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Physical Exercise Increases Hippocampal Cell Loss in a Model of Epilepsy

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Abstract of the Dissertation

Physical Exercise Increases Hippocampal Cell Loss in a Model of Epilepsy

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The hippocampus is especially vulnerable to damage from a variety of insults. Administration of the neurotoxin kainic acid (KA) can produce a pattern of hippocampal sclerosis consistent with human temporal lobe epilepsy. Cell loss in this model is thought to be the downstream effect of severe behavioral seizures (*status epilepticus*). In animals, cell loss is often studied only after status epilepticus (SE), whereas in humans, SE is less common. Two studies were carried out to better understand the relationship between SE and cell loss, and the potential influence of lifestyle factors in the form of exercise. In the first study, we explore when you do and do not achieve hippocampal cell loss resulting from ICV KA. In the second study, we explore how environmental factors impact on cell loss.

To test whether SE is necessary for cell loss, KA was infused into the right cerebral ventricle of male rats (n=11). Subjects were categorized for seizure intensity and cell loss. While only 2/11 rats progressed to SE, cell loss was widely expressed in non-SE animal, suggesting that SE was not necessary to produce cell loss.

The present study tested whether experience in the form of exercise could influence such outcomes. CA3 cell layer length was measured following intracerebroventricular (ICV) injection of KA in sedentary control animals (n=14) and in rats that were pre-conditioned with 42 days of voluntary running (n=15). The cell layer length in both hemispheres was measured. Whereas only 2/15 of exercise animals progressed to SE compared to 5/14 of controls, cell loss was evident in proportionally more VX animals, especially among non-SE runners compared to non-SE controls (p=.040). Non-SE exercise animals exhibited shorter CA3 cell layer length in both hemispheres.

In conclusion, while exercise reduces the likelihood of animals progressing to SE and expressing the widespread cell loss associated with intense seizures, a higher proportion of VX animals exhibited more subtle cell loss resulting from sub-SE seizures. This pattern of findings paradoxically suggests that exercise spares the hippocampus from extensive neuronal damage, but increases the likelihood of moderate CA3 cell loss when KA is administered ICV.

In loving memory of
Gabriel Samuel Mitchell

CONTENTS

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1. GENERAL INTRODUCTION

1.1 Physical exercise influences hippocampal function

Physical activity in the form of regular exercise has long been recognized for contributing to good health and reducing the occurrence of many diseases. The human body is genetically equipped for a physically active lifestyle of hunting and gathering in order to ensure survival (Booth, Chakravarthy, & Spangenburg, 2002). However, the relatively sedentary nature of modern living precludes such behavior. In part, physical activity must affect health via its effects at the cellular level. Lack of activity potentially disrupts cellular homeostasis, ultimately impacting upon the pattern of gene expression in cells (Booth, Chakravarthy, & Spangenburg, 2002) and potentially on cell fate.

Whether the "sedentary" or "exercise" condition signifies a *true* experimental manipulation remains a topic of debate. There is some evidence to suggest that the relatively sedentary nature of modern life represents a condition inconsistent with optimal cellular homeostasis (Booth, Chakravarthy, & Spangenburg, 2002; Cotman & Berchtold, 2002; Cotman & Engesser-Cesar, 2002; Trejo, Carro, Nunez, & Torres-Aleman, 2002). In this manner, physical activity in the form of exercise may be considered as the control condition. By contrast, in experimental studies, exercise is often operationally defined as the *addition* of physical activity in both human (Colcombe et al., 2003; Colcombe et al., 2006; Kramer & Erickson, 2007; Kramer, Erickson, & Colcombe, 2006) and animal (Arida, de Jesus Vieira, & Cavalheiro, 1998; Arida, Scorza, dos Santos, Peres, & Cavalheiro, 1999; Carro, Nunez, Busiguina, & Torres-Aleman, 2000; Ramsden,

Berchtold, Patrick Kesslak, Cotman, & Pike, 2003; Trejo, Carro, Nunez, & Torres-Aleman, 2002; Vaynman, Ying, & Gomez-Pinilla, 2004) subjects. Consistent with the methodology utilized in previous studies, exercise will be defined as an increase in physical activity for the purposes of the present investigation. Additionally, given the low-cost and ready availability of exercise to most of the population, it is worthwhile to experimentally investigate the effect of added physical activity in both wellness and disease conditions.

A large human study of Alzheimer's Disease and other age-related dementias revealed that prevalence of these pathologies is significantly reduced with greater physical activity in older adults (Laurin, Verreault, Lindsay, MacPherson, & Rockwood, 2001). Recent findings suggest that exercise may reverse frontal lobe volume loss normally seen in aging (Colcombe et al., 2003; Colcombe et al., 2006), and cognitive deficits associated with this volume loss (Kramer & Erickson, 2007; Kramer, Erickson, & Colcombe, 2006). In this way, exercise may impact on brain structure and function in ways that can have implications both for the healthy brain and for disease conditions. To understand both the causal relationships, and to understand the mechanisms through which exercise may exert effects on the brain, animal models can be used.

A number of studies in animals report exercise effects on brain function and a number of brain variables. Exercise affects metabolic support in a number of motor related areas of the brain. It increases capillary density in the cerebellum (Isaacs, Anderson, Alcantara, Black, & Greenough, 1992), and increases metabolic capacity in the motor cortex and striatum (McCloskey et al., 2003). Physical exercise has been shown to reduce stroke-related hippocampal and striatal damage in gerbils (Stummer,

Weber, Tranmer, Baethmann, & Kempski, 1994). Several investigators have shown exercise induced plasticity in the hippocampus. The hippocampus – the major brain region associated with learning and memory – exhibits electrophysiological activity related to movement (Bland & Vanderwolf, 1972; Czurko, Hirase, Csicsvari, & Buzsaki, 1999), thus long-term plasticity in response to exercise may not be so surprising.

In the rat hippocampus, gene expression is altered by physical activity (Farmer et al., 2004; Neeper, Gomez-Pinilla, Choi, & Cotman, 1996; Tong, Shen, Perreau, Balazs, & Cotman, 2001)**,** including genes related to synaptic function (Tong, Shen, Perreau, Balazs, & Cotman, 2001). Long-term potentiation – an alteration of synapse strength – is reported to be enhanced in the hippocampus of exercising animals (van Praag, Christie, Sejnowski, & Gage, 1999). Hippocampal-dependent learning also improves after exercise. Hippocampus-dependent spatial learning and memory in the radial arm maze (B. J. Anderson et al., 2000) and Morris water maze (Ahmadiasl, Hojjatallah, & Hanninen, 2003; Fordyce & Wehner, 1993; van Praag, Christie, Sejnowski, & Gage, 1999) is enhanced in animals following a regimen of physical activity. Thus, exercise enhances normal activity in the hippocampus, making this area of particular interest in further understanding the relationship between physical activity and brain function. The hippocampal formation is vulnerable to damage, especially damage caused by seizures, ischemia, and early deterioration of the type seen in Alzheimer's disease. If exercise causes hippocampal plasticity that supports learning, then it may also affect how cells respond to damaging events.

1.2 Human temporal lobe epilepsy

Ten percent of Americans will experience a seizure in their lifetime, most during childhood. About one-third of these individuals go on to later develop epilepsy. The condition can be a response to high fevers, exposure to toxins, or previous head injury. One particular class of epilepsy, temporal lobe epilepsy (TLE), is associated with abnormal excitatory neuronal activity originating in the hippocampus, the major brain region involved in spatial learning and memory, and spreading to adjacent brain regions. The physiological occurrence is accompanied by the behavioral seizures that characterize the condition. Permanent cell loss in discrete segments of the hippocampal formation is seen in human patients, and is believed to be principally responsible for the generation of recurrent seizures. The neuronal atrophy is thought to lead to synaptic reorganization and subsequent alterations in transmitter systems, both of which are then believed to contribute to the development of spontaneous seizures.

Approximately 50-70% of patients undergoing resection of the temporal lobe for the treatment of TLE show a pattern of neuronal loss in the hippocampus that corresponds to that first described by Sommer more than 120 years ago (1880). This mostly unilateral atrophy of the hippocampus has come to be known as Ammon's horn sclerosis (or hippocampal sclerosis) and is distinguished by segmental loss of neurons in CA1, CA3, and the hilus regions of the hippocampus (see Figure 1-1), while selectively sparing the neurons of CA2 and the dentate gyrus (DG) regions (Margerison & Corsellis, 1966) (see Appendix A for a brief review of hippocampal anatomy). The cell loss is accompanied by gliosis in the affected segments, resulting in shrinking and hardening of the hippocampal tissue.

Although hippocampal sclerosis is widely accepted as the source of epilepsy, there have been suggestions that the role of hippocampal sclerosis in epileptogenesis is overstated because of the relative ease of identifying cell loss in this brain region, and that instead atrophy of other structures (especially the amygdala) may be equally important in TLE pathology (Gloor, 1991). Margerison and Corsellis (1966) described atrophy in structures outside of the hippocampus, and noted that this was only present in conditions where cell loss in the hippocampus was expansive. Extra-hippocampal atrophy is virtually non-existent with conservative loss of neurons in the hippocampus, suggesting that hippocampal sclerosis may be the central histopathological feature of TLE. Thus, the hippocampus remains the focus of research on epilepsy. Current theories as to why neuronal loss in the hippocampus leads to seizures have been developed from observations in animal models.

1.3. Animal models of TLE

Animal models of epilepsy seek to model the behavioral and histopathological features of the human condition (see Appendix B for an extensive review). TLE is characterized behaviorally by the spontaneous, recurrent seizures that hallmark most forms of epilepsy. In addition to producing the hallmark behavioral seizures, animal models of TLE must also display a pattern of neuronal damage consistent with that seen in human TLE. Namely, patients undergoing resection of the temporal lobe for treatment of epilepsy frequently exhibit cell loss in the hippocampus, especially the CA3 and CA1 divisions (Meldrum, 1997; Meyer, Falconer, & Beck, 1954). In human patients with no prior medical history of epilepsy, SE has been shown to induce extensive hippocampal

damage (Fujikawa, Itabashi, Wu, & Shinmei, 2000). For this reason, the ability to produce hippocampal cell loss has become the central histological component of assessing animal models of epilepsy.

Two common animal models of epilepsy are kindling and chemical induction of high-intensity seizures, termed *status epilepticus* (SE). In kindling, the hippocampus/amygdala are directly stimulated via an electric current, resulting in behavioral seizures similar to those exhibited by human epileptics (Sato, Racine, & McIntyre, 1990). While kindling is a useful tool in the laboratory for producing seizures on demand, it does not reliably produce hippocampal damage consistent with the human pathology. In contrast, application of a single dose of a chemical convulsant (*chemoconvulsant*) is often sufficient to produce both the continuous seizures and resulting cell loss central to TLE. Two common such substances are pilocarpine and kainic acid (KA). Pilocarpine is a muscarinic acetylcholine agonist while KA is an analogue of the major excitatory neurotransmitter, glutamate. Both substances work by ultimately increasing excitatory, glutamatergic activity at synapses in the hippocampus. Chemoconvulsant-treated animals show hippocampal cell loss comparable to that observed in resected tissue from human epileptics, making them preferable for examining the development and progression of epilepsy.

The hippocampus is especially vulnerable to damage resulting from KA administration as this brain region expresses high levels of receptors (*kainate* receptors) with a high affinity for KA. The binding of kainate receptors on the large mossy fiber terminals of the hippocampus results in the release of copious amounts of glutamate onto

the pyramidal cells of CA3 (Ben-Ari, 1985). Glutamate in such large quantities can have a toxic and, even fatal, effect on this cell population.

1.4. Mechanism of cell death in TLE

Because of its high energy demand and continuous reliance on oxygen-dependent metabolism of glucose, the brain is especially vulnerable to the toxic challenge of KA administration and subsequent hyper-excitability caused by excessive glutamate release. The resulting loss of energy for the ion pumps that normally maintain the resting-state membrane potential creates an aberrant accumulation of ions on both sides of the cell membrane (Kristian & Siesjo, 1997), weakening the electrical gradient and resulting in depolarization of the cell membrane. This action ultimately leads to the activation of second messenger systems and signaling cascades, resulting in the liberation of intracellular Ca^{2+} stores. Toxic levels of intracellular Ca^{2+} exert damage through irreparable activation of caspases, lipases, proteases, and other intracellular mechanisms that further increase the cell's demand for oxygen (Lyden & Wahlgren, 2000). Synthesis of these "free radical" species leads to the dismantling of the mitochondrial electron-transport chain responsible for the production of the cell's energy source (ATP) and the ultimate death of the cell due to energy deprivation. At the same time, the rise in intracellular calcium induces the release of vesicular neurotransmitters into the extracellular milieu, especially glutamate. The excess glutamate release, in turn, triggers the same damaging intracellular events in the target neuron population.

1.5. Exercise influences cell death

Evidence that physical exercise may influence cell survival was first presented in animal models of stroke. Similar to the excitotoxic cell death in epilepsy, obstruction of cerebral blood flow in stroke results in the activation of Ca^{2+}-dependent cellular damage that can ultimately result in cell death. Exercise before and after brain insult has been shown to attenuate cerebral damage resulting from ischemia (Stummer, Baethmann, Murr, Schurer, & Kempski, 1995; Stummer, Weber, Tranmer, Baethmann, & Kempski, 1994; Wang, Yang, & Yu, 2001; Yang, Wang, Wang, & Yu, 2003). Animals given the opportunity to exercise in running wheels *ad lib* for two weeks followed by bilateral occlusion of the carotid arteries show a more than 4-fold greater survival rate two weeks following the ischemic episode compared to that of non-running control animals (Stummer, Weber, Tranmer, Baethmann, & Kempski, 1994). Histological examination revealed that exercise attenuated cell loss in several brain regions, including the hippocampus (Stummer, Weber, Tranmer, Baethmann, & Kempski, 1994). Wang and colleagues (2001) found that rats trained for varying numbers of weeks on treadmills followed by bilateral middle cerebral artery occlusion (MCAO) show a smaller volume of cerebral cell loss than non-running animals. Additionally, they found that the amount of cell loss is inversely related to the duration of the training regimen, such that cell loss was least pronounced in the animals running the greatest number of weeks (Wang, Yang, & Yu, 2001). Exercise during the recovery phase may also be beneficial. Rats subjected to bilateral MCAO followed by daily training on a treadmill show reduced cerebral cell loss volume and enhanced recovery of neurological function compared to non-running rats (Yang, Wang, Wang, & Yu, 2003). In this manner, an exercise paradigm initiated before

or after an isolated neurological challenge may influence the extent to which neuronal damage is present in the brain.

Consistent with the idea that exercise training may affect cellular events during and after a damaging event, previous findings in animal models of TLE suggest that exercise influences the pathology at two distinct stages: i. susceptibility to seizure; ii. recovery and cell loss following SE. Physical activity has been shown to reduce susceptibility to seizures in both the kindling and chemoconvulsant models of epilepsy. Rats exposed to long-term daily exercise (forty-five days of treadmill training) are more resistant to kindling-induced SE than short-term exercise (one day of treadmill training) and sedentary control animals. Specifically, long-term exercise rats required a relatively greater number of stimulations to reach the highest level of seizure-behavior in the kindling paradigm (Arida, de Jesus Vieira, & Cavalheiro, 1998; Michalakis et al., 1998). These findings suggest that the amount of exercise determines the degree of susceptibility to seizures. This effect may not be limited to the kindling paradigm. Using the KA paradigm, we have observed a lower frequency of high-running rats entering SE than their low-running and non-running control counterparts (B. Anderson & McCloskey, 2004). Additionally, more high-running rats persist in low-stage seizures, never entering near-SE stages 3 and 4, than low-running and non-running controls (B. Anderson & McCloskey, 2004). Taken together, these findings suggest that susceptibility to seizure is influenced by physical activity, and that the extent of seizure behavior may be related to the degree of exercise.

During the silent recovery period following SE, there is continued apoptosis-like cell loss and rewiring of local circuitry in hippocampal subregions – especially CA3 and

the DG (Routbort, Bausch, & McNamara, 1999; Sutula, Cascino, Cavazos, Parada, & Ramirez, 1989). These alterations are suspected to be necessary for the production of the later spontaneous seizure behavior that defines the epileptic condition. Exercise may affect cellular events in this period as well. Arida and colleagues (1999) showed that rats exposed to daily exercise following pilocarpine-induced SE had fewer recurrent seizures after the silent phase than non-exercising controls. The exercise regimen was initiated following the first post-SE spontaneous seizure. Additionally, exercising animals continue to show fewer seizure episodes following the cessation of the exercise regimen (Arida, Scorza, dos Santos, Peres, & Cavalheiro, 1999). This finding suggests that exercise introduced shortly following the initial spontaneous seizure may be able to attenuate recurrent seizures and subsequent additive damage to the hippocampus. Furthermore, a relatively brief period of physical training (45 days in this study) introduced immediately following the silent period may attenuate the occurrence of future spontaneous seizures, even after the termination of the exercise regimen. This suggests that by reducing the frequency of seizure occurrence in a critical time period following the initial insult, short-term physical exercise may serve to lessen the future appearance of spontaneous recurrent seizures.

Several investigators have also tested whether exercise affects the second stage, recovery and cell loss following an initial damaging event. Carro and colleagues (2001) reported a reduction in hippocampal cell loss in male mice exposed to treadmill running followed by an IP injection of domoic acid – a KA analogue naturally occurring in phytoplankton that acts as a neurotoxin when ingested in seafood. Cell counts in this study were determined 5-7 days following domoic acid administration, when the toxin is

known to have maximal effect on hippocampal neurons. The finding of an exercise-induced attenuation of cell loss at this time phase further suggests that physical activity can counteract processes in the post-SE phase of TLE. In this manner, moderate levels of physical exercise (as approximated by daily treadmill training) may be able to both reduce the occurrence of spontaneous seizures and reduce continuing cell loss in the silent period following the initial SE seizure. In this study, the animals were trained to run for one hour daily on a motorized treadmill at a low speed, for a reported total of 840 meters. By contrast, voluntary exercising animals have been reported to run up to 12km in a single day (Ramsden, Berchtold, Patrick Kesslak, Cotman, & Pike, 2003) . The neuroprotective relationship described by Carro and colleagues (2001)may not be sustained with the lower intensity, sporadic daily physical activity observed with voluntary exercising animals.

Contradictory to previous reports of exercise-induced neuroprotection, a single published study has provided evidence that physical exercise may exaggerate damage to hippocampal cells in animals subjected to a chemoconvulsant model of epilepsy. Using female rats exposed to twenty-eight days of voluntary exercise in the running wheel followed by unilateral intra-cerebroventricular (ICV) injections of KA, Ramsden and colleagues (2003) reported greater cell loss in these animals compared to their non-exercising counterparts. In this case, exercising animals showed significantly more cell loss in the CA2, CA3, and hilus regions of the hippocampus than non-exercising animals (Ramsden, Berchtold, Patrick Kesslak, Cotman, & Pike, 2003). The authors offered the suggestion of two factors that might explain this finding: i. this was the first study to use female rats, adding a hormonal component not previously examined in the exercise-

epilepsy relationship; ii. though voluntary, the amount of exercise was greater than that typically seen in male subjects. Unlike the systemic administration of drugs in the previous studies, these authors used ICV injections of the chemoconvulsant. The effect of different routes of administration have not been well described for KA, making it difficult to generalize between findings using systemic and ICV injections of the drug.

Furthermore, the animals in this study were injected while anesthetized with sodium pentobarbital, a drug commonly administered to inhibit seizures in animal models of TLE. The interaction between KA and sodium pentobarbital make it difficult to determine whether the exercise training amplified cell loss by increasing the excitotoxic effect of KA, or by reducing the efficacy of sodium pentobarbital to inhibit neuronal excitation in the exercise animals. Additionally, if the assumption that cell loss in this model results from damaging processes initiated during SE holds true, then the finding by Ramsden and colleagues suggests that exercise may have increased the number of animals entering SE. The administration of KA to the animals under general anesthesia inhibits the assessment of behavioral seizures in the critical time period following drug delivery. In this manner, it is impossible to determine whether the reported increase in cell loss is the direct result of the initial intense seizures or if it is the case that hippocampal cell loss can occur in the absence of SE and that physical exercise may in some conditions exacerbate this effect.

1.6. Aim of current study

This dissertation comprises two experiments conducted with the ultimate aim of further understanding the effect physical exercise has on hippocampal cell survival using

a chemoconvulsant model of epilepsy. The first study was conducted to determine if SE was necessary to obtain discernable hippocampal cell loss. To avoid an interaction between the trauma related to surgery and the response to KA, cannulae were implanted two weeks before injecting KA ICV in awake rats. This allowed us to record any behaviors consistent with KA-induced seizures immediately following drug administration. Furthermore, this also permitted us to code whether or not animals progressed to the highest stage seizures. If cell loss was selectively associated with SE, we would expect to see discernable cell loss only in animals that progressed to the hgh-stage seizures in the coding period. Conversely, cell loss in non-SE animals would suggest that SE in not a central feature of hippocampal atrophy consistent with human TLE. This later view is supported by findings in human subjects that hippocampal cell loss can occur without a the prior experience of SE (Fujikawa, Itabashi, Wu, & Shinmei, 2000)

To better understand the interaction between physical exercise and seizures in producing hippocampal cell loss, we administered KA ICV to unanesthetized male rats that were pre-conditioned with voluntary exercise in a running wheel or held in identical cages lacking access to running wheels and coded for seizure progression. Accordingly, we could test whether exercise protected from SE induced by ICV injections similar to our earlier studies using i.p. administration of KA. We were specifically interested in determining if the aberrant finding reported by Ramsden and colleagues (2003) was generalizable to all animals receiving an intracerebroventricular (ICV) injection of KA – i.e. if *male* animals given access to voluntary exercise would show an increase in hippocampal cell loss similar to that previously reported in *female* animals (Ramsden,

Berchtold, Patrick Kesslak, Cotman, & Pike, 2003). If gender-induced hormonal differences account for the findings by Ramsden *et al*, then male rats exposed to long-term voluntary exercise followed by ICV KA administration of KA will show hippocampal cell loss equivalent to or less than that seen in control animals. Alternatively, if male voluntary runners show increased cell loss as compared to control animals, this may suggest that the effect of exercise in exaggerating cell loss is not gender-specific.

We hypothesize that exercise will reduce the occurrence of intense seizure behavior acutely following KA administration, but that the rate of cell loss observed 7 days later will be increased in the exercise group. The production of behavioral seizures in animal models of epilepsy is ultimately due to the downstream excitation of the motor cortex, leading to the activation of motor neurons, and, in turn, contraction of muscles. The acute resistance of exercise animals to seizure production may be representative of motor training. Likewise, exercise conditioning may influence cellular response to hyperexcitation such that hippocampal cell loss may not necessarily be related to seizure production. Physical exercise may increase the likelihood of hippocampal cell death when faced with a challenge.

1.7. Figures

Figure 1-1: Human SE

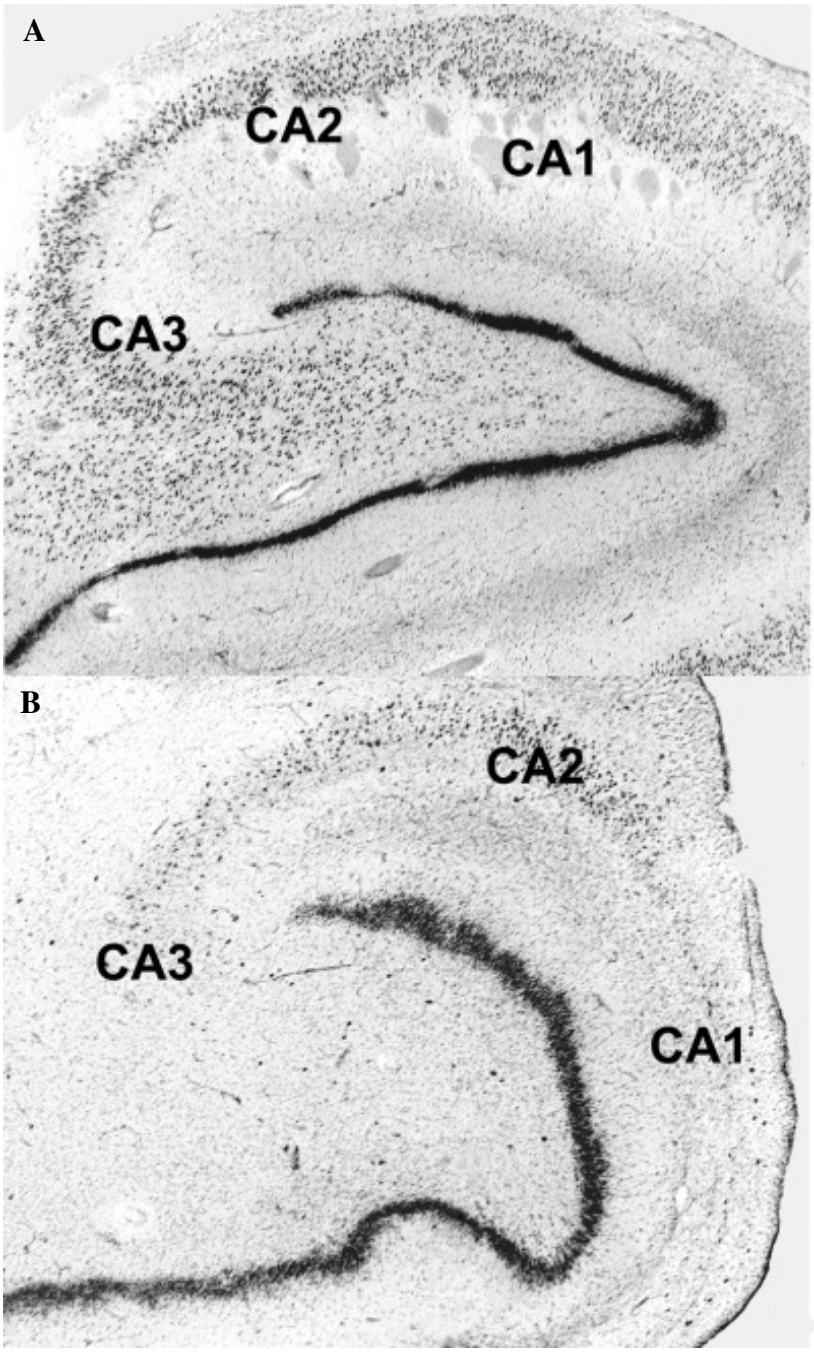

Figure 1-1: (A) The laminar structure of the human hippocampus is well defined in the control brain. (B) SE produces extensive ablation of the pyramidal cell layer, especially in CA3 and CA1, but CA2 appears to be spared. This is consistent with human TLE pathology. Adapted from (Swartz et al., 2006).

2. IS PROLONGED STATUS EPILEPTICUS NECESSARY FOR THE PRODUCTION OF HIPPOCAMPAL CELL LOSS?

2.1. Introduction

Status epilepticus (SE) has been described as a continuous period of at least 30 minutes of seizure activity without recovery between subsequent seizures (Treiman, 1997) capable of inducing a permanent epileptic condition (Browne & Holmes, 2001; Clark & Wilson, 1999; Treiman, 1997). In most cases, this is accompanied by the absence of consciousness in the inter-ictal period (Delgado-Escueta & Enrile-Bacsal, 1983). SE is considered a medical emergency due to the acute possibility of mortality and the longer term risk of developing the spontaneous recurrent seizures associated with temporal lobe epilepsy (TLE).

In animal studies, SE is commonly determined by assessing behavioral indicators of seizure that are consistent with behavioral indicators of human SE. Specifically, animals exhibiting continuous high-stage seizures as described by the Racine Scale (Ben-Ari, 1985; Racine, 1972) are described as being "in status epilepticus." Behavioral SE in animals corresponds to epileptiform discharges in the hippocampus/amygdala that are analogous to discharges observed in the human condition (Clark & Wilson, 1999; Sanchez et al., 2006; Williams et al., 2006).

Prolonged SE in experimental animals can result in a pattern of neuronal damage frequently observed in epileptic patients (Ben-Ari, Tremblay, Ottersen, & Meldrum, 1980; Nadler, Perry, & Cotman, 1978; Sloviter, 1999). Hippocampal sclerosis is the

most common histological feature of temporal lobe epilepsy. Up to 75% of patients undergoing resection of the temporal lobe for medically untreatable epilepsy display the characteristic loss of pyramidal cells in the CA1, CA3, and hilar regions of the hippocampus that hallmark the condition. There is some evidence to suggest that hippocampal cell loss may be present in patients who do not have a history of the severe behavioral seizures associated with SE (Bower, Kilpatrick, Vogrin, Morris, & Cook, 2000; Thom, Zhou, Martinian, & Sisodiya, 2005). This suggests that exposure to moderate insult can result in hippocampal cell loss, and the subsequent development of spontaneous seizures consistent with human epilepsy.

Cell loss and rewiring are believed to support the development of spontaneous seizures. Since seizures, including spontaneous seizures that are not characterized as SE, are believed to contribute to the increased likelihood of subsequent spontaneous seizures, one would predict that sub-status seizures might also cause cell loss. Few studies have addressed this possibility. In the present study, we explore the possibility that neuronal damage consistent with human TLE may be produced by SE limited to a maximum of 60 minutes. Additionally, we wanted to determine if exposure to an excitotoxin in the absence of behavioral SE can produce sufficient damage to result in cell loss similar to that in human epileptics.

2.2. Methods

Subjects

Twelve twenty-one day old male Sprague-Dawley rats (Taconic) were used for this study. They were housed two-three per standard tub cages for 2 weeks with a 12:12

light:dark cycle (lights on at 10 am) and free access to food and water. During this time, they were habituated to handling. Animals were then individually housed in standard tub cages for four weeks. All animals were handled twice weekly for the 42-day duration of the study. After four weeks, cannulae were implanted, and after two weeks of recovery, KA was injected, and seizures were coded. All animals were euthanized one week following drug administration.

Surgery

Under Ketamine (50mg/kg) and Xylazine (10mg/kg) sedation, a 24-guage stainless steel hypodermic cannula (Small Parts, Inc., Miami Lakes, FL) 1.7 cm long was implanted into the right lateral ventricle (from Bregma, -0.8 AP, +1.5 ML, -4.0 DV, (Paxinos & Watson, 1986) of each rat. Four small screws anchored in the skull and quick-set epoxy were used to secure the cannula and a section of plastic tubing (~1.5 cm long) protecting the cannula. All animals were kept warm until they demonstrated wakefulness and the ability to move around the cage, at which point they were returned to the colony.

Drug Dosage

Kainic acid was mixed in a single-batch, aliquoted, and stored in microfuge tubes at −20°C. On each injection day, a single microfuge tube was thawed at room temperature and vortexed briefly to ensure thorough mixture of the drug. The drug was administered via a 1.95 cm injector connected to a 10µL Hamilton syringe (Hamilton Co., Reno, NV) over 1-minute. The injector was left in place for one minute following

KA delivery to allow for diffusion. KA was administered to conscious animals during the first half of their dark cycle.

Preliminary tests were conducted to determine an optimal dose of intracerebroventricular (ICV)-administered KA for inducing behavioral seizures while minimizing mortality. According to Cámon *et al* (2001), ICV administration of 2.3 nmol KA (approximately .50 μg) produces seizure activity in 81% of sedentary Wistar rats with a 78% survival rate at 24 hours. In our lab, we failed to produce discernable seizure activity in Sprague-Dawley rats injected with the 0.50 μg dose. Therefore, the dose was increased to 0.55 μg per animal, at which point we were able to observe behavioral changes associated with KA administration (increased locomotor activity, wet dog shakes, and behavioral seizures) at rates that were comparable to that observed with intraperitoneal (i.p.) administration. Based on these preliminary findings, each animal in the present study received an injection of 4.4 μL of a kainic acid solution (0.125 μg/μL) into the right lateral ventricle, for a final dosage of 0.55 μg KA.

Behavioral Coding

Following drug administration, each animal was coded by an investigator blind to the treatment conditions for behavioral seizures according to a modification of Racine's Scale (Racine, 1972) as previously described by Ben-Ari (1985) for intra-peritoneal administration of KA. Table 2-1 shows further modification of this scale consistent with the progression of seizure behavior observed with ICV administration of KA. Status epilepticus was operationally defined as a period of uninterrupted convulsive activity with an initial onset of five minutes during which the animal displays no voluntary

movement between behavioral seizures. Animals exhibiting a catatonic posture – i.e. absence of continuous convulsive behavior characterized by facial twitching and head bobbing – following behavioral seizures were not considered to be in SE. We have previously found that animals tend to enter SE within one hour of receiving ICV KA at the present dose (unpublished finding). All animals received a single injection of an anticonvulsant one hour after entering SE or three hours after the initial KA injection, whichever occurred first. We delayed sedating non-SE animals until 3-hours following KA administration as this was the earliest point at which we could ensure that these animals would not enter SE,.

Sodium pentobarbital is commonly administered to control seizure activity in animal models of TLE. However, previous findings suggest that ketamine may be more effective than sodium pentobarbital at controlling prolonged SE lasting 60 or more minutes (Borris, Bertram, & Kapur, 2000). Therefore, all KA animals were administered 100mg/kg of ketamine intra-peritoneally at the end of the seizure-coding period. All animals were continuously monitored during the sedation period and were returned to the colony upon waking. Following KA administration, each animal was evaluated daily for the presence of behavior consistent with post-SE activity previously observed in our laboratory (unpublished) – especially enhanced startle and hyper-reflexive response to touch.

Tissue Processing and Anatomical Analysis

Seven days following the injections, all animals were decapitated under sodium pentobarbital (70 mg/kg) sedation. This time frame was selected in order to maximize

the appearance of cell loss resulting from apoptotic and necrotic processes and to allow for the removal of debris resulting from cell death. Brains were immediately dissected out, quick-frozen by immersion in isopentane (2-methylbutane) cooled to -45°C with dry ice, and stored at -70°C till further processing. Tissue was later cryostat-sectioned at 30μm, collected in a one-in-four series on gelatin-coated slides maintained in the cryostat, and stored at -4°C until stained. Slides were air-dried at room temperature 2-4 days, stained with methylene blue/azure II (0.16% and 0.1%, respectively), dehydrated in an alcohol series, and cover-slipped with Permount.

Slides were scanned at 3200 dpi (Epson Perfection 4870 Photo) and hippocampi of interest were cropped from scanned brain sections (Adobe Photoshop Elements 2.0). All anatomical measures were limited to the dorsal hippocampus of the injected (ipsilateral) hemisphere. Regions of interest included the first coronal section containing both the inferior and superior blades of the dentate gyrus. Regions of interest continued from the previous anterior point to the caudal boundary of the dorsal hippocampus, which was defined as the posterior-most section in which the ventral and dorsal portions of the hippocampus were still visibly discontinuous (see Fig. 2-1). The total number of sections within the dorsal hippocampus was divided into the *proximal* and *distal* dorsal hippocampus – such that the proximal sub-division represented the caudal half of the dorsal hippocampus and the distal subdivision represented the rostral dorsal hippocampus (see Fig. 2-2). Anatomical measures were averaged across each subdivision of the hippocampus.

Based on the method previously described by Conrad *et al.* (2004), cell loss was assessed as an inverse function of the visible CA3 cell layer length, such that shorter cell

layer lengths reflect more cell death. CA3 contains a high density of kainate receptors; therefore, this region shows the highest extent of KA-induced damage (Ben-Ari, 1985). The visibly undamaged portion of the pyramidal cell layer was measured from the lateral CA1 border to the medial-most intact point of the CA3 (see Fig. 2-3). Furthermore, as methylene blue/azure II staining does not distinguish between CA3 and CA2, the distal segment of CA3 for the purposes of this study included CA2 pyramidal cells.

Statistical Analysis

Statistical analyses were conducted using SPSS for Windows, version 9.0. Levene's test for equality of variance was performed to determine if there was comparable variance in CA3 cell layer length between SE and non-SE animals in the proximal and distal dorsal hippocampus. Independent-samples t-tests were then conducted to test for group differences in mean CA3 cell layer length between SE and non-SE animals. This examination was made across both divisions of the dorsal hippocampus. Equal variance was not assumed for tests of group means. Pearson's correlation was used to test for a relationship between SE and CA3 cell layer length in the proximal and distal dorsal hippocampus.

2.3. Results

One rat died shortly following KA administration and was eliminated from the study. Among the remaining 11 animals, 2/11 reached criteria for SE according to the modified Racine Scale (Ben-Ari, 1985; Racine, 1972). Levene's test for equality of variances revealed significant differences between SE and non-SE animals in both the

proximal [$F_{(1,10)}$= 15.904; p=.003] and distal [$F_{(1,10)}$= 10.195; p=.011] subregions. It is interesting to note that non-SE animals showed the greatest variance in CA3 cell layer length, while cell layer length in SE animals was consistent across both regions of the dorsal hippocampus. There was a significant effect of seizure progression on CA3 length in the proximal sub-region [$t_{(1,8.023)}$=3.071; p=.015] and a trend in the distal division [$t_{(1,4.017)}$=2.221; p=.091] with SE animals exhibiting shorter CA3 cell layers than non-SE animals (see Figure 2-4). When the relationship between SE and CA3 cell layer length was examined, there was a strong negative correlation in the proximal dorsal hippocampus [r=–.420; p=.198] and in the distal dorsal hippocampus [r=–.409; p=.211]. While these statistics represent sizeable correlational effects, they failed to reach significance due to the low number of animals entering SE.

2.4. Discussion

The present study evaluates the appearance of hippocampal cell loss in male rats that were injected with a single ICV micro-injection of KA at a dose of 0.55 μg per animal. Animals that progressed to SE were allowed to remain in SE for a maximum of one hour, while non-SE animals were sedated 3-hours following the KA injection. Near ablation of the CA3 cell layer was evident in those animals progressing to SE. Additionally, among the non-SE animals, neuronal damage – as assessed from CA3 cell layer length – varied broadly in response to moderate seizures. These findings suggest that KA injected directly into the lateral ventricles may produce discernable hippocampal cell loss without necessarily inducing the prolonged seizures thought to be a central feature of this pathology. The observed damage to the CA3 cell layer resulting from ICV

KA administration suggests that cell loss of the type associated with epileptogenesis in TLE can be induced with duration of only one hour of behavioral SE, or with moderate seizures that do not reach the level of behavioral SE. Given that SE does not appear to be a mandatory component of human epilepsy, this finding has implications for the majority of epileptic patients who develop the pathology from sub-SE neuronal insult.

When Alfred Meyer and colleagues first described hippocampal sclerosis in patients who had undergone temporal lobectomy for the successful treatment of complex partial seizures (Meyer, 1956; Meyer, Beck, & Shepherd, 1955; Meyer, Falconer, & Beck, 1954), most of their patients had reported a history of prolonged seizures during childhood. This finding was consistent with the then prevalent idea that hippocampal sclerosis was the direct cause of prolonged generalized seizures. Subsequently, it was recognized that cell loss of the type observed in TLE can result from seizure-induced cell death *or* can be caused by some other event in the life of the brain not necessarily associated with SE (stroke, tumor, trauma, exposure to toxins, etc).

Consistent with the idea that SE is not a central feature of hippocampal cell loss in epilepsy, in the present study, KA was shown to have a toxic effect on pyramidal cell bodies of the hippocampus although the administered dose did not trigger the high-intensity seizures thought necessary to produce this level of cellular damage. The present results suggest that moderate behavioral seizures that do not typically manifest as a potentially harmful situation requiring immediate medical intervention, may result in discernable cell loss of the type seen in epilepsy histopathology. While the extent of cell loss necessary for the generation of spontaneous seizures is unknown, .this finding still

has implications for the severity of initial seizure in the later generation of spontaneous epileptic seizures.

2.5. Tables

Table 2-1: Modified Racine Scale based on acute behavioral response to 0.55-0.75 µg ICV dose of kainic acid

Stage	Characteristic Behaviors	Measurements
1. Jumping	Jumping, circling, rolling, intense agitation and wild running.	• Time to Jumping.
2. Hyperactive	Most difficult stage to recognize. Hyperactivity: frequent forelimb and head movements, incessant walking, chewing, and yawning. Myoclonic forelimb jerks.	• Clonus frequency.
3. Wet Dog Shake	Head nodding. Numerous wet dog shakes with increasing intensity and frequency (7-8/min) that eventually subsides.	• Time to first shake. Shake frequency.
4.Hypoactive/ Catatonic	~1 minute staring spells, crouching & akinesia (immobilized catatonic posture) occasional mild mouth and facial movements. Vigilant and hypoactive (unresponsive to stimuli). Ears cocked back.	• Time to first spell.
5. Rearing	Rearing on hindlimb begins for seconds and progresses to minutes. Salivation, jerks and contractions similar to stage 3. Increased forelimb clonic jerks. Foaming at the mouth.	• Frequency of episodes.
6. Rearing & Falling	Generalized myoclonic seizures (as in stage 4). Falling or loss of balance while rearing. Foaming at the mouth.	• Number of falls. Fall frequency.

(Ben-Ari, 1985; Racine, 1972; Sperk, 1994)

2.6. Figures

Figure 2-1: Anterior and posterior borders of the region of interest

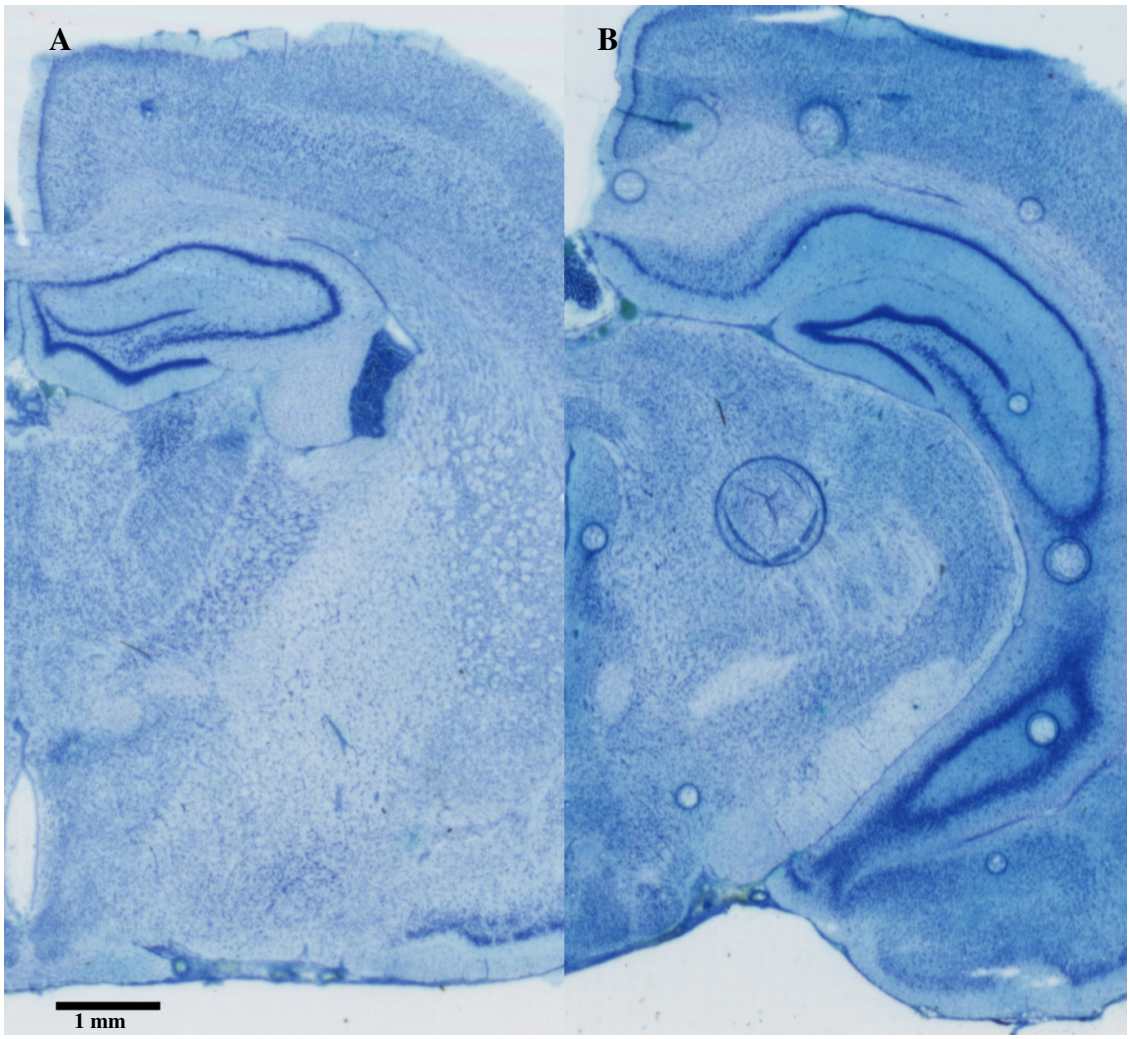

Figure 2-1: The anterior component of the CA3 division of the hippocampus was functionally defined as being bordered on the rostral end (A) by the first section in which the upper and lower blades of the dentate gyrus were both visible and connected, and on the caudal end (B) by the last section in which the ventral CA3 is still discontinuous from the rest of the pyramidal cell layer.

Figure 2-2: Proximal and distal subdivisions of the dorsal hippocampus

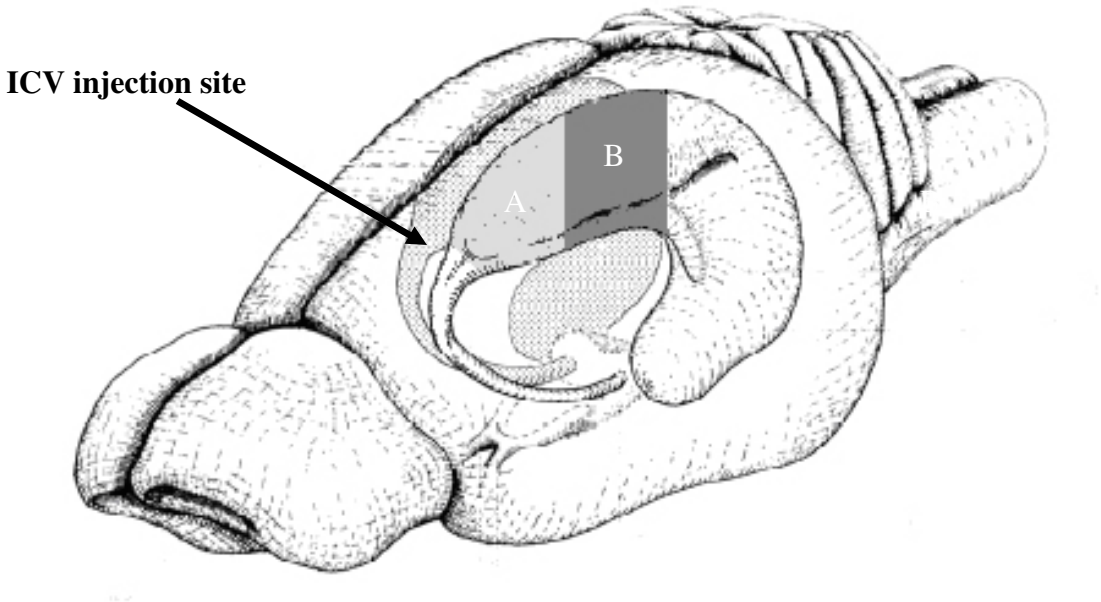

Figure 2-2: The total shaded area (A and B) comprises the dorsal division of the rat hippocampus. This region was bisected in each animal into the *proximal* (A) and *distal* (B) subdivisions. The arrow represents the approximate injection site, adjacent to the anterior end of the hippocampus. Adapted from Cheung and Cardinal (2005).

Figure 2-3: CA3 cell layer length

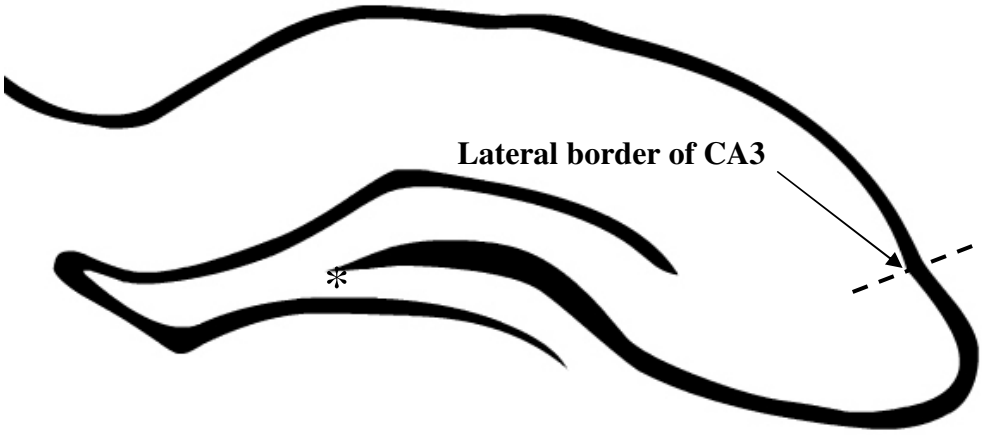

Figure 2-3: CA3 cell layer length was measured along the pyramidal cell layer of the hippocampus from the medial end of CA3 (*) nearest to the dentate gyrus to the lateral border of CA1 (dashed line)

Figure 2-4: Correlation between seizure progression and CA3 cell layer length

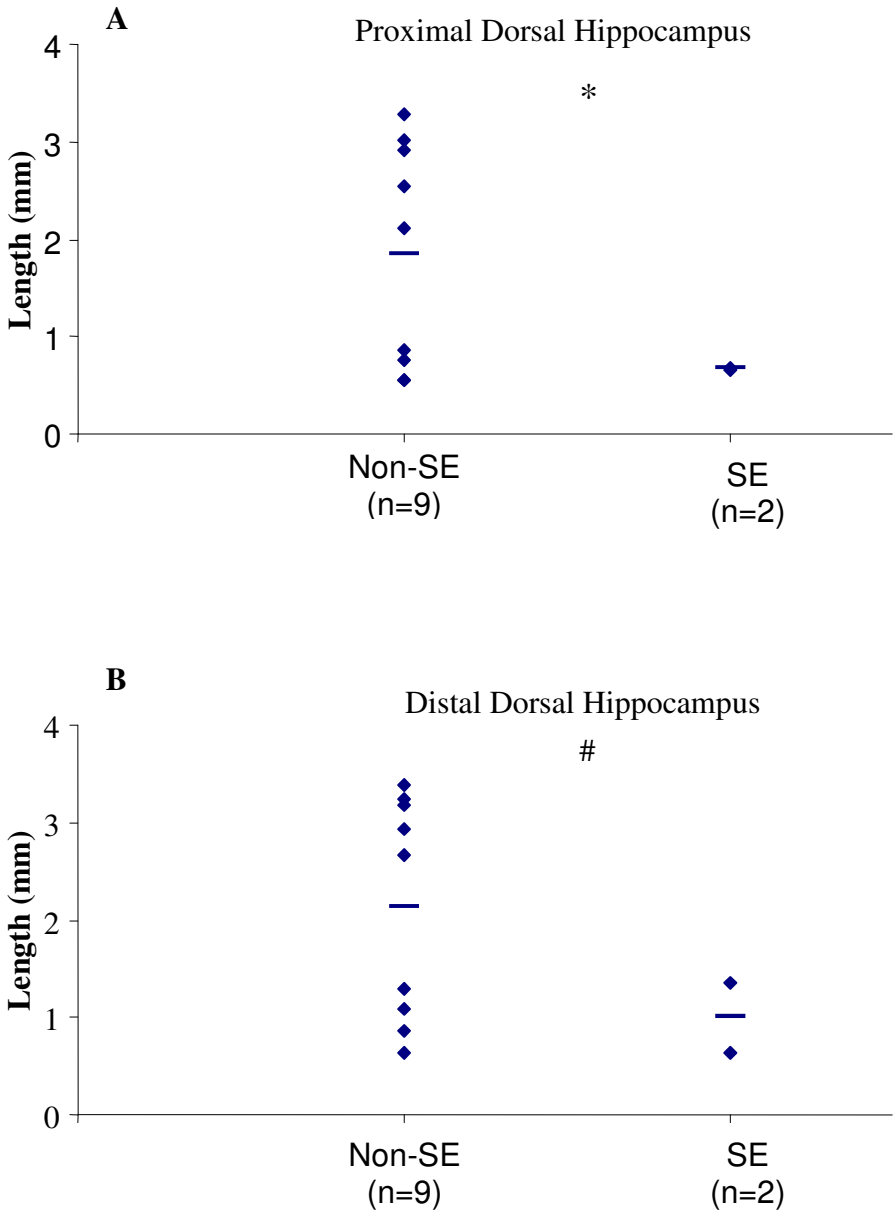

Figure 2-4: CA3 cell layer length were significantly shorter in SE animals compared to non-SE animals in the (A) proximal dorsal hippocampus [$t_{(1,8.023)}$=3.071; p=.015] and showed a trend toward shorter CA3 cell layer lengths in the (B) distal dorsal hippocampus [$t_{(1,4.017)}$=2.221; p=.091]. Bars represent group means.

3. PHYSICAL EXERCISE INCREASES HIPPOCAMPAL CELL LOSS IN AN ANIMAL MODEL OF TEMPORAL LOBE EPILEPSY

3.1. Introduction

Approximately 34% of people who experience a seizure will suffer a second one within 5 years (Hauser, Rich, Annegers, & Anderson, 1990). Previous findings suggest that the outcome of an initial seizure has strong implications for subsequent seizures and, ultimately, the development of the recurrent, spontaneous seizures associated with clinical epilepsy (Stefan et al., 2006). During seizures, cells are challenged metabolically. The high rate of activity exceeds metabolic capacity, which can allow excess calcium entry. The events are often followed by cell loss. Afferent axons, no longer capable of reaching their lost target cell populations, have been proposed to innervate their site of origin, leading to the possibility of recurrent connections that support the development of spontaneous seizures, which comprise epilepsy (Babb, Kupfer, Pretorius, Crandall, & Levesque, 1991; Blumcke, Beck, Lie, & Wiestler, 1999). This is consistent with the idea that *seizures beget seizure*s. With each subsequent seizure, there is the potential for cell loss, and further aberrant circuits that support seizures. Understanding factors that may impact upon cell fate in response to neuronal insult plays a significant role in developing potential interventions to conditions like epilepsy, as well as other conditions involving metabolic challenges that are hallmarked by abnormal cell death within discrete brain regions (e.g., Alzheimer's Disease, and Huntington's Disease). Likewise, identifying circumstances, including environmental

factors, in which cells may be more vulnerable to damage is equally important in ultimately reducing the prevalence of these conditions.

In clinical populations, there is a great deal of variance in response to injuries that lead to epilepsy, and variance in subsequent seizure frequency and duration. Whereas much of the variance will be accounted for by genetic factors, or the initial damage, it is possible that lifestyle factors may also moderate seizure frequency, duration and intensity, which may be related to the amount of cell loss. To experimentally address the role lifestyle factors such as exercise play in cell vulnerability, animal models are valuable. In these models, there is a growing body of experimental studies suggesting that physical exercise influences cell survival.

Support for the hypothesis that exercise can moderate neuronal damage first emerged in studies of ischemia. Cells of the hippocampus are highly vulnerable to the disruption of blood flow and oxygen supply. Exercise pre-conditioning in gerbils was shown to reduce the size of the infarct area in the cortex and in the CA3 region of the hippocampus following forebrain ischemia (Stummer, Weber, Tranmer, Baethmann, & Kempski, 1994). Similar protection was reported in the striatum following middle cerebral artery occlusion (Li, Luan, Clark, Rafols, & Ding, 2004). Later studies of models of epilepsy added support to the idea that physical exercise can alter the fate of cells facing an acute challenge. Male mice exposed to 1 km per day of treadmill running exhibited a reduction in hippocampal cell loss 5-7 days following seizures induced by a single intraperitoneal (i.p.) injection of the glutamate analog domoic acid (Carro, Trejo, Busiguina, & Torres-Aleman, 2001).

By contrast, it has also been suggested that exercise may in some conditions increase the likelihood of neuronal death in response to an insult. Following 28-days of voluntary exercise in running wheels, female rats subjected to a microinjection of kainic acid (KA) directly into the hippocampus showed an increase in cell loss within the CA2, CA3, and hilus regions of the hippocampus (Ramsden, Berchtold, Patrick Kesslak, Cotman, & Pike, 2003). This was the first published study to suggest that physical exercise may negatively impact upon cell fate in some circumstance.

The authors of the latter study suggested that sex might have played a role in the outcome, as this was the first study using female animals in testing the effect of exercise on cell survival (Ramsden, Berchtold, Patrick Kesslak, Cotman, & Pike, 2003). The authors were unsure of whether there was a significant hormonal factor, or whether the outcome was related to higher levels of voluntary exercise in female rats. Whereas these factors differ from earlier studies using exercise, they are not the only notable differences that may account for the reported outcome. The latter study used intracerebroventricular (ICV) injections of the neurotoxin KA rather than i.p. injections. ICV injections are preferable because they avoid the possibility that the groups differ in the rate of drug clearing. Additionally, ICV injections have been previously shown to produce a pattern of seizure activity more consistent with human epilepsy than seizure activity elicited in response to i.p. injections (Babb, Pereira-Leite, Mathern, & Pretorius, 1995). Thus the different route of administration may also explain the conflicting direction of effect seen in the exercise group.

To further test whether exercise is neuroprotective, or whether exercise increases neuronal vulnerability, animals in the present study were housed in control conditions or

with running wheels. After 4 weeks, cannulae were implanted into the lateral ventricle, and they were then given two additional weeks to exercise. After six weeks of exercise, KA was injected into the right lateral ventricle of each animal, and seizure progression was coded. Histological analysis was performed to assess cell loss in the dorsal hippocampus. Like Ramsden *et al.* (2003), we used ICV injections, but we injected KA while the animals were awake rather than under anesthesia. Additionally, we used male rats, in contrast to female animals. We hypothesize that the exercise animals will show an increase in hippocampal cell loss similar to that reported by Ramsden *et al.* (2003). Such an outcome would imply that sex is not a primary factor driving the previously reported finding (Ramsden, Berchtold, Patrick Kesslak, Cotman, & Pike, 2003), and that the ICV nature of the injection may instead play a major role in increasing the appearance of cell loss among exercise animals.

The high-intensity seizures of SE are commonly held to be a necessary component of producing the hippocampal cell loss that defines temporal lobe epilepsy (TLE) histopathology. However, we have previously shown that moderate (non-SE) seizures induced by ICV administration of KA can produce discernable hippocampal cell loss in rats (unpublished finding). Since SE is believed to be a prerequisite for cell loss, it is possible that SE frequency differed across groups in the study reported by Ramsden *et al*. (2003). Ramsden *et al.* (2003) administered KA to rats under general sedation, precluding the coding of behavioral seizures. In previous studies using i.p. injections of KA, we found that exercise reduced the frequency of animals that progressed to SE (B. Anderson & McCloskey, 2004; McCloskey et al., 2003). If greater cell loss in the current study was related to an increase in SE frequency among exercising rats, this would

contradict our earlier observations that exercise protects against SE. To test the potential effect of exercise on SE frequency and its downstream relationship to cell loss, KA was administered in awake animals so that seizures progression to SE could be coded.

3.2. Methods

Animals

Twenty-nine male Sprague Dawley rats were obtained at 21 days-old (Taconic, Germantown, NY) and housed 2-3 per cage for two weeks with a 12:12 light:dark cycle (lights on at 10 a.m.) and *ad lib* access to food and water. Each animal was handled twice a week during this fourteen-day period. On day 14 of the study, they were divided into two groups matched for body weight. Fifteen animals were assigned to the *voluntary exercise group* (VX) and fourteen to the *control group* (CT). Additionally, body weight and food/water consumption were monitored weekly throughout the study.

Treatment Conditions

Beginning on day 14 of the study, CT animals were singly-housed in standard tub cages, whereas VX animals were singly-housed in standard tub cages with an adjoining free-rotating running wheel. The treatment conditions lasted a total of 42 days, including the recovery period following surgery (below). The wheels were interfaced with a computer that recorded the number of wheel rotations daily.

Surgery

On day 42, all animals in the study underwent surgery to implant a cannula into the right lateral ventricle at a position adjacent to the head of the hippocampus. Under ketamine (100 mg/kg) and Xylazine (10 mg/kg) sedation, a small incision was made on the top of the skull and a 17 mm long, 24-guage stainless steel hypodermic cannula (Small Parts, Inc., Miami Lakes, FL) was implanted at −0.8 AP, +1.5 ML, −3.5DV from Bregma (Paxinos & Watson, 1986), . Four stainless steel screws were inserted into the skull surrounding the cannula, and the whole assembly was secured with quick-set epoxy. Tygon tubing (approximately 15 mm long) was then mounted around the cannula and held in place by quick-set epoxy. All animals were kept warm until they exhibited wakefulness and the ability to move about the cage. VX animals were returned to the running wheel-equipped cages 24-hours following surgery. In order to minimize the effects of surgical trauma, and allow VX animals to return to peak running rates, all animals were permitted 14 days to recover prior to KA administration.

Drug administration

Kainic acid was administered following the protocol we described in an earlier (unpublished) study. On day 56 of the study, all animals received a single microinjection of KA (0.55µg in 4.4µL saline) into the right lateral ventricle via the implanted cannula. The KA was mixed in a single-batch, then aliquoted into microfuge tubes and stored at −20°C till needed to ensure consistency in dosage across injection days. Additionally, CT and VX animals were injected alternately by group to control for the potential effect of drug administration order on seizure progression. Injections were delivered over 1-

minute using a 10µL Hamilton syringe (Hamilton Co., Reno, NV) attached to a 19 mm injector. The injector was held in place for an additional minute to prevent the drug from moving up the cannula tract. Stained sections were later examined to ensure that there was no physical damage to the hippocampus resulting from the cannula and injector.

Seizure coding

KA was administered during the first half of the dark cycle to awake animals. All animals were coded for 3 hours acutely following drug administration according to a modification of the Racine Scale (Ben-Ari, 1985; Racine, 1972). *Status epilepticus* was defined as a period of continuous generalized seizure activity without visible recovery, which in these studies was almost always initiated by rearing and falling, and included foaming at the mouth. In the present study, SE was indicated by an initial period of 5 minutes of seizure behavior without the appearance of voluntary movement between subsequent seizures. All animals reaching full criteria according to the Racine Scale were allowed to remain in SE for 1 hour. After one hour, they were injected with Ketamine (100 mg/kg) to stop the seizure. If the animal did not progress to SE, they were sedated with Ketamine (100mg/kg) three hours after the KA injection. All animals were returned to standard tub cages so that any exercise effects would be attributed to exercise pre-conditioning.

All animals were closely monitored daily for the remainder of the study to determine behavior consistent with post-SE (especially hyper-responsiveness to being touched). Animals not displaying sufficient feeding behavior in the post-KA period were syringe-fed twice daily with a mixture of powered rat chow in a 5% sucrose solution until

they regained the ability to feed independently. This also served to hydrate them. Additionally, all animals were orally administered a single 1mL dose of mineral oil 24-hours following drug administration, which (from prior experience in our laboratory) reduced mortality following KA administration.

Tissue processing

All animals were deeply anesthetized (sodium pentobarbital, 70mg/kg) and sacrificed by decapitation 7 days following KA administration. The brains were quickly dissected on ice, frozen by immersion into 2-methylbutane cooled to −45°C with dry ice, and stored at −70°C for later analysis. Thirty-micrometer sections were collected with a cryostat on gelatin-coated slides at 120μ intervals. The slides were air-dried for 2-4 days, stained with methylene blue/azure II (0.16% and 0.1%, respectively), dehydrated in an alcohol series, and cover-slipped with Permount.

Region of interest

Anatomical examination of cell loss was limited to the dorsal portion of the injected (ipsilateral) and non-injected (contralateral) hemispheres. This region was operationally defined as beginning on the anterior end at the first hippocampal slice in which the upper and lower blades of the dentate gyrus were both fully visible in stained sections. The posterior border was defined as the caudal-most section in which the lateral component of CA3 was visibly disconnected from the ventral CA3 (see Figure 3-1). The dorsal hippocampus was further divided into two subregions, based on proximity to the site of injection – i.e. relative to the anterior-most end of the hippocampus. The rostral-

most portion of the anterior hippocampus was termed the *proximal* dorsal hippocampus, while the caudal subdivision was termed the *distal* dorsal hippocampus (see Figure 3-2). The number of sections between these two points was divided into the proximal and distal halves so that the region of interest consisted of the proximal dorsal hippocampus, and distal dorsal hippocampus. Both regions were analyzed for both the hemisphere ipsilateral and contralateral to the injection site.

Anatomical analysis

All slides were scanned at 3200 dpi (Epson Perfection 4870 Photo), and hippocampal sections within the inclusion region were individually cropped and coded. CA3 cell layer length measures were collected using MCID software (InterFocus Imaging Ltd., UK). Length was collected by two independent researchers blind to the condition and sequence of sections, so that measures on all sections were independent of measures taken in surrounding sections. Values of CA3 cell layer length were averaged across collectors for each subdivision of the anterior hippocampus.

Based on the method previously described by Conrad and colleagues (Conrad, Jackson, & Wise, 2004), the amount of cell loss in the current study was inversely related to CA3 length such that shorter cell layer lengths reflected more cell death. As methylene blue/Azure II does not distinguish between neurons in the CA2/CA3 segments of the hippocampus, the distal border of CA3 for the purposes of this study includes the CA2 region. Cell loss was defined as the visible lack of large, stained pyramidal cell bodies in the CA3 division of Ammon's Horn (see Figure 3-3). CA3 length was operationally defined as the measure along Ammon's Horn bordered on the medial

surface by the end of the pyramidal cell layer nearest to the inner apex of the DG, and on the lateral surface by the CA2/CA1 border – visualized by the dramatic change in the cell layer thickness at this junction (see Figure 3-4).

Statistical analysis

All statistical analyses were conducted using SPSS for Windows, version 9.0. A repeated-measures ANOVA was conducted with treatment condition entered as the between-subjects condition, and hemisphere and subdivision of the anterior hippocampus (i.e. proximal *vs.* distal) as the within-subjects conditions. Correlations were computed to test for relationships between all possible combinations of body weight at the time of KA administration, animals entering SE, and CA3 length across the region and subregions of interest. Additionally, the number of rotations in the 24-hour period prior to the injection was correlated with the length of CA3 cell layer 7 days later.

T-tests were conducted *post-hoc* to test for group differences in CA3 cell layer length in each sub-division of the anterior hippocampus. Equal variance was not assumed for all tests of group mean. Further, the relationship between SE and cell loss was tested with a point-biserial correlation coefficient.

3.3. **Results**

Three CT animals died during SE and were, therefore, excluded from the anatomical measures in the current study. Among VX animals, running rates peaked at day 10 of exercise training with a daily average of 1725.13 rotations [standard error of the mean (SEM)=234.99; $p<.001$]. Similarly, running rates 24-hours prior to KA

administration averaged 1579.27 [SEM=199.44, p<.001] rotations, approximately 92% of peak running rates. This supports the idea that VX animals resumed voluntary running in the two week recovery period following stereotaxic surgery. In the VX group, number of rotations one day prior to KA administration was not strongly related to the tendency to enter behavioral SE [r=.303; p=.272]. Additionally, among non-SE VX animals, running rates were not significantly correlated with CA3 cell layer length in the ipsilateral proximal [r=−.039, p=.891], ipsilateral distal [r=−.103, p=.715], contralateral proximal [r=.018, p=.949], or contralateral distal [r=−.082, p=.773] subdivisions of the anterior hippocampus.

Voluntary exercise appeared to strongly reduce the frequency of animals entering SE in the 3-hour period acutely following a single 0.55 µL injection of KA into the right lateral ventricle. CT animals, relative to the VX group, had a two-fold greater probability of progressing to behavioral SE in response to KA administration (5/14 *vs.* 2/15, respectively). However, this effect failed to reach significance [X^2=1.981, p=.159] (see Figure 3-5) due to the overall low number of animals entering behavioral SE.

Cell layer length across all regions of interest was strongly correlated with SE (see Table 3-1). SE produced near-complete ablation of CA3 in both CT and VX animals (see Figure 3-6). When SE animals of both groups were compared to animals that did not progress to SE (non-SE), the SE animals exhibited more extensive cell loss (CT and VX animals combined). CA3 was significantly shorter in animals progressing to SE than in non-SE animals in the ipsilateral hemisphere [$t_{(1, 8.904)}$=4.572; SEM=0.496; p=.001], and a trend toward shorter CA3 in the contralateral hemisphere [$t_{(1, 4.683)}$=2.356; SEM=0.981;

p=.06]). Overall, inter-rater reliability was strong for CA3 cell length in both the ipsilateral [r=.941; p<.001] and contralateral [r=.945; p<.001] hemispheres.

Since SE was a strong predictor of the extent of cell loss, group comparisons were restricted within types of seizures. As so few exercise animals progressed to SE, a group comparison for animals with this stage of seizure was not possible. In contrast, for non-SE animals, cell layer length was compared across groups. A repeated-measures ANOVA with two within-subject factors (hemisphere and region of CA3 – i.e. proximal/distal sub-division) and one between-subjects factor (group) revealed a significant effect of region [F(1,20)=5.738, p=.027] and an interaction between region and group [F(1,20)=5.750, p=.026; see Figure 3-7] among non-SE animals. Post-hoc contrast comparisons revealed that the VX group had significantly shorter cell layer length than the CT group in the ipsilateral dorsal CA3 (p=.018), and a tendency for shorter CA3 cell layer length in the VX relative to CT group in the contralateral proximal dorsal CA3 (p=.073). In the distal half of the dorsal hippocampus, there was a similar tendency for a group effect on CA3 length in the ipsilateral hemisphere [p=.103], but not in the contralateral distal dorsal hippocampus (p=.221). The pattern of results suggest that the greatest cell loss among non-SE animals occurred in the exercise group in the proximal dorsal hippocampus in the injected hemisphere, followed by cell loss in the proximal dorsal hippocampus in the contralateral (non-injected) hemisphere. There was some indication of cell loss in the distal dorsal hippocampus of the injected hemisphere, but no indication of cell loss in the distal dorsal hippocampus of the contralateral hemisphere. Additionally, there was greater variability in CA3 length

across all subdivisions of the dorsal hippocampus in VX animals compared to CT animals (see Figure 3-7).

Non-status VX animals were more likely to have cell loss than control non-SE animals. Correlations revealed that SE accounted for 59.3% of animals with cell loss observed in the CT group (p=.006), but only for 10.2% of animals with cell loss in their VX group (p=.245) (see Figure 3-8). Among non-SE exercisers, this resulted in greater variance in cell loss, reflected in the range of CA3 cell layer length across all subregions of interest (see Table 3-2).

Overall, these findings suggest that while physical exercise may reduce the probability of an animal entering SE following administration of KA, it increases the likelihood that animals may exhibit hippocampal cell loss. More dramatically, VX animals that did not progress to SE were likely to undergo cell loss, whereas CT animals not progressing to SE exhibited CA3 cell loss less frequently.

3.4. Discussion

The purpose of the current study was to determine whether voluntary physical exercise could influence cell survival after a seizure. Kainic acid can induce several stages of seizure, the most severe being SE. SE has typically been associated with cell loss, so we categorized animals as exhibiting SE or not exhibiting SE. Cell loss was assessed in the anterior hippocampus following a single intracerebroventricular injection of 0.55µg KA into the right lateral ventricle. Length of the cell layer of the anterior division of CA3 was measured 7 days after kainic acid administration. Acutely following the KA injection, the proportion of exercise animals that entered status epilepticus was

lower than that of control animals entering status epilepticus, suggesting that exercise had an anti-convulsant effect. SE was strongly associated with extensive cell death in both CT and VX animals, and exercise conferred no visible protection from cell loss among those animals entering SE. When non-SE animals were analyzed separately for cell loss, exercise animals exhibited neuronal loss whereas control animals showed relatively very little. Within the exercise condition, the amount of exercise did not correlate with neuronal loss.

There was a strong relationship between SE and CA3 cell loss. This was illustrated by the finding that in control animals, cell loss was significantly correlated with progression to SE. Likewise, in both the CT and VX groups, the CA3 cell layer length was significantly associated with SE. Similarly, SE animals had significantly shorter CA3 pyramidal cell layers than animals that did not progress to SE. Although the low numbers of animals progressing to SE prevented statistical comparisons of group differences, there were no qualitative indications that exercise protected SE animals from cell loss. In all animals that progressed to SE, regardless of group, there was near completed ablation of CA3 pyramidal cells. We have consistently seen that exercise produces lower rates of SE following KA injections than control conditions. This effect is persistent regardless of route of administration (B. Anderson & McCloskey, 2004; McCloskey et al., 2003) or dose (data not shown).

The current study categorized SE based on behavioral seizure activity. Seizures were coded according to the stages described by Racine (1972), and modified by Ben-Ari (1985). SE was characterized as continuous seizure behavior initiated by a 5-minute period during which animals fail to show voluntary movement between subsequent

seizures. Animals that failed to progress to SE did not exhibit generalized seizures lasting longer than 5 minutes. Non-SE seizures included all characteristic behaviors normally associated with KA administration (wet dog shakes, myoclonic seizures, and generalized seizures), but that were followed by periods of voluntary movement. Whereas electrophysiological activity would be ideal to confirm our behavioral categorization of SE, several additional sources of data supported our coding methods. Post-SE behaviors were assessed daily following KA injections. Only those animals that were initially coded as entering SE exhibited hyper-reflexivity associated with SE. Likewise, the cell loss seen in SE animals was significantly greater than the cell loss seen in animals that did not progress to SE, and included total ablation of the CA3 cell layer in both hemispheres of the anterior hippocampus. The correspondence between the behavioral indicators of seizure, post-KA behavior, and cell loss support the accuracy of our coding methods for categorization of SE.

Hippocampal cell death in the current study was determined by measuring the length of the CA3 pyramidal cell layer. Shorter CA3 lengths reflect more cell death within a given section of the anterior hippocampus. Although this method has a lower resolution than counting cells, it has been used to measure loss following KA, because affected areas of CA3 cell layer suffer complete loss of the pyramidal cell layer. The accuracy of cell counting methods would far exceed what is needed for studies of cell loss following kainic acid. Accordingly, we have adopted the method previously described by Conrad *et al.* (2004). In the current study, cell loss is assessed as a function of the remaining CA3 cell length.

In the present study, exercise increased the likelihood that animals that received KA, but did not progress to SE, exhibited cell loss. The non-SE animals in the control group were highly unlikely to exhibit cell loss, whereas nearly half of the exercise group exhibited cell loss. We have previously reported a non-selective relationship between SE and cell loss in non-exercising animals following a single ICV injection of KA (unpublished data). In the present study, when cell loss occurred in non-SE animals, it was less severe than in the SE animals. These findings are consistent with the idea that hippocampal cell loss can occur in human epileptics with no prior medical history of SE (Bower, Kilpatrick, Vogrin, Morris, & Cook, 2000; Thom, Zhou, Martinian, & Sisodiya, 2005).

In the current study, the correlation between SE and cell loss in CT animals was stronger than in our earlier (unpublished) report. This may be due to more conservative injection coordinates in the present study, such that KA was administered at a site slightly further from the body of the hippocampus relative to the prior study. In the latter study, we observed mechanical damage due to the precise location of the injection site, resulting in an increased presence of CA3 cell loss (relative to the current study). In order to minimize the potential added effect of mechanical damage on CA3 cell death, KA was delivered in the present study to a location in the right lateral ventricle slightly further from the hippocampus than in our earlier (unpublished) study. In this manner, the effect of KA exposure in the present study reflects a more subtle effect of excitotoxicity related to the local concentration of KA at the level of the hippocampus nearest to the injection site.

For the present study, KA was injected into the right lateral ventricle, adjacent to the anterior-most area of the hippocampus. By injecting into the ventricles, any differences between the exercise and KA condition could not be confounded by peripheral differences in the ability to clear the drug. This route of injection has previously been shown to produce a pattern of seizure activity more consistent with human epilepsy than with peripheral administration of the neurotoxin (Babb, Pereira-Leite, Mathern, & Pretorius, 1995).

Assessment of neuronal damage was limited to the hippocampus in the present study. While kainate-dependent cell loss is known to occur in other brain regions (Ben-Ari, 1985), this is thought to represent extensive damage, secondary to cell loss observed in the hippocampus (Gloor, 1991; Margerison & Corsellis, 1966). Whereas other structures may exhibit more subtle damage in response to KA (e.g. reduced density of cell bodies), the laminar organization of the hippocampus and the rich availability of kainate receptors in this structure results in highly discernable cell loss, especially in the CA3 division. Taken together, this supports the idea that hippocampal damage is a central pathological feature of TLE, and that subtle histological alterations related to epileptogenesis can well be visualized in this structure.

In non-SE animals, exercise significantly increased the appearance of neuronal damage in the hippocampus. The greatest effect of KA administration was expected at sites proximal to the injection location because of the highest concentration of drug nearest the kainate receptors within this brain area (Ben-Ari, 1985), followed by regions that receive the strongest downstream excitatory afferent connections, the proximal region of the contralateral hemisphere, and within the ipsilateral hemisphere, the area just

posterior to the region closest to the injection site (i.e., the distal half of the anterior hippocampus). This was consistent with the pattern of observed cell loss such that the amount of damage was greatest in the ipsilateral proximal anterior hippocampus followed by the contralateral proximal hippocampus with the distal anterior hippocampus showing the least damage in both hemispheres. Cell loss from non-SE seizures is at least partially related to the finding that a higher proportion of animals in the exercise condition exhibited cell loss relative to the control condition. This was demonstrated by the contrast between groups in the relationship between cell loss and SE. Whereas SE predicted cell loss in the control group, it was not selectively associated with cell loss in the exercise group.

There was an effect of location within the anterior on CA3 cell loss in both groups of animals. Specifically, the greatest damage was observed in the proximal anterior hippocampus of the injected hemisphere followed by the contralateral proximal anterior hippocampus then the ipsilateral distal anterior hippocampus. According to Amaral (1995), the hippocampus maintains conservative inter-hemispheric and intra-hemispheric circuitry such that the CA3 at a given level sends afferents to the same position within the contralateral hemisphere and a weaker afferent in the rostral-to-caudal direction of the ipsilateral hemisphere. Consistent with this idea, we observed a similar pattern of regional damage, suggesting that the administration of KA produced cell loss of the type previously described (Ben-Ari, 1985) and in the direction predicted by the anatomy of this brain region (Amaral, 1995). Toxicity resulting from drug spread alone does not account for the observed pattern of hippocampal cell loss. If direct exposure to KA was the principal cause of cell loss, we would expect to see a pattern of damage progressing

from the rostral to caudal divisions of the ipsilateral (injected) hemisphere, then equally to both divisions of the contralateral anterior hippocampus. Such a sequence would suggest that KA is spread in a proximal-to-distal fashion within the injected hemisphere via the CSF, then across to the contralateral hemisphere. Instead the damage was greatest in the proximal division of the injected hemisphere followed by the proximal-most division of the non-injected hemisphere. The distal division of both hemispheres was relatively undamaged, consistent with the idea that this brain region receives less excitation from rostral-more positions within the hippocampus.

Taking into consideration that only one published study to date reported that exercise increased, rather than decreased, neuronal death following administration of a toxin (Ramsden, Berchtold, Patrick Kesslak, Cotman, & Pike, 2003), it is necessary to allow for factors that might account for differing outcomes on cell survival in exercising animals exposed to an excitotoxin. Most notably, route of drug administration may play a role in how the toxin is metabolized and, subsequently, how it may impact on cells of the CNS. In the two previously published studies in which excitotoxins were administered outside the nervous system (Arida, Scorza, dos Santos, Peres, & Cavalheiro, 1999; Carro, Trejo, Busiguina, & Torres-Aleman, 2001), physical exercise reduced the appearance of hippocampal cell loss. By contrast, in the current study – as in the report by Ramsden *et* al. (2003) – exercising animals received an injection of KA directly into the ventricles. Ramsden *et al*. (2003) concluded that the greater exercise in female rats may have accounted for the difference in the direction of the effect. In the present study, exercise again increased the likelihood of damage, but only in non-SE animals. Yet in this study, male rats that typically exercise at lower rates than female rats were used. It

is also important to note that Ramsden et al. (2003) injected KA while the animals were anesthetized and when they underwent stereotaxic surgery. The anesthesia would have prevented behavioral seizure coding, but it seems unlikely the animals progressed to SE because the anesthesia administered is often used to suppress seizures. It seems reasonable then to assume that their animals would not have progressed to SE, and are instead similar to our non-SE condition. It should be noted that our non-SE animals also received anesthesia, but not until 3 hours after the KA administration, and far beyond the point at which they would have progressed to SE. Given the consistent direction of the effects regardless of sex and amount of exercise, it seems likely that the effect is related to the route of KA administration. Earlier studies using both KA (B. Anderson & McCloskey, 2004) and pilocarpine (Carro, Trejo, Busiguina, & Torres-Aleman, 2001) reported exercise protection with routes of administration outside the CNS. The findings suggest that when hippocampal neurons are directly exposed to glutamatergic excitotoxins, as in the case of ICV administration of KA, physical exercise increases the likelihood of triggering intracellular pathways that lead to cell death. In contrast, if KA is administered i.p., then drug clearing may differ, leading to contradictory results.

In the present study, anesthesia was administered after one hour of SE or three hours following KA infusion in animals that did not progress to SE. Since the anesthesia may interact with KA, and subsequently affect cell loss, we analyzed SE and non-SE animals separately – i.e. animals that were anesthetized one hour after the onset of SE or animals that were injected three hours after KA administration). Furthermore, whereas there might be an underlying interaction between KA and anesthesia, anesthetics are commonly used in humans to suppress seizures. Therefore interactions may be relevant

for clinical conditions. Remarkably, in non-SE animals, which had the longest exposure to KA without seizure suppression by anesthesia, there was less cell loss than in the SE animals that were sedated following KA administration.

In VX animals, running rates did not significantly predict either SE or extent of CA3 cell loss. In a previous study, we reported that seizure progression was related to the amount of voluntary running when KA was administered i.p. (B. Anderson & McCloskey, 2004). In the current study, we administered KA ICV to reduce the possibility that peripheral effects of physical conditioning may impact on drug metabolism and, subsequently, on seizure production and the development of neuronal damage. The absence of a significant correlation between amount of running – as assessed by number of rotations – and both SE and cell loss suggests that that relationship may not necessarily hold true when KA is administered ICV. Although not significant, the correlation between running rates and SE would be considered to constitute a medium effect from a statistical perspective. While the sample size in the current study precluded the detection of such an effect at a significant level, the possibility of such a relationship remains open.

Environmental enrichment in the form of having access to the running wheel space and visual complexity does not likely account for the outcome on SE and cell loss in VX animals. Environmental enrichment has been previously reported to reduce seizure production and apoptotic cell loss in response to KA (van Praag, Kempermann, & Gage, 2000; Young, Lawlor, Leone, Dragunow, & During, 1999). If the findings of the present study were accounted for only by environmental enrichment provided by the physical wheel space, we would expect the relatively lower rate of SE to be accompanied

by less cell loss in VX animals (compared to controls). Furthermore, when enrichment has been directly compared to exercise, exercise was found to induce greater hippocampal plasticity (van Praag, Kempermann, & Gage, 1999). The present outcome suggests that physical exercise had an opposite effect on cell loss than previously described from environmental enrichment alone.

There is some suggestion that a more active lifestyle in the form of exercise may represent the "natural" condition – i.e. that the human body optimally requires a level of activity that is not often present in modern life. Given that a relatively sedentary lifestyle is increasingly prevalent, it is beneficial to understand the wide range of effects that physical conditioning can have on health and behavior, both in the healthy brain and in disease conditions. Physical exercise has been previously shown to impact on brain structures with potential functional implications. In humans, exercise has been reported to increase cortical thickness in older adults (Colcombe et al., 2006), while in animals, physical exercise has been shown to increase neurogenesis, especially in the dentate gyrus of the hippocampus (van Praag, Christie, Sejnowski, & Gage, 1999; van Praag, Kempermann, & Gage, 1999). While the functional implications of neurogenesis have not been fully described, there is the possibility that an exercise effect on this process may impact on brain function. However, in the present study, cell loss was most prominent in the CA3 division of the hippocampus. To date, neurogenesis has not been described in this brain region, precluding the idea that the effect described in the current study may be due to exercise altering the size of the CA3 division of the hippocampus by inducing cell proliferation in this area.

To conclude, while physical exercise reduced the likelihood that animals would express the high-intensity seizures associated with SE, VX animals exhibited increased CA3 cell loss in response to moderate seizures. When the extent of cell loss was compared across the hippocampal regions proximal and distal to the infusion site, VX animals were shown to have significantly more cell loss in the proximal anterior hippocampus of the injected hemisphere, followed by a trend toward more cell loss in the contralateral proximal anterior hippocampus and the ipsilateral distal anterior hippocampus. Overall, exercise still appears to be neuroprotective. The unexpected cell loss from non-SE seizures in nearly half of the exercise animals is outweighed by the protection exercise provided against SE and its corresponding severe cell loss. The cell loss following moderate seizures is of great interest nonetheless with regard to clinical conditions, because far more seizure in humans would be similar to the moderate seizures described than to SE as it is modeled in rats.

3.5. Tables

Table 3-1: Summary of correlation coefficients

	SE	Ipsilateral Hemisphere	Contralateral Hemisphere	Rostral Ipsilateral	Caudal Ipsilateral	Rostral Contralateral	Caudal Contralateral
Weight at KA injection		Cell layer length	Cell layer length	Cell layer length	Cell layer length	Cell layer length	Cell layer length
Overall	0.011	**0.086**	**0.060**	**0.145**	**0.017**	**0.103**	**0.006**
CT	-0.302	0.248	0.237	0.242	0.245	0.228	0.247
VX	0.286	-0.383	-0.386	-0.296	-0.440	-0.311	-0.450$^{\#}$
SE							
Overall	—	**-0.771*****	**-0.659*****	**-0.698*****	**-0.799*****	**-0.651*****	**-0.634*****
CT	—	-0.958***	-0.840**	-0.960***	-0.918***	-0.855***	-0.786**
VX	—	-0.724**	-0.725**	-0.588*	-0.804***	-0.662**	-0.759**

$^{\#}p < .10$

$^{*}p < .05$

$^{**}p < .01$

$^{***}p < .001$

Table 3-1: Weight at time of KA injection was not significantly correlated with susceptibility to SE or CA3 cell layer length in either the VX or CT animals. SE was negatively correlated with CA3 cell layer length across all sub-regions, so that SE was associated with shorter CA3 lengths across all sub-regions of the anterior hippocampus.

Table 3-2: Range of CA3 length (mm) in ipsilateral and contralateral anterior hippocampus of KA-injected animals

	Rostral Anterior Ipsilateral CA3	Caudal Anterior Ipsilateral CA3	Rostral Anterior Contralateral CA3	Caudal Anterior Contralateral CA3
SE+/CL+				
Overall	**0.307 - 0.772**	**0.198 - 2.081**	**0.360 - 2.386**	**0.385 - 2.921**
CT	0.627 - 0.772	0.783 - 2.081	0.949 - 2.386	2.224 - 2.291
VX	0.307 - 0.679	0.198 - 0.464	0.360 - 0.666	0.385 - 1.188
SE−/CL+				
Overall	**0.643 - 3.539**	**1.282-3.565**	**1.030-3.349**	**1.132-3.454**
CT	2.464 - 2.464	3.186 - 3.186	3.000 - 3.000	3.085 - 3.085
VX	0.643 - 3.539	1.282-3.565	1.030-3.349	1.132-3.454
SE−/CL−				
Overall	**2.511-3.467**	**3.125-3.676**	**2.813-3.416**	**2.914-3.581**
CT	2.924 - 3.467	3.194 - 3.676	2.813 - 3.322	2.914 - 3.467
VX	2.511-3.458	3.125-3.536	3.046-3.416	3.180-3.581

Table 3-2: Range of CA3 cell length in sub-divisions of the anterior hippocampus in SE animals (SE+/CL+), non-SE animals exhibiting visible cell loss (SE−/CL+), and non-SE animals expressing no visible indication of cell loss (SE−/CL−).

3.6. Figures

Figure 3-1: Rostral and caudal border of dorsal hippocampus

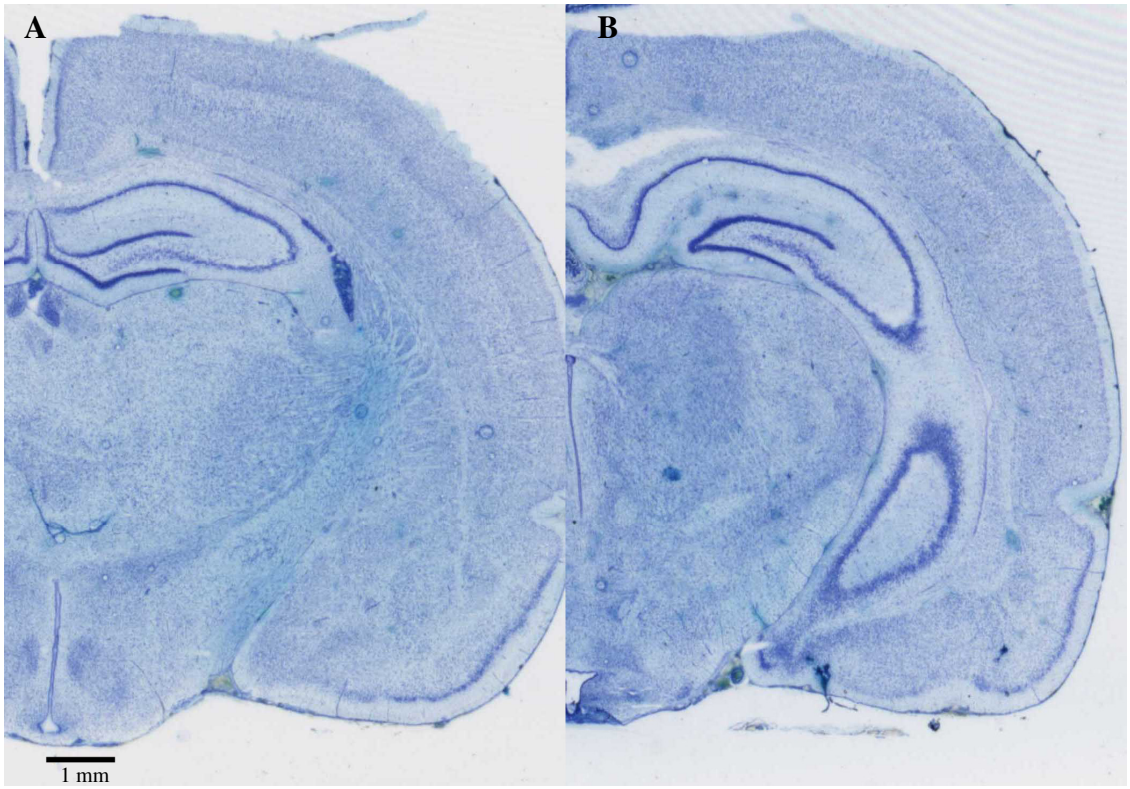

Figure 3-1: (A) The anterior border of the region of interest is the first section in which the upper and lower blades of the dentate gyrus are both fully visible. (B) The posterior border is the caudal-most section in which the ventral portion of CA3 is visibly discontinuous from Ammon's Horn.

Figure 3-2: Proximal and distal subdivisions of the dorsal hippocampus

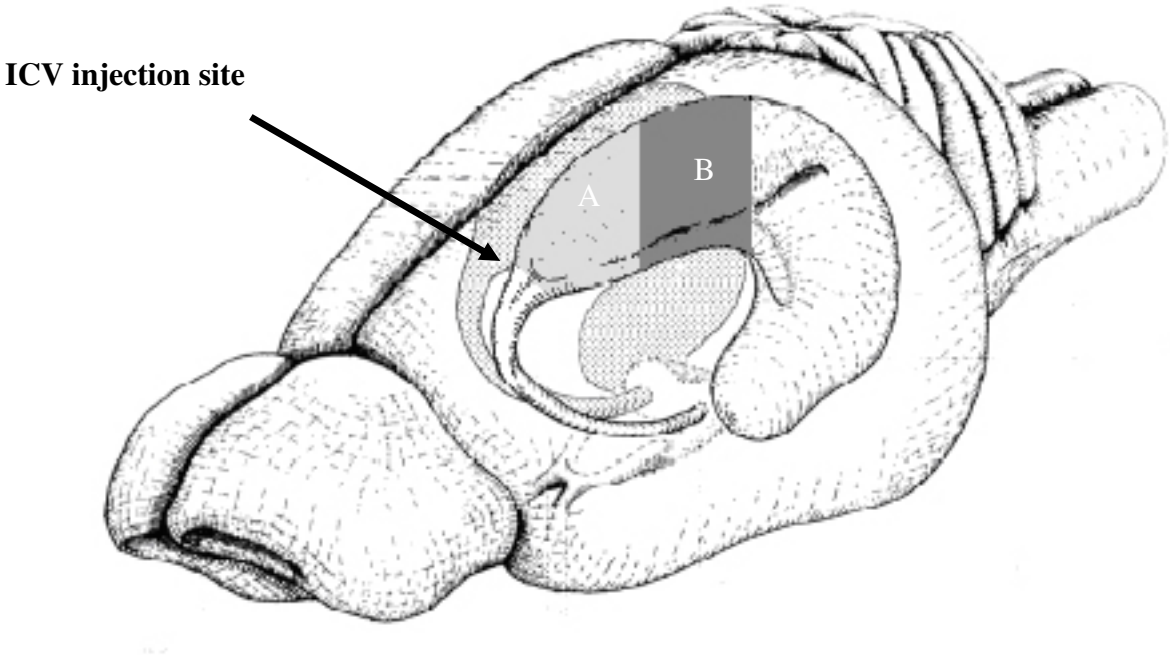

Figure 3-2: The entire shaded area (A and B) represents the dorsal division of the rat hippocampus. This region was further divided in each animal into the *proximal* (A) and *distal* (B) subdivisions based on the total number of dorsal hippocampal sections. The arrow represents the approximate injection site, near the anterior end of the hippocampus. Adapted from Cheung and Cardinal (2005).

Figure 3-3: Cell loss in CA3 of the dorsal hippocampus

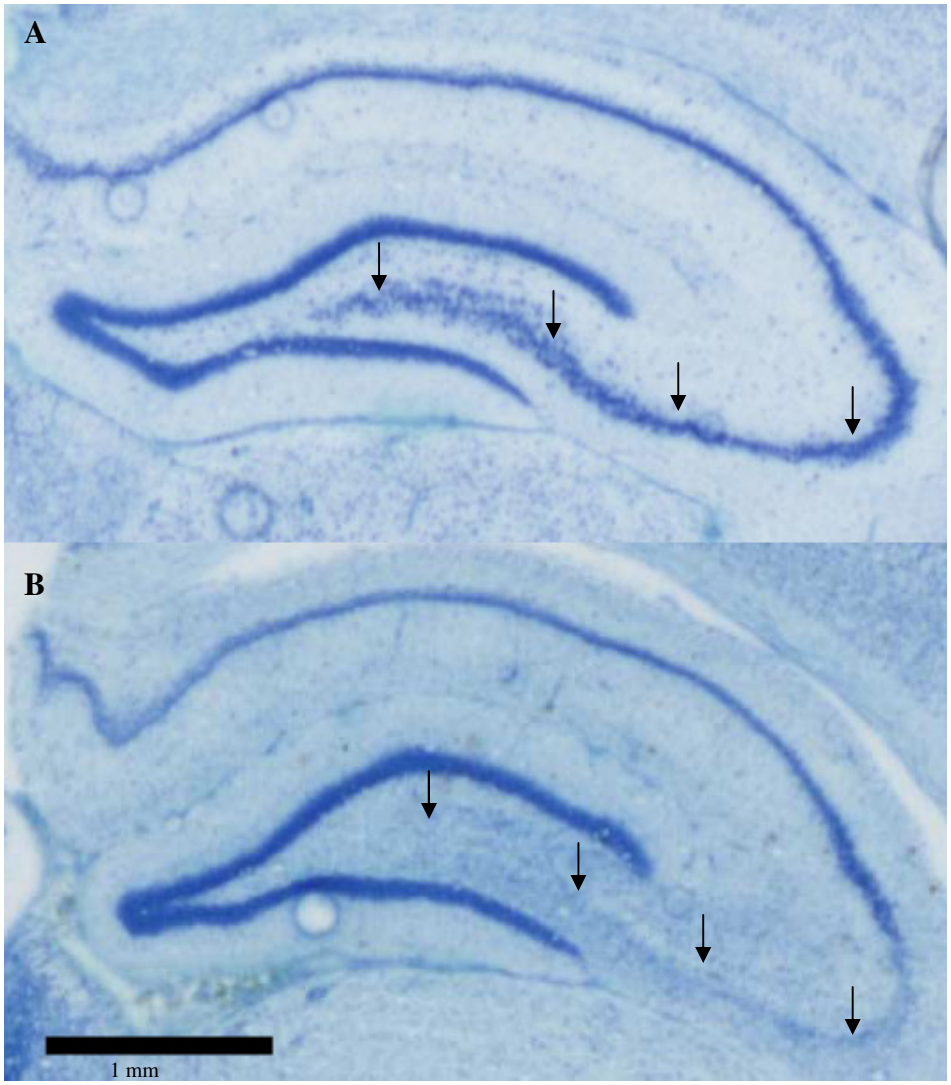

Figure 3-3: (A) Arrows indicate the CA3 cell layer in methylene blue/Azure II-stained hippocampal sections. (B) Cell loss was operationally defined as the absence of the pyramidal cell layer (arrows) in this division of the hippocampus in both the injected (ipsilateral) and non-injected (contralateral) hemispheres.

Figure 3-4: Lateral border of CA3 cell layer

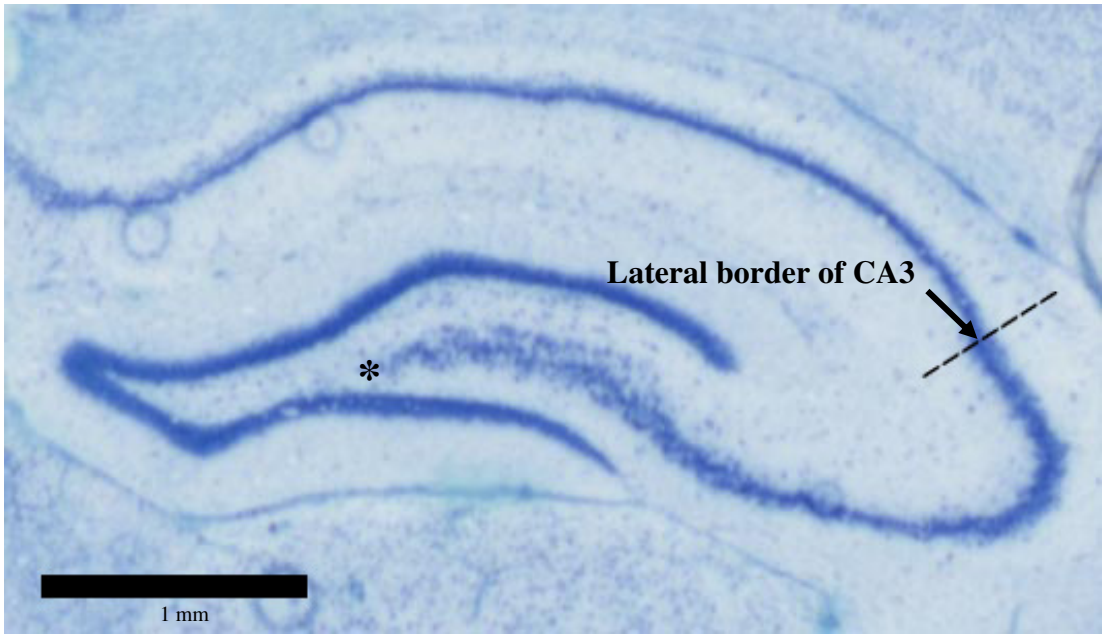

Figure 3-4: Dashed line represents the lateral border of the CA2/CA3 hippocampal cell layer. CA3 length measurements were taken from the medial-most point along the pyramidal cell layer (*) to the lateral border.

Figure 3-5: Percentage of animals exhibiting behavioral SE

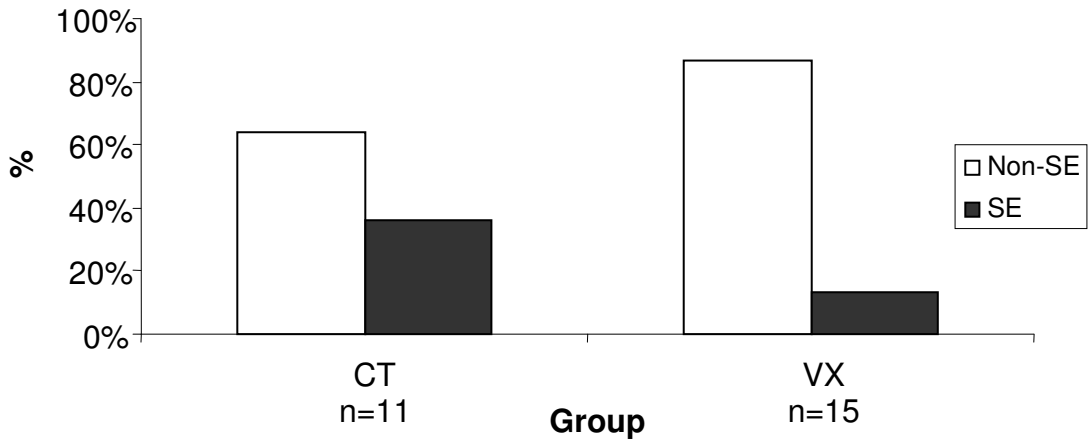

Figure 3-5: Percentage of animals entering SE within 3-hour period following KA administration did not differ significantly between CT and VX groups [X^2=1.981, p=.159].

Figure 3-6: Extent of cell loss was related to SE progression

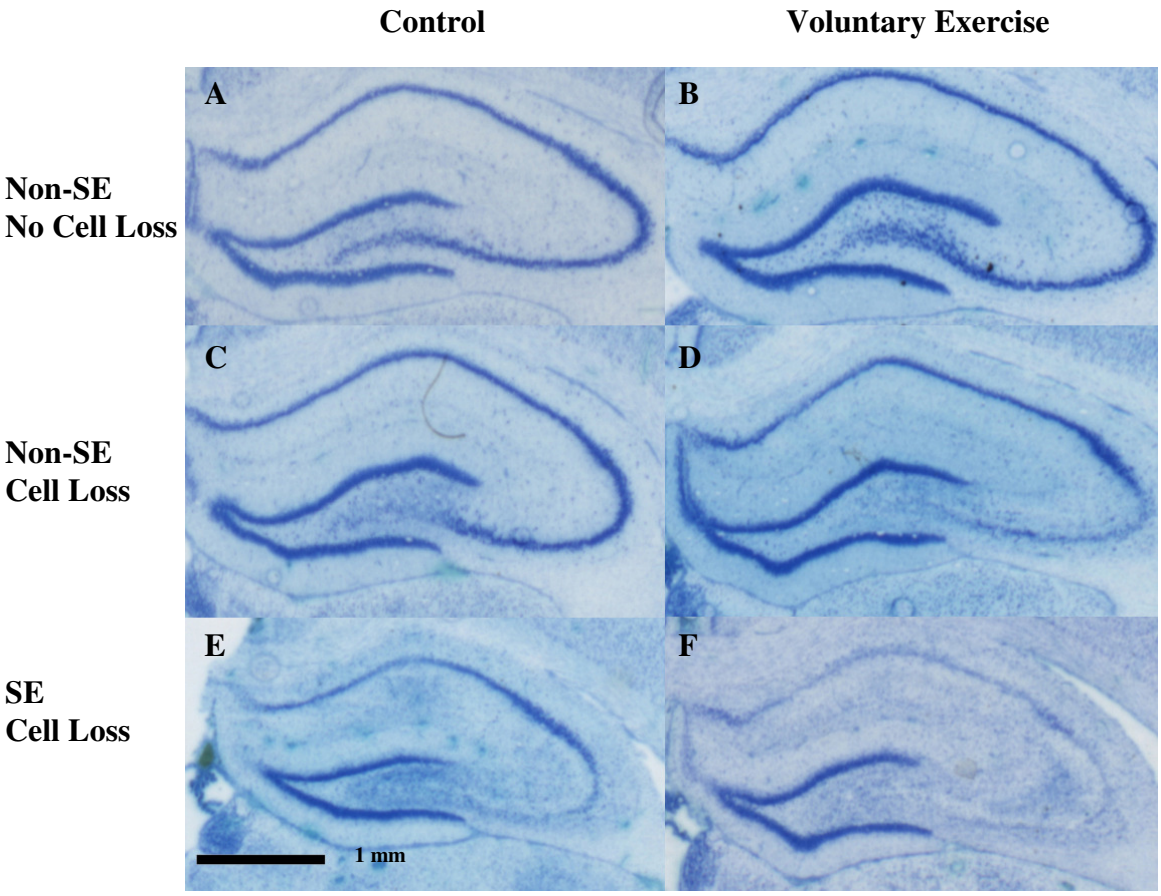

Figure 3-6: The extent of cell loss was related to seizure progression following KA administration. Among non-SE CT and VX animals not exhibiting CA3 damage (A and B, respectively), the pyramidal cell layer remained intact within this brain region. An intermediate level of cell loss was evident in a small proportion of non-SE CT animals (C) and a large proportion of non-SE VX animals (D). By contrast, SE produced extensive CA3 cell loss in both CT and VX animals (E and F, respectively). In all instances of visible cell loss, the pattern of loss preceded in a medial-to-lateral manner, such that the greatest amount of damage was evident in the medial-most area of CA3, while damage in the lateral component of the area of interest represented more extensive cell loss, reflected in shorter CA3 cell layer lengths among these animals (C – F).

Figure 3-7: Mean CA3 length in subregions of the dorsal hippocampus of non-SE animals

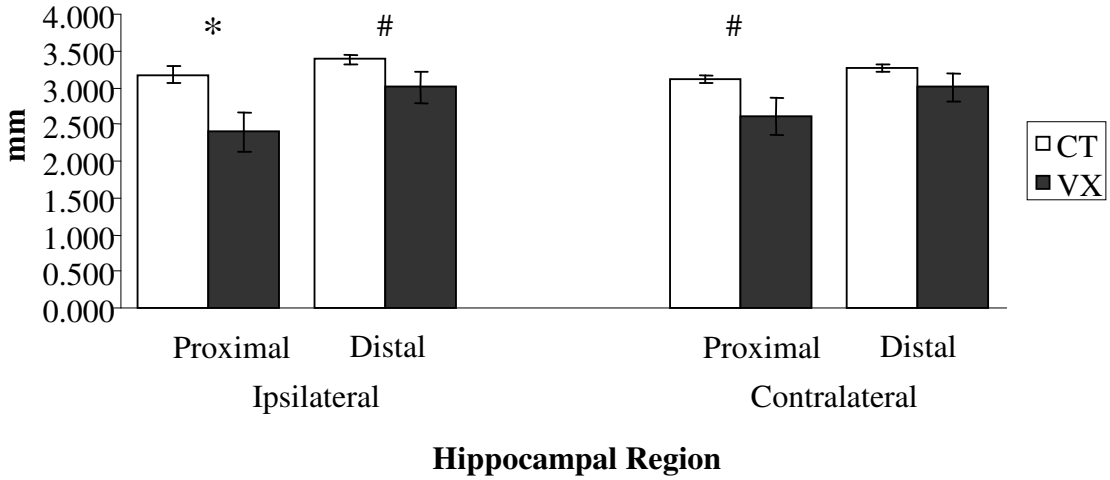

Figure 3-7: CA3 length in the dorsal hippocampus was measured in the proximal and distal subdivisions of both the injected (ipsilateral) and non-injected (contralateral) hemispheres of animals that did not progress to SE (non-SE animals). Physical exercise resulted in significantly shorter CA3 cell lengths in the proximal component of the ipsilateral dorsal hippocampus and a trend toward shorter cell layers in the distal subdivision of the ipsilateral dorsal hippocampus and across both subregions of the contralateral dorsal hippocampus [*=p<.05, #=p≤.10].

Figure 3-8: Correlation between cell loss and SE

CT animals

	CL−	CL+	
SE−	8	1	**9**
SE+	0	2	**2**
	8	**3**	**11**

r=.770, p=.006
r^2=.593

VX animals

	CL−	CL+	
SE−	6	7	**13**
SE+	0	2	**2**
	6	**9**	**15**

r=.300, p=.245
r^2=.102

Figure 3-8: CA3 cell loss was significantly correlated with SE in CT animals, but not in VX animals, so that CT animals exhibiting cell loss were more likely to have entered SE in the coding period following KA administration than voluntary exercisers (VX animals).

4. APPENDICES

Appendix A: Abridged review of hippocampal anatomy

The hippocampal formation is a laminar structure located in the medial temporal lobe. It is comprised of four major parts: the dentate gyrus, Ammon's horn (*or* the hippocampus proper), subiculum, and entorhinal cortex. Amaral *et al.* (1995), Bayer (1985), and O'Keefe & Nadler (1978) provide extensive reviews of hippocampal regions, cells and connections, and therefore served as the primary sources for this brief review. Circuitry of the hippocampal formation is unique in the CNS in that it consists mainly of a series of well-defined unidirectional projections. The DG is a 3-layered structure and receives its principal input from the entorhinal cortex via the perforant pathway. The superficial-most layer – the molecular layer – is cell-sparse and populated by dendrites of cells in the adjacent layer. Most of the perforant path-DG synapses are located in this layer. The middle layer – the granule cell layer – is comprised mainly of glutamatergic granule cells, but also contains GABAergic basket cells and a variety of small, inhibitory interneurons. The deep-most layer – the polymorphic cell layer *or* hilus – contains large, triangular cells (called *mossy cells*) and a number of interneuron types. Granule cells have prominent axonal projections marked by large, vesicle-filled terminals called *mossy fibers*. Mossy fibers send out collateral projections that innervate the mossy cells of the hilus and the granule cells themselves, but a majority of the fibers project to the CA3 region of Ammon's Horn. Mossy cells of the hilus project back to the granule cells,

providing a feedback loop. In this way, the only output from the DG is via the MF connection to CA3.

Ammon's Horn is a 5 layered structure, which can be further divided into 3 linear regions: CA1, CA2, and CA3. The cytoarchitecture is relatively consistent between the three regions, with the exception of an additional morphological layer in the CA3 region. On visual examination, the most prominent layer of Ammon's horn is *stratum pyramidal*, containing the soma of the pyramidal cells, which is the dominant neuron type in Ammon's Horn. In addition to pyramidal cells there are GABAergic basket cells. Pyramidal cells of the hippocampus proper are arranged with a long prominent dendrite (termed *apical* dendrites) protruding from the apex of the cell body, and projecting toward the center of the hippocampus. Relatively smaller dendrites (termed *basal* dendrites) emerge from the rest of the soma, and project into a layer that wraps around the outside of this curved structure. Axons exit the cell bodies on the basal surface in *s. oriens* the layer immediately ventral to *s. pyramidal*. This layer also contains a number of small GABAergic interneurons. Persisting on, the axons continue superficially into *s. alveus*, where they turn and travel laterally, between the regions of Ammon's Horn and to extrahippocampal structures. In some regions of Ammon's Horn, collaterals of axons also turn and travel back through the neuropil to innervate the apical dendrites of pyramidal cells and small interneurons (see below). *Stratum radiatum* is immediately dorsal to the cell body layer and contains primary and secondary branches of apical dendrites, GABAergic interneurons, and the aforementioned axon collaterals. The deep-most layer, *s. lacunosum*, contains lower-order branches of the apical dendritic arbor, which are innervated by the perforant pathway as it courses through this layer of

Ammon's Horn *en route* to the molecular layer of DG. In CA3, interposed between *s. radiatum* and *s. pyramidal*, is a relatively small layer comprised of synaptic connections between MF terminals from the granule cells of DG and large, thorny excrescences on the base of apical dendrites. Prominent glutamatergic presynaptic terminals that provide immense excitatory input in close proximity to CA3 cells bodies mark these synapses. The quantity of glutamate released at these synapses ensures the generation of an action potential in CA3 pyramidal neurons.

Axons of CA3 bifurcate in *s. alveus*, with one stream traveling through CA2 and CA1 and continuing out to the fimbria. The second stream travels up through *s. oriens* and *s. pyramidal* and continues along *s. radiatum* (through CA1 and CA2) out to the lateral septal nucleus, branching *en route* to innervate basket cells in all three regions of Ammon's Horn. Additionally, this pathway innervates the apical dendrites of CA1 – via what is termed the *Schaffer collaterals* – such that each pyramidal cell of CA3 sends an excitatory afferent to the pyramidal cell of CA1. In this way, the CA3 region of the hippocampus serves to relay cellular signaling originating in the DG to all regions of the hippocampus proper and to coordinate the integration of this manifold signaling through its simultaneous excitation of pyramidal cells and GABAergic interneurons. Although similar in cell size and density, the CA2 region can be distinguished from CA3 by its lack of MF synapses and the *lacunosum* layer they form in CA3. There is some evidence that CA2 receives afferents from the hypothalamus and the mammillary area. CA2 connections are similar to those of CA3, with CA2 providing afferents to all subregions of CA1. The cells of CA1 are smaller and less densely packed than those of CA2/3. There are two major projections from the CA1 region: one to the subiculum and the other

to the entorhinal cortex. Thus, the general flow of information in the hippocampus is maintained in a unidirectional circuit. In addition to the intrahippocampal circuitry described here in brief, each region of the hippocampus sends a topographically well-preserved connection to its contralateral counterpart.

Appendix B: Animal models of epilepsy

Animal models of temporal lobe epilepsy (TLE) seek to model the human pathology in order to understand the causes of seizures and how the consequence of initial seizures leads to vulnerability to subsequent seizures. The validity of such models must be considered. The consequence of seizures in valid models can then be studied to develop an understanding of the mechanisms that lead to cell death, and the subsequent events that lead to susceptibility to recurrent seizures. Understanding these events has allowed investigators to test the influence of other factors on TLE. The effect of exercise on vulnerability to initial seizure (which would influence the frequency of the development of epilepsy) as well as response to the initial seizure (which will influence the severity of epilepsy) has been studied using animal models of TLE.

Valid models of TLE must meet the following criteria in order to be considered analogous to the human condition: i. central involvement of the hippocampus and amygdala in pathology; ii. pattern of neuronal damage consistent with Ammon's horn sclerosis; iii. spontaneous recurrent seizures; iv. relatively resistant to anticonvulsants (comparable to human TLE) (Ben-Ari, 1985). Generally, animal models of TLE are defined by their ability to induce status epilepticus (SE) – a generalized, convulsive seizure – later resulting in permanent recurrent seizures. These latter models of acquired epilepsy are specifically considered to be analogous to the condition seen in young children who develop epilepsy following febrile seizures, and patients who develop epilepsy in response to stroke, tumors, or head trauma (Annegers, 2001).

Two commonly used animal models of epilepsy are kindling and chemical-induced SE. In kindling, electrical stimulation is applied to the amygdala or the

hippocampus, eliciting physiological and behavioral seizure activity which closely mimics the clinical condition (Sato, Racine, & McIntyre, 1990). Stimulation of the amygdala specifically triggers physiological activity in the hippocampus that is consistent with the activity observed during SE. This effect is mediated by the amygdala's excitatory connection to the hippocampus via the perforant pathway (see Appendix A). In about 50% of rats, daily administration of kindling over several weeks or months results in SE, with the length of time required for this effect dependent on the exact site of kindling (Michalakis et al., 1998). Kindling is a useful tool in the laboratory in that seizures arising from a given brain region (the location of the electrode) can be generated on demand, but a major drawback of this method is the length of time required to generate the spontaneous seizures that typify the epileptic condition. While kindling is generally accepted as a model of TLE, it lacks some other anatomical features of clinical epilepsy, including sclerosis of hippocampal neurons and amygdalic damage (Morimoto, Fahnestock, & Racine, 2004; Tuunanen & Pitkanen, 2000).

In order to more closely approximate the type of cell damage seen in human patients, chemical convulsants (or *chemoconvulsants*) are frequently used as models of TLE. Administration of a single dose of these compounds is often sufficient to produce the sustained cellular depolarization necessary to induce SE. Two common such substances are pilocarpine and kainic acid (KA). Pilocarpine is an acetylcholine agonist that acts at muscarinic-type acetylcholine receptors throughout the body. Kainic acid is a glutamate analogue. Pilocarpine and KA are both believed to cause neuronal loss by ultimately inducing a calcium influx, resulting in the activation of apoptotic-like pathways in the affected cells. Pilocarpine is thought to lead to glutamate release

indirectly through hyper-activation of muscarinic receptors (Liu, Nagao, Desjardins, Gloor, & Avoli, 1994).

The pattern of neuronal damage observed with pilocarpine administration is similar to that seen with KA (described below), but there are some significant differences that distinguish the two substances in their use as models of epilepsy. Pilocarpine requires the administration of drugs such as scopolamine to block its effect on peripheral muscarinic receptors. Additionally, mortality rates during SE induced by pilocarpine (Cavalheiro et al., 1991; Liu, Nagao, Desjardins, Gloor, & Avoli, 1994) are much higher than with KA (Camon, de Vera, & Martinez, 2001). In light of these two observations, and because of its ability to consistently produce patterns of neuronal damage congruent to the hippocampal sclerosis seen in the human pathology, KA appears to be the preferable chemoconvulsant model of TLE.

Kainic acid is an analogue of the major excitatory neurotransmitter in the brain, glutamate, and binds selectively to a subset of glutamate receptors – termed, *kainate receptors*. While there is evidence for the location of KA receptors both on the pre-synaptic and post-synaptic membranes (Ben-Ari, 1985), the most significant action of KA in inducing seizures is thought to be the action of KA on receptors located on the membranes of pre-synaptic boutons of large mossy fiber terminals in the hippocampus. Their action facilitates the release of excitotoxic quantities of glutamate from these terminals. The presence of such large amounts of glutamate at the post-synaptic membrane leads to depolarization of the cell and a consequential increase in intracellular calcium from the concurrent opening of ligand- and voltage-gated ion channels in the cell membrane and the mobilization of intracellular calcium stores. Toxic quantities of

cytoplasmic calcium lead to activation of several enzymes that can break down cytoskeletal proteins and disrupt the membrane, which in turn lead to cell death. The resulting neuronal loss in the hippocampus following KA-induced SE is consistent with Ammon's horn sclerosis (Ben-Ari, 1985). Susceptibility to KA-induced cell damage varies across brain regions with the hippocampus – especially the CA3 and DG subregions – being the most vulnerable (Ben-Ari, 1985).

In the non-epileptic brain, a major afferent of the hippocampus is the entorhinal cortex, which innervates granule cells of the DG. Granule cells in turn make excitatory synapses onto pyramidal cells in the CA2 and CA3 subregions of Ammon's Horn and onto mossy cells of the hilus. Large, vesicle-dense pre-synaptic terminals that selectively form excitatory synapses onto pyramidal apical dendrites in stratum lucidum of CA3 mark these singular connections (termed *mossy fiber synapses*). By contrast, in the epileptic brain, complete loss of their CA3 and hilar targets leads to sprouting and redirection of mossy fiber afferents back into the inner portion of the molecular layer of the DG, forming recurrent synaptic connections onto the dendrites of the granule cells that gave rise to these aberrant processes (Routbort, Bausch, & McNamara, 1999; Scharfman, Sollas, Berger, & Goodman, 2003; Sutula, Cascino, Cavazos, Parada, & Ramirez, 1989). In the intact hippocampus, there is a small, reciprocal connection that normally provides feedback to the inner layer of the DG (Amaral, 1995). However, this connection is amplified in the epileptic hippocampus (Isokawa, Levesque, Babb, & Engel, 1993), representing a robust increase in recurrent MF innervation of the granule cell layer of the DG. These observations have led to the theory that this excitatory activity originating in the DG may indeed be the source of electrical spread in TLE. This

theory is consistent with findings that resection of the hippocampus in a number of human epileptics eliminates or greatly reduces the occurrence of seizures (Bailey & Gibbs, 1951; Engel J, 1987; Luders, 1991).

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