase: 15.17 $\pm$ 1.62 sec; control: 15.63 $\pm$ 2.38 sec) and the first trial of retention day (early phase: 19.52 $\pm$ 3.94 sec; late phase: 29.89 $\pm$ 8.68 sec; control: 26.48 $\pm$ 4.58 sec; Figure 14).

Discussion

Characteristics of CLA kindling

In the present study, I examined the behavioural consequences of kindling the anterior CLA. Histology confirmed that the electrodes were placed in the anterior CLA. Kindling of the anterior CLA resulted in the appearance of 2 distinct phases. The early phase of CLA kindling is similar to what is observed in neocortical kindling: short AD durations, forced motor responses (i.e., log rolls), short clonus durations, and rapid progression into bilaterally generalized seizures (Mohapel et al., 2000). The early phase quickly developed into late phase CLA kindling, characterized by much longer seizures and clonus durations, which resembled limbic-type (i.e., amygdaloid or hippocampal) seizures.

The anterior CLA is very susceptible to kindling, in that it required fewer kindling sessions to meet the criterion of 5 consecutive stage 5 seizures as compared to hippocampal (Hannesson et al., 2001a; Hannesson et al., 2001b) or amygdaloid kindling (Zhang et al., 2001). Furthermore, as expected, late phase kindling required more stimulations than early phase

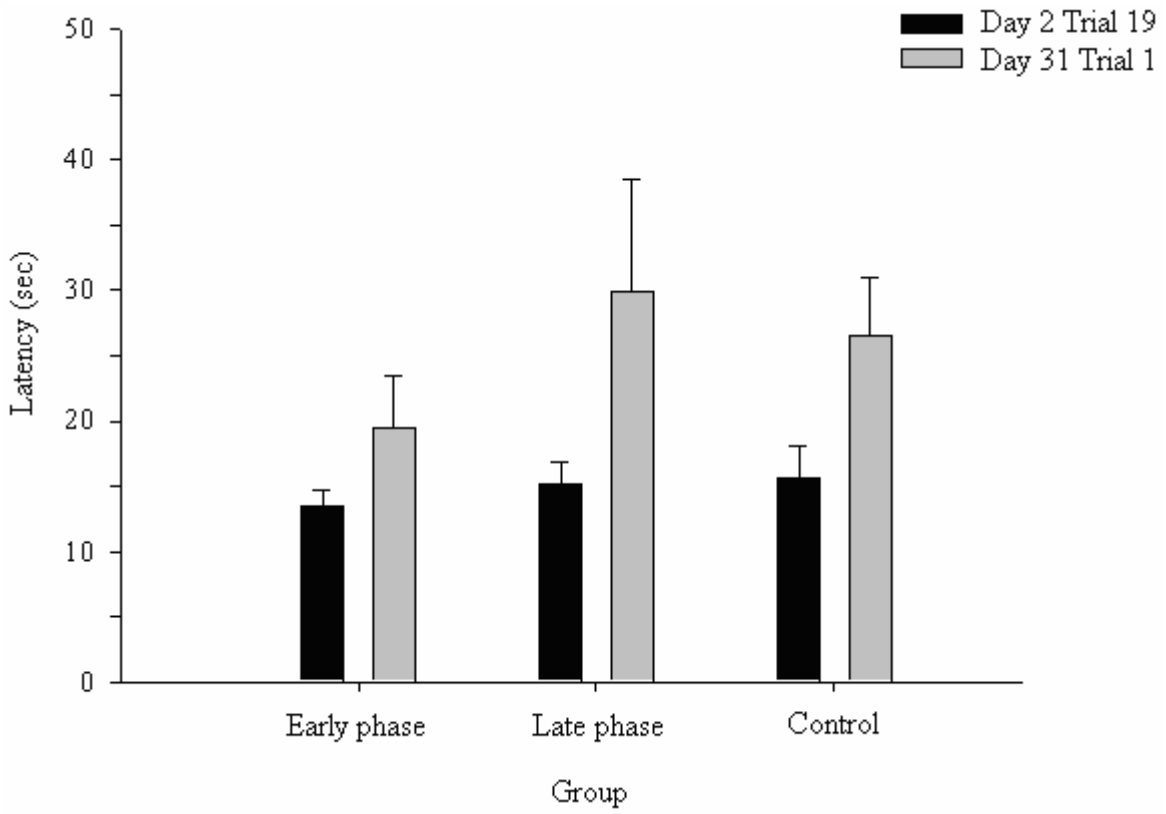

Figure 14. Latency to escape on the last acquisition trial and first retention trial. Across all groups, there was a significantly higher latency to escape on the first retention trial, 28 days after the last acquisition trial. The groups did not differ significantly from each other.

kindling. That is, a mean of 10.9 $\pm$ 0.6 stimulations was required to kindle the first early phase stage 5 seizure and a mean of 20.7 $\pm$ 1.2 stimulations to kindle the first late phase stage 5 seizure in this study (data not shown). These results vary somewhat from previous descriptions of CLA kindling. For example, Zhang et al. (2001) reported that an average of 5.6 $\pm$ 1.4 stimulations was required to kindle the first early phase stage 5 seizure and 9.3 $\pm$ 1.3 stimulations to kindle the first late phase stage 5 seizure with stimulation of the anterior CLA. Mohapel et al. (2001) kindled the posterior CLA and found that a mean of 13.7 $\pm$ 1.1 stimulations was required to kindle the first late phase stage 5 seizure. A reason for the discrepancy in kindling rates may be differences in electrode placements. Zhang et al.'s (2001) electrode placements were located within the central region of the anterior CLA, whereas the electrode placements in my study were in the dorsal-anterior CLA, bordering along the anterior forceps of the corpus callosum. Mohapel et al.'s (2001) electrodes were of course aimed at the posterior (i.e., dorsolateral) CLA.

In terms of latency to clonus, the early phase group displayed longer latencies than the late phase group. Clonus durations and AD durations were much longer in the late phase group, as expected. These findings are similar to what has been described by previous researchers (Mohapel, Zhang, Gillespie, Chlan-Fourney, Hannesson, Corley, Li, & Corcoran, 2001; Sheerin et al., 2004; Zhang et al., 2001) and are an indication of the more severe generalized seizures in the late phase group. These observations further indicate that the anterior CLA is extremely susceptible to kindling and are suggestive of the possibility that the CLA may play an important role in seizure generalization.

Rats in the early phase group did not displayed secondary AD or wet dog shakes. On the other hand, although there was considerable variability, the likelihood of observing secondary AD and wet dog shakes in rats belonging to the late phase group was much higher. Therefore,

because secondary AD and wet dog shakes are commonly seen in limbic-type seizures (e.g., amygdaloid and hippocampal kindled seizures), this would suggest that late phase kindling involves propagation of AD to limbic structures, leading to development of limbic-type seizures. Indeed, Sheerin et al. (2004) have reported that late phase CLA kindling is associated with strong limbic AD.

Behavioural effects of CLA kindling

Because late phase kindling in the CLA involves limbic-type seizures, and limbic kindling produces distinct and reliable behavioural effects, I hypothesized that late phase kindling, but not early phase kindling, would lead to changes in some of the behaviours affected by limbic kindling. The EPM, the open field task, the object recognition test, and the WM were used to test the hypothesis.

The performance on EPM, a test for anxiety-like behaviour, was very consistent: rats spent a majority of their time in the closed arms compared to the open arms. This reflects an aversion towards the open arms due to anxiety or fear, which was expected (Pellow, Chopin, File, & Briley, 1985). There were, however, no group differences. That is, kindled and control rats did not exhibit different levels of anxiety-related behaviours with regard to the duration, percentage of grooming, locomotor activity, rearing, and immobility in the closed versus open arms.

These results are in contrast to the effects of amygdaloid kindling, which has been shown to consistently produce avoidance of open arm entries, presumably reflecting elevated levels of anxiety (Adamec & Shallow, 2000a; Adamec & Young, 2000). As long-term kindling sessions continue to increase levels of anxiety, rats may become so fearful in the EPM that they spend significantly more time in the open arms searching to escape by jumping or leaping (Adamec &

McKay, 1993; Adamec & Morgan, 1994; Adamec & Young, 2000; Kalynchuk et al., 1998a; Kalynchuk, Pinel, Treit, & Kippin, 1997). Similarly, Hannesson et al. (2005) showed that full kindling of the anterior perirhinal cortex also results in enhanced anxiety-like behaviour and therefore lower open-arm entries in the EPM, whereas hippocampal kindling does not affect anxiety (Hannesson et al., 2001a). Thus, unlike amygdaloid and perirhinal kindling, early phase or late phase kindling in the anterior CLA did not alter anxiety-related behaviours.

Before I discuss further the remaining behavioural tests, it is important to explain what I mean by the extent of kindling. Hannesson & Corcoran (2000) divided the extent of kindling into 3 categories: partial, full, and extended. Partial kindling refers to kindling where generalized convulsions were not evoked (Hannesson & Corcoran, 2000). Full kindling involves 1 to 30 generalized convulsions (Hannesson & Corcoran, 2000). Extended kindling involves more than 30 generalized convulsions (Hannesson & Corcoran, 2000). It is noteworthy that partial or full kindling results in anatomically specific changes in behaviour, whereas anatomical specificity is lost with extended kindling.

The open field task, a test for anxiety and exploratory behaviours, indicated that from day 8 to day 10, habituation occurred. That is, more time was spent in the outer zone of the open field on day 8 and less time was spent in the middle or center zones. By day 10, rats were spending more time in the middle and center zones. Of interest, late phase kindled rats were spending more time in the middle and center zones than the other two groups. These results indicate that rats in the late phase group habituated more or had significantly lower levels of anxiety compared to rats in the early phase and control groups.

Another interesting finding in the open field was a difference in the percentage of time spent on locomotor activity on day 9. Rats in the late phase group displayed significantly more

locomotor activity in the middle and center zone than rats in the early phase and control groups. To characterize this further, I analyzed each group's mean velocity (measured as cm per sec; data not shown) and found that the late phase group travelled significantly faster than the other 2 groups ($p = 0.033$) in the outer zone on day 8. However, this analysis does not clarify the behaviour of the late phase kindled rats on day 9. Although no main effect of group was found in grooming, rearing, locomotor activity, and immobility, it may have been the case that late phase kindled rats simply spent less time performing each of these behaviours, thus permitting more time for travelling and exploring the open field. At the same time, however, the EPM provided no evidence that anxiety-like behaviour in kindled rats was different from controls. Likewise, there were no significant group differences in the total distance travelled in the EPM (data not shown). Thus, understanding of the late phase kindled rats' tendency to spend more time travelling or exploring particular zones on particular days in the open field will require further research.

The effects of CLA kindling differ from the effects of kindling some limbic sites. For instance, Kalynchuk et al. (1997) reported that extended amygdaloid kindling resulted in increased anxiety-like behaviour and thus decreased open field activity (for groups receiving 60 or 100 stimulations) and enhanced thigmotaxis (in groups receiving 20 or 100 stimulations). Similarly, full perirhinal kindling led to significant decreases in the number of entrances into the center zone of the open field, although total distance travelled between kindled and control rats did not differ (Hannesson et al., 2005). In contrast, full dorsal hippocampal (dHPC) kindled and control rats spent similar dwell times in the central zone of the open field, suggesting that anxiety levels were not affected (Hannesson et al., 2001a).

Anterior CLA kindling did not disrupt object recognition memory, because all groups displayed a similar tendency to investigate the novel object in the second trial. Full kindling of the dHPC also failed to affect object recognition memory, suggesting that non-spatial forms of cognition are spared (Hannesson et al., 2001a). However, perirhinal kindling results in disruption of object recognition memory, in that Hannesson et al. (2005) reported that kindled rats failed to show significant biases towards the novel object.

Kindling of the anterior CLA also did not affect spatial learning in the WM. All groups learned the location of the submerged platform within the first 6 trials (Block 1 and 2) of acquisition day. Probe trials also indicated that rats spent a higher percentage of their time in the quadrant where the platform was located. Likewise, on the first day of retesting after the 28 day break, all groups showed a comparable deterioration in memory.

These findings are similar to those seen with perirhinal kindling, which fails to affect spatial learning (Hannesson et al., 2005). In comparison, kindling of hippocampal field CA1 to 3 consecutive stage 5 seizures, but not partial kindling, leads to an impairment in learning the location of the submerged platform (Gilbert et al., 1996). It was also reported that evoking generalized seizures in kindled rats 25 - 45min before testing led to profound deficits in the latency to escape, whereas evoking nonconvulsive AD 25 - 45min before testing failed to affect performance (Gilbert et al., 1996). However, Gilbert et al. (2000) later reported that eliciting non-convulsive seizures in field CA1 25 - 45min before testing did produce deficits in acquisition. Full dHPC kindling has also been shown to disrupt spatial memory in the WM (Hannesson et al., 2001a).

Failure to see behavioural changes

There may be several explanations as to why I saw no evidence that anterior CLA kindling produces many changes in behaviour. For example, the ventral CLA of the rat and cat has reciprocal connections with olfactory structures and limbic system, whereas the dorsal CLA has bilateral projections to most of the neocortex and visual and motor areas (Cortimiglia et al., 1991). Thus, perhaps the electrode placements in the present experiment were all in the dorsal aspect of the anterior CLA, and kindling simply did not affect the limbic system sufficiently to produce noticeable changes in behaviour. Arguing against this interpretation, however, some researchers have claimed that injections of fluorescent retrograde tracers, fast blue and nuclear yellow in Wistar rats, revealed that neurons in the anterior and central CLA project to all limbic areas (Majak, Kowianski, Morys, Spodnik, Karwacki, & Wisniewski, 2000).

Conceivably, triggering 5 consecutive late phase generalized seizures was not enough to produce noticeable changes in behaviour. Thus behavioural changes might occur after extended kindling of the CLA. Note, however, that because repeated kindling leads to progressive amplification and propagation of AD to widespread regions in the brain (Hannesson & Corcoran, 2000), extended kindling beyond the late phase could result in the loss of anatomical specificity, making it difficult to determine the functional role of the CLA. For instance, it has been reported by Hannesson et al. (2005) that extended kindling of a variety of sites outside the hippocampus can lead to disruptions of spatial cognition, whereas full kindling of these sites would not produce the same behavioural effects (Hannesson & Corcoran, 2000).

Another consideration is our standard battery of tests. Use of the EPM, open field task, object recognition test, and WM permits comparison to the behavioural effects of kindling other sites previously conducted in our lab. But perhaps the behaviours sampled in these tests are not relevant to the functions of the CLA, and other behavioural tasks would have been more

sensitive in revealing the effects of CLA kindling. For example, Sloniewski et al., (1995) reported that subcutaneous injections of formalin into forepaws of rats increased radioactive glucose uptake in the contralateral CLA, anterior pretectum, ventral thalamic nucleus, somatosensory, insular and piriform cortices. Injections of morphine into the contralateral pretectum to the side of stimulation abolished glucose uptake in the CLA (Sloniewski et al., 1995). Their conclusion was that the CLA is a relay nucleus that seems to convey somatosensory information from the pretectum to the cerebral cortex, bypassing the thalamus, via the pretecto-claustrl-cortical pathways (Sloniewski et al., 1995). Hence, a test of reaction to painful stimulation might be more appropriate to reveal the effects of CLA kindling.

There may also be differences between propagated AD versus AD triggered by direct electrical stimulation. That is, kindling in the CLA results in propagation of AD to limbic and other sites (e.g., amygdala, hippocampus, perirhinal cortex), but largely fails to affect behaviours that are altered when AD is triggered in these sites by direct application of electrical stimulation to the sites. Therefore, neuronal behaviour underlying AD triggered by direct stimulation may differ in significant ways from neuronal behaviour underlying propagated AD – for example, in the duration of burst discharges, the number of neurons displaying bursting, or the intensity of inhibitory influences – and these differences in cellular behaviour could account for the different behavioural consequences of AD triggered in a site versus propagated secondarily from the CLA. Sophisticated electrophysiological techniques would be required to evaluate this hypothesis.

Finally, and related to the point above, Sheerin et al. (2004) suggested that the closer a structure is functionally to sites intimately linked to seizure generalization, the less kindling is required and the less likely kindling of the structure is to drive plastic changes at distant sites. In other words, structures distant from substrates of seizure generalization may require more

kindling than sites closer to the substrates, and the more distant structures may be resistant to transfer (whereby kindling one site subsequently leads to faster kindling of another site) from closer substrates (Sheerin et al., 2004). As a result, one could expect that the limbic system would require more kindling than the CLA, which arguably is closer to substrates of seizure generalization (Sheerin et al., 2004). In addition, limbic sites would show transfer from other limbic sites, but not from the CLA (Sheerin et al., 2004). Consequently, eliciting and propagating AD from the CLA and other structures closely involved in the seizure generalization network may not be sufficient to drive plastic changes in limbic sites and trigger noticeable changes in behaviour.

Implications

Recording, lesion, and pharmacological studies have consistently shown that the amygdala is involved with emotional function, the hippocampus with spatial function, and the perirhinal cortex with object-related cognitive function (Hannesson & Corcoran, 2000). Correspondingly, amygdaloid kindling produces changes in anxiety-like behaviours, tested with the EPM and open field, without affecting object recognition memory; hippocampal kindling produces deficits in learning and memory related to spatial cognition, tested using the WM, while sparing object recognition; and perirhinal kindling disrupts object memory, tested with the object recognition test, while sparing spatial cognition. The general conclusion of these studies is that the effects of kindling are site specific. The results of my research support the idea that the CLA is closely linked to substrates of seizure generalization. More important, and contrary to expectations, late phase kindling of the anterior CLA did not result in changes in behaviour, at least in the behaviours measured in our standard battery of tests. This reinforces the idea that behavioural changes after kindling are not caused by the seizures themselves, but must be

attributed to changes in the local circuitry of the area stimulated. Furthermore, such changes apparently do not occur when AD is propagated after full kindling of the CLA. An important topic for future research will be to investigate the differences in neuronal behaviour during propagated versus directly triggered AD, because this may help us to understand the mechanisms underlying the behavioural effects of epileptogenesis.

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