UNIVERSITY OF CALIFORNIA, SAN DIEGO

Rats with confirmed temporal lobe epilepsy have impaired pattern separation behavior on 8 arm radial maze

A Thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Biology

by

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2017

The Thesis of Ario Ramezani is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2017

DEDICATION

This thesis is dedicated to my family and friends

for their endless love and support.

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LIST OF ABBREVIATIONS

IACUC	Institutional Animal Care and Use Committee
MTL	Medial temporal lobe
CNS	Central nervous system
GCL	Granule cell layer
IML	Inner molecular layer
TLE	Temporal Lobe Epilepsy
CTE	Chronic Traumatic Encepalopathy
fMRI	Functional Magnetic Resonance Imaging
RAM	Radial Arm Maze
KA	Kainic Acid
PBS	Phosphate-buffered saline

ACKNOWLEDGMENTS

I would like to thank Jill Leutgeb and Laura Ewell for their mentorship and experimental design of the project. Thank you to J. Leutgeb, additionally, for the opportunity to work in the Leutgeb Lab.

I would also like to thank Veronica Piatti for donating her data on the dentate lesion experimental group that was used for comparison within this thesis. To the UCSD students—Gabrielle Suarez, Kimia Nourmahnad, Manpreet Bangian, Danielle Esteban, and Christopher Luong—thank you for your contributions toward behavioral testing, video monitoring, histology, and microscopy throughout the course of the past year.

Thank you to the entire Leutgeb Lab for your help and advice on various aspects of this project. Lastly, I would like to thank my additional thesis committee members, Stefan Leutgeb and Daniel Donoghue, for their kind support.

The results section of the thesis is currently being prepared for publication in which I will be a co-author alongside Jill Leutgeb and Laura Ewell.

ABSTRACT OF THE THESIS

Rats with confirmed temporal lobe epilepsy have impaired pattern separation behavior on 8 arm radial maze

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Ario Ramezani

Master of Science in Biology

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Professor Jill Leutgeb, Chair
Professor Stefan Leutgeb, Co-Chair

Episodic memories are a unique subtype of memory that encode the when, the where, and the what. The dentate gyrus within the hippocampus is vital for mediating pattern separation: the brain's distinct ability to distinguish very similar memories from one another. Epilepsy, a disease characterized by hyper-excitation of neuronal circuits, is correlated with neuronal reorganization of the dentate gyrus. Previous studies from our lab have demonstrated that rats induced with chronic temporal lobe epilepsy through a low-dose kainate model have impaired behavioral performance on an 8 arm radial maze that tests for pattern separation. In this project, we asked whether these behavioral deficits were a precursor to, or a result of, chronic epileptogenesis. We introduced a 24-hour video monitoring system, which allowed us to review recorded video of induced animals in a vivarium to observe and score seizures. This data was then compared to the data collected from behavioral trials and we found that in the adjacent condition of the 8 arm radial maze (pattern separation condition), those with confirmed epilepsy had a statistically significant increase in trials necessary to reach criterion when compared to the control group. Their performance was comparable to a dentate gyrus lesion group, which controlled for inhibited pattern separation function. In the non-adjacent 8 arm radial maze condition, there was no significant difference in performance between groups since this condition is not dentate gyrus-dependent. Holistically, we conclude that chronic epileptogenesis results in impaired pattern separation behavior. Histological analysis of Timm stained tissue further supports that mossy fiber sprouting is positively correlated with impaired pattern separation behavior.

Introduction

Episodic memories are a unique subtype of memory that quickly forms to encode information about what, when, and where something occurred. The formation of these memories, those that capture and consolidate vivid details from our surroundings at the time of incidence, is hypothesized to be a distinct function of the hippocampus. Within the hippocampus exists a complex network of neuronal pathways that provide the succinct opportunities to retrieve the millions of memories housed in its circuitry. The dentate gyrus, a subregion of the hippocampus, receives excitatory inputs from the cerebral cortex and filters these stimuli before relaying the information to the CA3 subregion. This mechanism allows for the retrieval of stored episodic memories and the facilitation of a unique function called pattern separation.

Pattern separation is our brain's ability to convert similar patterns of input stimuli into less similar outputs (Madar et al., 2016). In other words, it is the ability to distinguish very similar memories from one another. With this neuronal process, we are able to discern the intricate details that distinguish varying memories in order to minimize confusion between past and present stimuli. Previous studies have investigated the role of the dentate gyrus by inducing lesions of this area and examining pattern separation behavior (Morris, Churchwell, Kesner, Gilbert, 2012). In this study, the investigators discovered that lesion of the dentate gyrus led to rats having impaired pattern separation behavior. This was quantified by observation of the increase of trials it took for dentate lesion animals to learn a pattern separation behavioral task in comparison to control animals injected with a vehicle. Such studies have not demonstrated that the dentate

gyrus is necessary for pattern separation, but show strong support for it playing a significant role in efficient pattern separation capabilities.

When presented with visual stimuli that we have encountered before, our brains work to decode the stimuli and make sense of our surroundings. In this situation, we begin to recall other times when we have been in a similar scenario and attempt to segregate the unique details that discern one experience from the next. The more similar the stimuli, the more difficult it is to accurately retrieve the desired memory. Thus, the hippocampus has developed in such a way to optimize pattern separation and associated memory retrieval. Prior research has made use of behavioral, histological, fMRI, and electrophysiological recordings in humans and rodents to further demonstrate that the dentate gyrus does, in fact, mediate pattern separation (Bakker, Kirwan, Miller, and Stark, 2008; J. Leutgeb, S. Leutgeb, M. Moser, E. Moser, 2007; Yassa et al., 2010; Chawla et al., 2005; Marrone, Adams, and Satvat, 2011).

Epilepsy is a neurological disease described as hyper-excitation of neurons in the central nervous system (CNS), which has been known to decrease certain cognitive functions, including memory. Many retrospective analyses of clinical and animal studies have shown evidence of temporal lobe epilepsy (TLE) being the result of a "precipitating event" early in life (Patterson, Baram, and Shinnar, 2014). Examples of such events may include recurrent febrile seizures in early childhood, which could induce hippocampal neuronal reorganization and sclerosis, providing a foundation for development of chronic temporal lobe epilepsy later in life.

In recent years, two methods of inducing chronic TLE have become widely used and accepted by the scientific community. The first is electric stimulation—kindling—of

temporal lobe to induce seizures. The other involves intraperitoneal, subcutaneous, or intracranial injections of drugs such as kainic acid. The latter, used in this project, subjects rats to an acute phase of chronic epileptogenesis where animals enter status epilepticus characterized as recurrent seizures. The frequency of these seizures is enough to produce strong activation of the temporal lobe and induce the restructuring of hippocampus, which is similar to the pathology seen in human patients with epilepsy. The completion of this acute phase marks the beginning of a latent phase, which lasts for several weeks and is characterized by no seizure activity. Once chronic epileptogenesis reaches the final phase, the chronic phase, the induced animals exhibit spontaneous seizures from that point onward (Maguire, 2016).

As previously mentioned, the dentate gyrus receives inputs from upstream neurons and filters these inputs as a "gateway" to the downstream CA3. The dentate gyrus has been of profound interest in studies investigating mechanisms underlying epileptogenesis in TLE. These studies have generated a number of hypotheses to provide an explanation of the molecular and cellular basis of epileptogenesis. Among these hypotheses is that the development of newly excitable circuits that follow mossy fiber sprouting can lead to hyper-excitability in the temporal lobe, thus constituting seizure activity. Repetitive acute seizures that are induced with a recurrent injection of the chemotoxin, kainic acid, serve as a method of causing neuronal loss in the dentate gyrus. This neuronal death is hypothesized to contribute to mossy fiber sprouting in the granule cell layer of the hippocampus (Dudek and Sutula, 2007). Thus, the presence of mossy fiber sprouting implies neuronal death has occurred within the dentate gyrus. With the loss of these neurons in this hippocampal subregion, the"gateway" of a tri-circuit

hippocampus, we anticipate dentate gyrus-dependent behavioral function to become impaired. In this study, we further examine the relationship between mossy fiber sprouting and pattern separation behavior. We hypothesize that the severity of quantified sprouting is proportional to the severity of impairments observed in subjects learning a dentate gyrus-dependent behavioral task.

Our lab has previously demonstrated that the low-dose model of inducing chronic TLE can be used to generate similar behavioral deficits on a pattern separation behavioral task as seen with dentate lesion animals. Moreover, they showed that kainic acid (KA) induced rats exhibited impaired pattern separation when compared to control animals. In this next experiment, we implemented a 24-hour video monitoring system that would allow for continuous observation of animals throughout the period that behavioral trials were run. With this system, we were able to identify induced subjects that reached the chronic phase of epileptogenesis who had sporadic seizures and compare their results to healthy animals, those with a dentate lesion, and other KA induced rats without observed seizures.

With these set experimental parameters, we were able to investigate whether these behavioral impairments are a precursor to, or an effect of, epilepsy. The distinction between KA induced rats with or without confirmed epilepsy via 24-hour video monitoring provided a model to answer our question. In the case that the pattern separation behavioral impairments were a precursor to epilepsy, we wondered if a pattern separation task could be used as a prognostic or diagnostic tool to evaluate if at-risk patients were developing epilepsy.

In this project, we also analyzed histological data in order to assess the presence of a correlation between pathology associated with epilepsy and behavioral performance of animals on various conditions of an 8 radial arm maze. The hippocampus of all animals was visualized using a Timm stain and further analyzed for epileptic pathology. The dentate gyrus is comprised of two unique cell layers called the granule cell layer (CGL) and inner molecular layer (IML). Previous histological analysis of kindled rat tissue that was Timm stained showed evidence of mossy fiber neuronal reorganization (Cavasos, Golarai, Sutula, 1991). This pathology, common among epileptic subjects, is evidence of a deteriorating mossy fiber pathway that links the dentate gyrus to the CA3 of the mammalian hippocampus. The severity of the sprouting in each section was scored and these results were cross reference with results from behavioral trials in order to determine existence of a possible correlation.

Materials and Methods

Subjects

The project utilized 48 male Long Evans rats that were housed individually on a 12 hour light and 12 hour dark schedule with lights off at 7:00 A.M. Behavioral experiments were run during the animals' dark phase. The protocols and procedures conducted during the course of this experiment were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego. All experiments performed were further in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Low Dose Kainate Induction

At 40 days postpartum, six of twelve Long Evans rats from the same litter were induced with temporal lobe epilepsy using a low dose kainate model (Hellier and Dudek, 2005). 50 mg of kainic acid was purchased from Tocris Bioscience and dissolved in 5 mL of sterile saline to create a 10 mg/mL solution of kainate. The solution was left on a high speed shaker for 1 hour. Rats were weighed and a 5 mg/kg dose of kainate was prepared in a syringe designated for each animal. An intraperitoneal injection of the kainate was delivered to each rat every hour. The subjects were observed for seizures until status epilepticus was achieved. Status epilepticus is defined as continuous stage IV or stage V seizures. Once status was reached, the animals remained in this state for 2 hours. After the 2 hour mark, the induction was terminated by an intraperitoneal injection of 35 mg/kg of Pentobarbital. The induced subjects were monitored periodically over the next day to ensure good health. One induced subject (n = 1) died during the induction. The remaining

six rats that were not induced represent the age-matched control groups in each round of behavior.

Video Monitoring

At 6 months of age, the induced animals were observed on a 24-hour Q-See surveillance system set up in the vivarium. The recorded video was observed for seizures and the seizures were scored according to the Racine scale (Racine, 1972). Seizures were classified on a scale of I through V with the following criteria: (stage I) face and/or jaw convulsions; (stage II) stage I criteria and head nodding; (stage III) forearm clonus; (stage IV) stage III criteria with animal rearing on its hind legs; (stage V) stage IV and the animal falling over. Since stages I and II are limited to facial abnormalities, these stages were not typically visible with the video monitoring system, and thus, could have been overseen. Furthermore, while all induced animals achieved status epilepticus during the low dose kainate induction, only 8 of these subjects (n = 8) had confirmed epilepsy from the video monitoring.

Behavioral Apparatus

This project assesses spatial pattern separation. To assess such behavior, the rats were trained on an 8 Arm Radial Arm Maze (RAM) in a room rid of any obvious spatial cues. This RAM consists of 8 arms arranged around an octagonal stem. Each arm is spaced 45 degrees apart for its adjacent arms. The RAM measures approximately 188 centimeters in diameter and is elevated approximately 36 centimeters above the ground. Transparent Plexiglas walls were placed around the central stem of the maze and attached with velcro in order to block unused arms and designate the two arms that were open per trial. The Plexiglas measured approximate 30 centimeters in length by 0.6 centimeters in

width. Uniform, chrome, silver bowls were taped to the end of each arm.The bowl of the reward arm (arm 1) was the location of the food reward for all behavior testing performed during this experiment. The food reward consisted of Kellogg's Cocoa Puffs and Cocoa Pebbles.

Behavioral Diet

One week prior to the start of habituation, the animals were placed on a strict food restricted diet. On the first day of this diet, each animal was weighed and their baseline weight recorded. This initial weight was used to calculate the goal behavior weight of each animal (85% of baseline weight). Animals were weighed every day and provided food as necessary to maintain the goal weight. The purpose of this diet was to facilitate animal motivation on the maze and optimize behavioral performance. During this food depravation week, the subjects were introduced to their food reward every third day within their home cages. One rat (n = 1) was removed from behavioral trials after being fed during the food restriction period.

Habituation and Behavior: Setup

Each rat was brought up from the vivarium to the behavior room at approximately the same time every day. Some fluctuations in habituation and behavior time occurred, but these fluctuations were strictly kept within a four hour time frame of the animal's daily behavior time to ensure consistency. Once brought up to the behavioral testing room, each rat was given a food reward in its cage. The stem and three arms that were used for the behavioral testing were cleaned with 70% ethanol. Each animal was weighed and their weight and any health observations were logged on a health monitoring sheet.

At the end of that day's experiment, the animal was placed back in its home cage, fed if appropriate for maintaining the goal weight and returned to the vivarium.

Habituation

At age 6 months, the rats began habituation on the 8 arm RAM. After completion of the preliminary steps outlined in *Habituation and Behavior: General Logistics*, each animal was individually placed onto the stem of the RAM and covered by an opaque bucket while the experimenter removed Plexiglass from the three arms that the animal would be required to run on throughout the course of behavioral testing. The experimenter would remove the opaque bucket and remain in the room to collect observations regarding habituation. Habituation was conducted in three phases. The first phase involved placement of a single food reward at the stem of the maze, midway down the three experimental arms, just outside the silver bowls, and inside each bowl. The animal was given 10 minutes to explore this phase of habituation. Rats were removed from the maze at the end of the 10 minutes and put back in their cage. If the animal ate all 10 rewards within the 10 minute frame, he moved to phase 2 the following day. If this criterion was not met, the animal would repeat phase 1 until passing. The second phase is characterized by placement of the food reward within the bowls and just outside the bowls of the same three arms. The animal once again had 10 minutes to eat all the food rewards. Successful completion of this would forward the animal to phase 3 the next day. The third phase is set up with only food in the 3 bowls. The subjects only had 5 minutes to complete this phase since it is most representative of the actual behavioral task. Once criterion was met for this phase, the animal was habituated and began day 1 of behavioral trials the next day. At the end of each habituation day, the animal was placed back in its

home cage, fed if appropriate for maintaining the goal weight and returned to the vivarium.

Behavior: Adjacent Condition

Trials were conducted with control (n = 25), kainate induced animals (n = 18), and dentate lesion (n = 11) animals. The protocol below is based on those from Morris et al 2012. After completion of the preliminary steps outlined in *Habituation and Behavior: General Logistics,* each animal was individually placed onto the stem of the RAM and covered by an opaque bucket while the experimenter set up the maze for the first trial of the day. One arm was designated as the reward arm and the arms immediately left and right of the reward arm were deemed non-reward arms (Figure 1). The two arms specified by protocol had their Plexiglass removed of the maze. The experimenter removed the opaque bucket and the rat would make his choice. The criterion for "choice" here is when the animal places all four legs onto one arm. Thus, if the animal chose the reward arm, but did not go down the entire length of the arm, he was still given a reward. After each decision, the animal would return to the stem and be covered with the bucket for approximately one minute while the next trial was prepped. If the rat took over 5 minutes to make a decision, went on a blocked off arm, or transversely crossed arms, the trial was restarted. Ten trials were run per animal, per day. Each adjacent, non-reward arm was used 5 times among the 10 trials in a pseudo-random order to ensure that the rat was utilizing pattern separation rather than a right-left strategy when making its decision. Between trials, the RAM was wiped down with a damp towel in order to prevent the animal's sense of scent from guiding their decisions as opposed to their memory. Between animals, the maze was thoroughly cleaned with 70% ethanol. At the end of that

day's experiment, the animal was placed back in its home cage, fed if appropriate for maintaining the goal weight, and returned to the vivarium. No animals were run on the maze within 2 hours of having a seizure in order to avoid collection of false data as a product of post-seizure amnesia. One KA induced animal (n = 1) was removed from the study due to having severe chronic epilepsy and having seizures while on the maze.

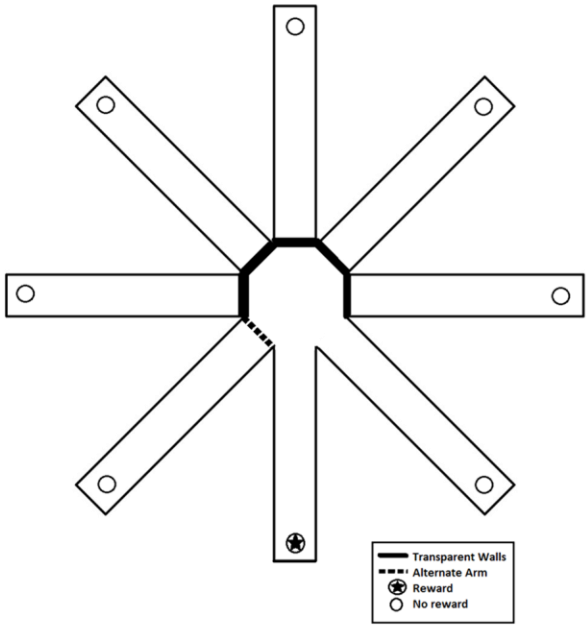

Figure 1. Maze Configuration for the Adjacent Condition. Control (n = 25), KA induced (n = 18), and Dentate Lesion (n = 11) rats were placed on the orthogonal stem of an 8 arm radial maze at the beginning of every habituation and behavioral trial day. One arm is designated as the reward arm for the duration of behavioral trials. One of the two adjacent arms is pseudo-randomly designated as an alternate arm for each trial. If the rat stepped all four of his feet onto the reward arm, he received the reward for that trial. If the rat stepped all four feet onto the alternate arm, he did not receive the reward.

Behavior: Non-Adjacent Condition

Trials were conducted with control (n = 15) and kainate induced (n = 17) subjects. All protocols were identical to those outlined above in *Behavior: Adjacent Condition.* However, in the non-adjacent condition, the non-reward arms were three arms away from

the reward arm (Figure 2). No animals were run on the maze within 2 hours of having a seizure in order to avoid collection of false data as a product of post-seizure amnesia.

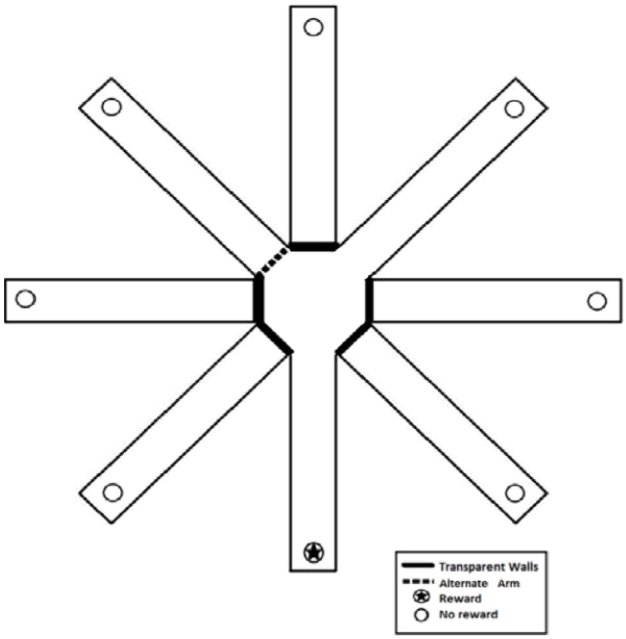

Figure 2. Maze Configuration for the Non-Adjacent Condition. Control (n = 15) and KA induced (n = 17) rats were placed on the orthogonal stem of an 8 arm radial maze at the beginning of every habituation and behavioral trial day. One arm is designated as the reward arm for the duration of behavioral trials. One of the two arms located 120 degrees away from the reward arm (3 arms away) is pseudo-randomly designated as an alternate arm for each trial. If the rat stepped all four of his feet onto the reward arm, he received the reward for that trial. If the rat stepped all four feet onto the alternate arm, he did not receive the reward.

Perfusions

After completion of behavioral testing, subjects were placed in an anesthesia chamber filled with gaseous isoflurane. Once anesthetized, the rat was given a lethal injection of Pentobarbital (0.8 mL – 2 mL). The rat's pain response was tested by pinching its feet. If no reflex, which indicated pain, was exhibited, then the perfusion was carried forward. Rats were, first, systemically perfused with 0.37% sulphide solution for

5 minutes and then with 4% paraformaldehyde (PFA) for another 10 minutes or until the tissue were fixed. Each brain was carefully extracted and submerged in 4% PFA and refrigerated for 24 hours. After this, the brains were transferred to 30% sucrose in 1X PBS solution and placed back into the refrigerator until being used for histology within 1 month of the perfusion date.

Tissue Sectioning

The right hemisphere of each brain was sectioned using a Leica microtome. The left hemisphere was kept in a 0.02% Sodium Azide in PBS (Na-Azide) solution for preservation. We began collecting sections once the early hippocampus was visible. 144, 40 um coronal sections were obtained and stored in 0.02% Na-Azide until mounting.

Timm Stain

Every sixth coronal section (240 um) was mounted onto Fisherbrand Superfrost Plus Microscope Slides with a gelatin and PBS mounting solution and left to dry over night. Slides were placed in distilled water for 3 minutes and then incubated in developer solution in the dark. The developer solution is composed of: 120 mL of Gum Arabic (100 g/200 mL), 10 mL of filtered citrate buffer (51g $C_6H_8O_7$/200 mL; 47g $C_6H_5Na_3O_7 \bullet 2H_2O$/200 mL), 60 mL of 1.7% hydroquinone solution, and 1 mL of 0.09% light-sensitive, silver nitrate solution. After 30 minutes of incubation, the developer solution was mixed and the tissue was re-submerged for at least another 30 minutes. If the stain was not dark enough, the tissue was left in solution until the stain was complete. The slides were then dipped twice in warm distilled water (50° C) and placed in room temperature distilled water for 15 to 30 minutes. Next, the slides were fixed in 5% sodium thiosulfat pentahydrat for 30 seconds. Slides were dehydrated in the following

series of ethanol concentrations: 70%, 80%, 90%, 100%, and 100% again. Dehydrated slides were placed in xylene and coverslipped with Permount. Once finished, the slides were left to dry overnight.

Timm Scoring

Timm stained slides were visualized using a Leica CTR 6000 microscope and analyzed for mossy fiber sprouting—reorganization of mossy fibers, which is pathology associated with epilepsy. The dorsal hippocampus was imaged on 10X magnification. Each image taken with the microscope was subject to blind scoring by two experimenters. The reorganization ("sprouting") of mossy fibers was scored according to the following scale: (0) no granules in the supragrangular region; (1) minimal granules in the supragrangular region; (2) several granules in the supragrangular region; (3) confluent patches of granules; (4) confluent patches of granules that connect into a dense laminar band; (5) criteria for (4) with increased width often extending into the inner molecular layer (Cavazos et al, 1991)

Results

Behavior Results

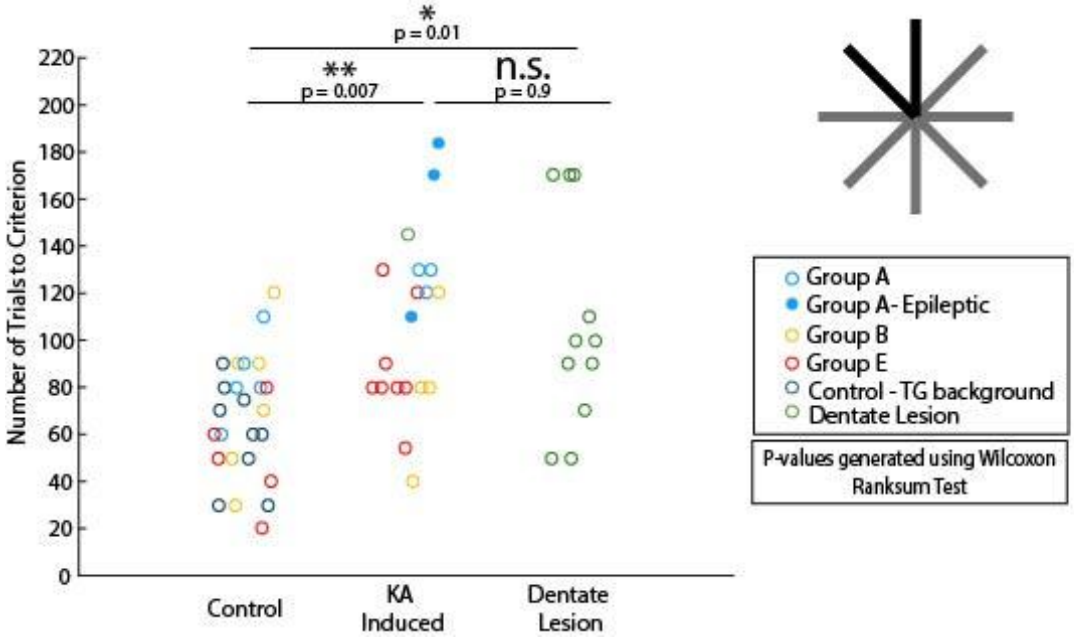

Figure 3. Rats induced with low-dose kainate model of temporal lobe epilepsy have impaired pattern separation behavior on the adjacent condition of an 8 arm radial maze. The scatter plot above shows the number of trials for each rat to reach criterion, which is defined as choosing the reward arm in 9 out of 10 trials for 2 consecutive days. The dentate lesion, transgenic background control, and group E data were gathered prior to the start of 24-hour video monitoring. Groups A and B behavioral trials were conducted concurrently with 24-hour video observations. Animals with confirmed chronic temporal lobe epilepsy (2 or more observed seizures during behavior) are represented as filled in points on the plot. KA induced rats required a significantly greater number of trials to reach criterion in comparison to controls (control n = 25, KA induced n = 18, Wilcoxon Ranksum Test p = 0.007). The dentate lesion group (n = 11) performed comparably to the KA induced group (p = 0.9). However, the dentate lesion group took significantly more trials to reach criterion than control animals (p = 0.01).

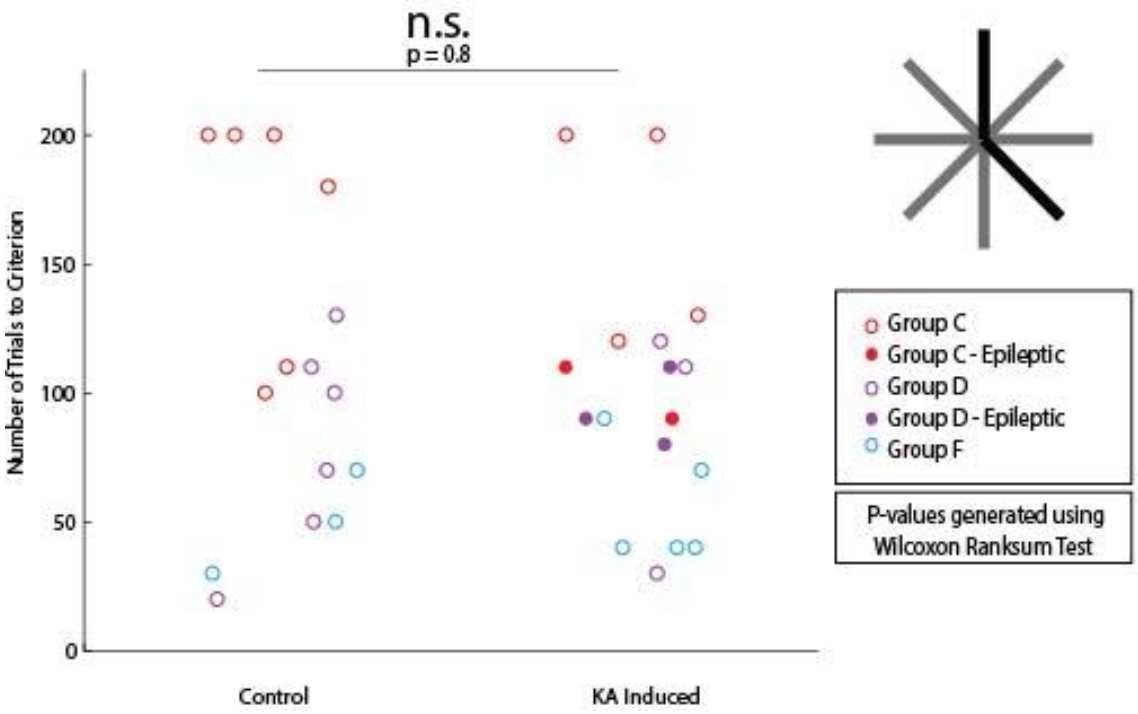

Figure 4. Rats induced with low-dose kainate model of temporal lobe epilepsy have normal pattern separation behavior on the non-adjacent condition of an 8 arm radial maze. The scatter plot above shows the number of trials for each rat to reach criterion, which is defined as choosing the reward arm in 9 out of 10 trials for 2 consecutive days. Group F data was gathered prior to the start of 24-hour video monitoring. Groups C and D behavioral trials were conducted concurrently with 24-hour video observations. Animals with confirmed chronic temporal lobe epilepsy (2 or more observed seizures during behavior) are represented as filled in points on the plot. There was no statistically significant difference ($p = 0.8$) between the performance of control animals ($n = 15$) and KA induced animals ($n = 17$).

Figure 3 summarizes the behavioral results from animals that were run on the adjacent condition of the 8 arm radial maze. In this condition, the animals were presented with a choice of two arms to go down. One arm was held constant as the reward arm. The significant overlap in distant spatial cues in this condition allows for assessment of pattern separation behavior. We compared the number of trials it took for animals within each group to reach criterion, which is defined as two consecutive days of getting 9 out of 10 trials correct. The dentate lesion group data, donated by V. Piatti, showed no

statistically significant difference in performance on the maze when compared to the KA induced rats. In both conditions, rats required up to 170 trials to reach criterion. Contrary to this, the dentate lesion group showed significant difference in the number of trials required to reach criterion when compared to the control group since animals in the latter required a maximum of 120 trials to learn. Control animals took significantly less trials to learn than KA induced animals. Three animals in the KA induced group had confirmed chronic temporal lobe epilepsy. One of these subjects took the greatest number of trials to learn between the three groups.

Figure 4 depicts the behavioral results from rats that were run on the non-adjacent condition of the 8 radial arm maze. In this condition, the subjects were presented with a choice of two arms that were 120 degrees apart from each other, and thus, contained less overlap of spatial stimuli. One arm was held constant as the reward arm for all trials. No dentate lesion data was gathered for this condition. However, the remaining groups (C and D) were both ran concurrently with the 24-hour video monitoring system. From this, we were able to identify five induced subjects that were confirmed with chronic temporal lobe epilepsy. The data in Figure 4 suggests that there is no significant difference in performance on the task when comparing KA induced and control animals.

Histology Results

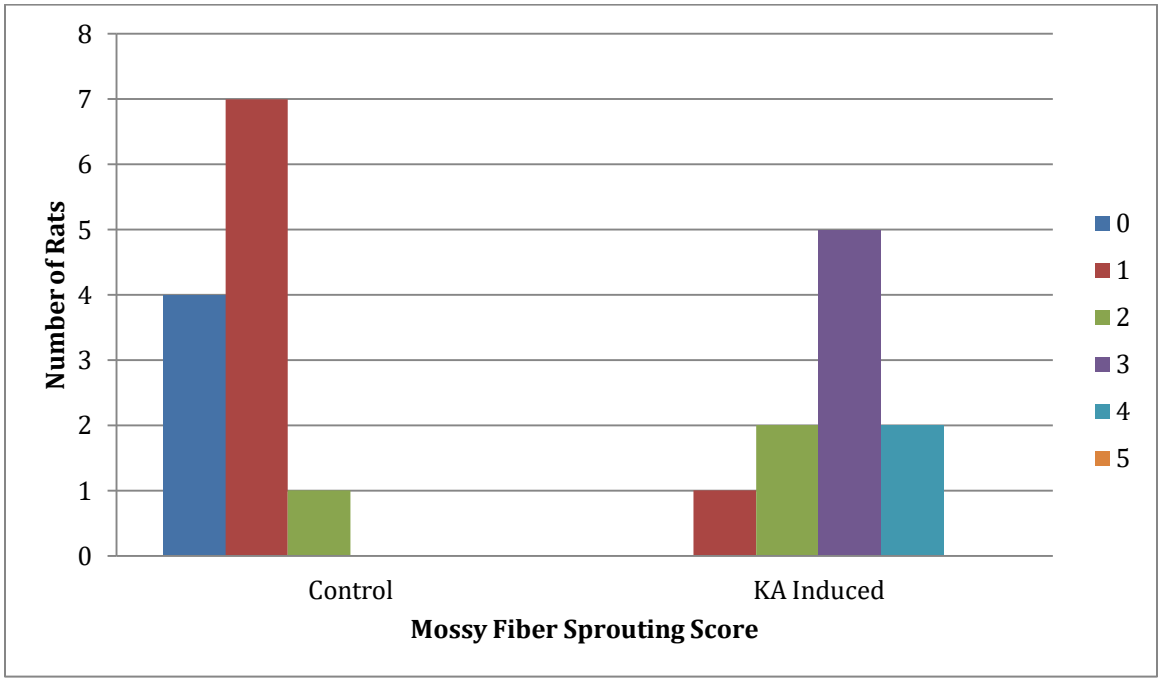

Figure 5. Adjacent condition Timm scoring results for mossy fiber sprouting in the dorsal dentate gyrus. Behavioral subjects tested within the adjacent condition were scored for mossy fiber sprouting in the dorsal dentate gyrus in accordance with the guidelines from Cavasos et al, 1991. Each of approximately sixteen 40 um thick, coronal sections were scored and the mode for each animal was used to illustrate the data above. The bar graph represents how many animals had each mode score within the control and KA induced conditions for behavioral groups A and B.

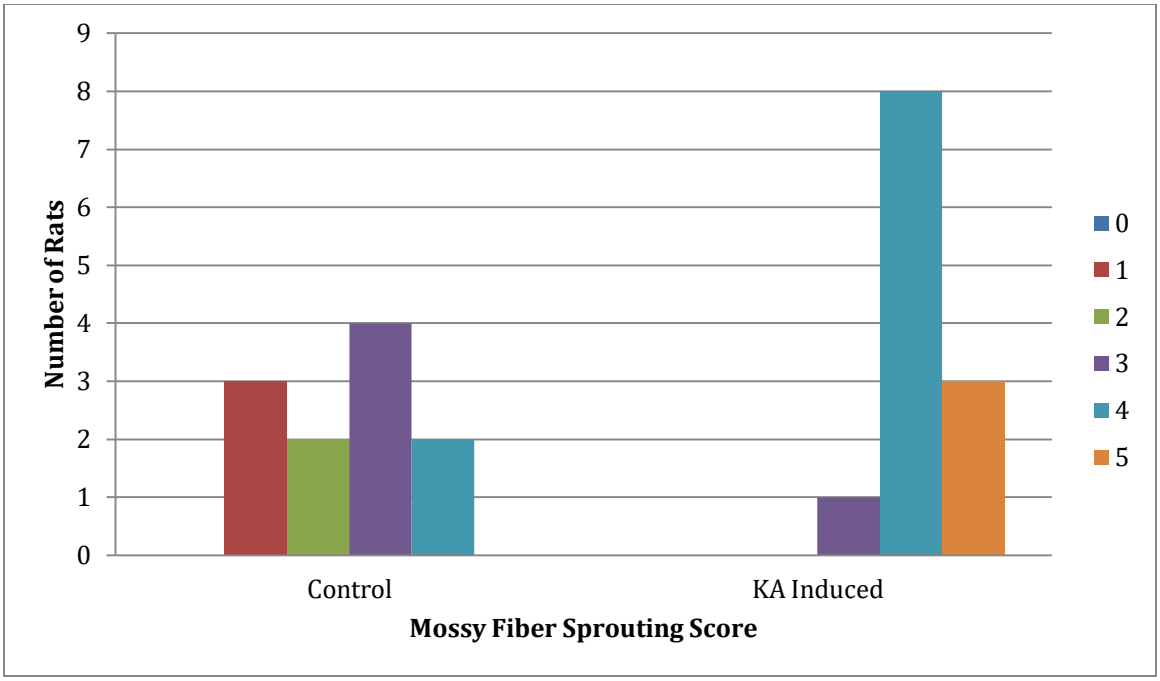

Figure 6. Non-adjacent condition Timm scoring results for mossy fiber sprouting in the dorsal dentate gyrus. Behavioral subjects tested within the non-adjacent condition were scored for mossy fiber sprouting in the dorsal dentate gyrus in accordance with the guidelines from Cavasos et al, 1991. Each of approximately sixteen 40 um thick, coronal sections were scored and the mode for each animal was used to illustrate the data above. The bar graph represents how many animals had each mode score within the control and KA induced conditions for behavioral groups C and D.

Once animals finished behavior, they were subject to perfusion with a 0.37% sulphide solution and 4% PFA. The sulphide solution allowed for visualization of the mossy fibers within the dentate gyrus by use of the Timm stain. Each mounted section for each animal was scored for mossy fiber sprouting in the granular layer of the hippocampus on a scale from 0 to 5 (least to most severe). Approximately 16 scores were assigned to each animal and the mode of the scores was used for representation in Figures 5 and 6.

In Figure 5, we plotted this data specifically for control and KA induced animals run on the adjacent condition of the 8 radial arm maze. We observe that the greatest

fraction of control animals scored a mode of 1 and had a range of scores from 0 to 2. KA induced animals scored in the range of 1 to 4 on Cavasos' scale, but most commonly had a mode of 3.

Figure 6 presents similar data to Figure 5, but for the non-adjacent condition. We see that there is a wide range of scores for the control animals (1 to 4). Although a score of 3 was the most frequent for non-adjacent control animals, the prominence of a single score is not as striking as we've seen in the other conditions. The results from Figure 2 provide an explanation for this unexpected finding. We see that in Figure 2, there is not as much clustering However, in the KA induced group for the non-adjacent task, we see a strong prominence of score 4 among the subjects and a range of scores from 3 to 5.

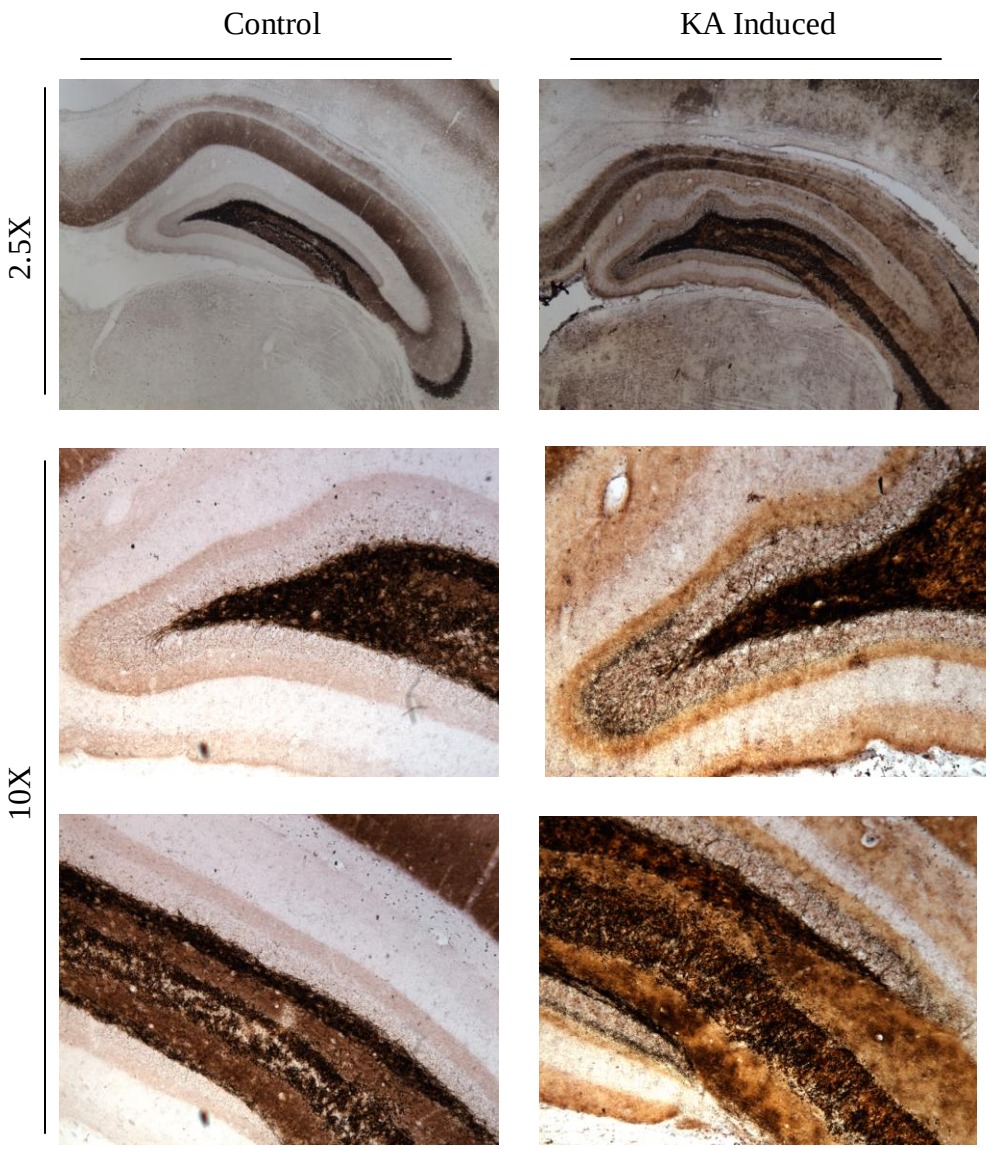

Figure 7. Timm staining indicates presence of mossy fiber sprouting in KA induced animals ran on the adjacent condition of the 8 radial arm maze. 40 um thick, coronal sections of the right hemisphere from control and KA induced rats were mounted and Timm stained to visualize mossy fiber sprouting in the dorsal region of the dentate gyrus. The select images depict severe sprouting in a KA induced rat with confirmed epilepsy from the 24-hour video monitoring, but no sprouting in the control animal.

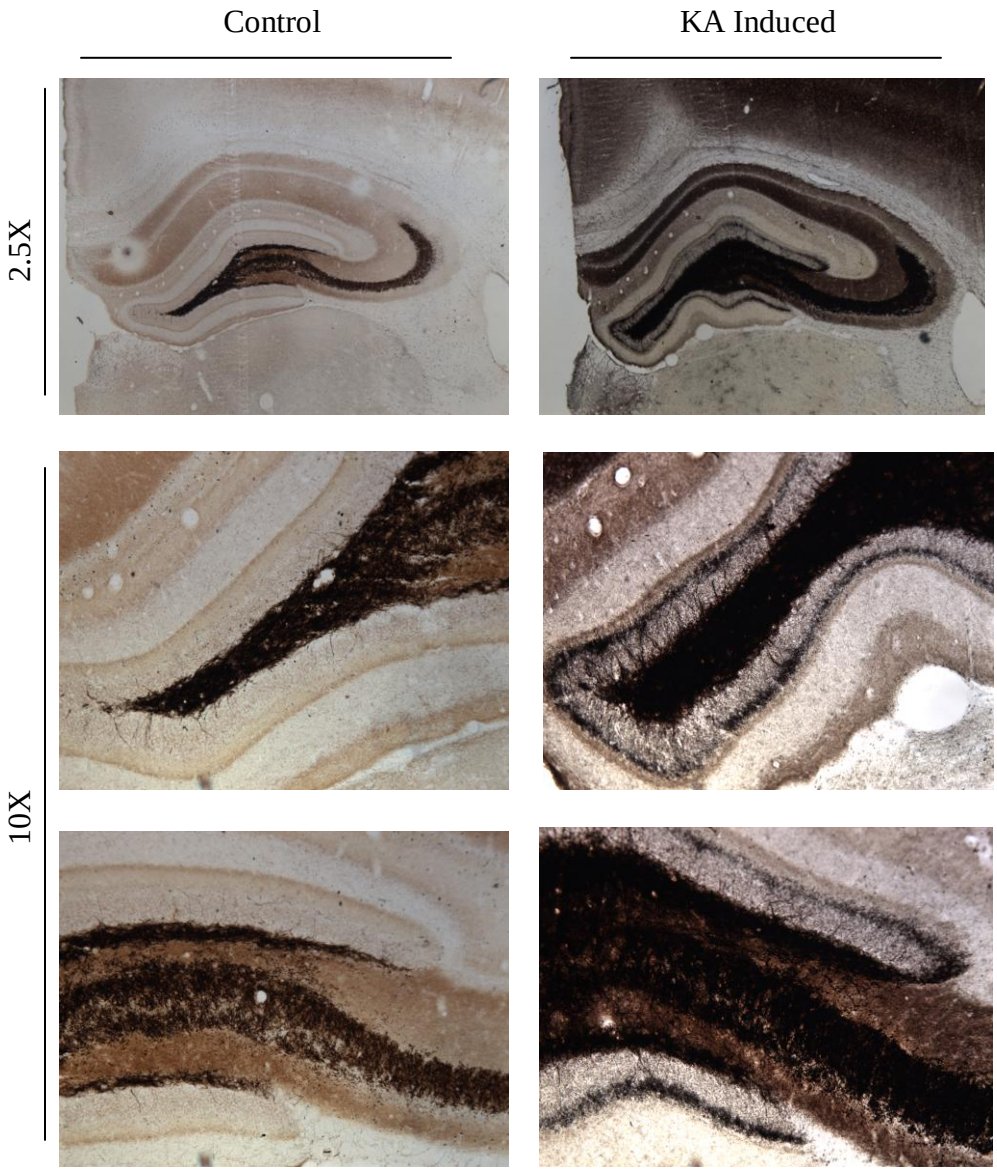

Figure 8. Timm staining indicates presence of mossy fiber sprouting in KA induced animals ran on the non-adjacent condition of the 8 radial arm maze. 40 um thick, coronal sections of the right hemisphere from control and KA induced rats were mounted and Timm stained to visualize mossy fiber sprouting in the dorsal region of the dentate gyrus. The select images depict severe sprouting in a KA induced rat with confirmed epilepsy from the 24-hour video monitoring, but no sprouting in the control animal.

Figures 7 and 8 feature select images of Timm stained sections from the adjacent and non-adjacent conditions, respectively. These images were taken at 2.5X and 10X to provide diverse perspectives of the scored dorsal dentate gyrus. Figure 7 depicts a control

animal in the left column that was given a mode score of 1 for mossy fiber sprouting. The right column depicts a KA induced rat's mossy fiber sprouting at approximately the same morphological stage of the hippocampus as the control subject. The KA induced rat had confirmed epilepsy from the 24-hour video monitoring and received a mode score of 4 for mossy fiber sprouting. The dark, laminar band forming at the apex and tails of the dorsal hippocampus is evidence of pathology associated with epilepsy.

In Figure 8, the control animal (left) received a mode score of 0 for mossy fiber sprouting. The KA induced rat (right), which had confirmed epilepsy via 24-hour video monitoring, exhibits severe mossy fiber sprouting which was given a mode score of 5 for increased thickness of these granular, laminar bands in the granule cell layer of the hippocampus.

The results section of the thesis is currently being prepared for publication in which I will be a co-author alongside Jill Leutgeb and Laura Ewell.

Discussion

This study investigated if the impairment on the pattern separation behavioral task was a precursor to, or a result of, epilepsy. To evaluate this, we subjected rats first to complete behavioral trials on an adjacent condition of the 8 arm radial maze (Figure 1). Pattern separation—the brain's ability to distinguish similar memories from one another—is manipulated as two adjacent arms on a radial maze. In the rat's line of vision, two adjacent arms overlap immensely in the context of surrounding, distant spatial cues. Epilepsy, which induces neuronal reorganization within the dentate gyrus, would thus alter normal pattern separation behavior. Rats were habituated to choose an arm, reward or non-reward, and learn that only their decision of going down the reward arm would allow them to obtain a food reward in that trial. In Figure 3, we are able to cross-analyze the results of performance across the multiple groups of animals run on this standardized condition. Here, the dentate lesion group represents a positive control since the results of this condition are what are expected for subjects lacking proper functionality of pattern separation. Previous studies including that of Yassa and Stark, 2012, have suggested that dentate gyrus-lesioned rats have increased difficulty discriminating between training and testing environments. In other words, they have behavioral deficits with pattern separation. Thus, we would expect there to be an increased dependent variable (trials to learn; trials to reach criterion) for these subjects in comparison to control subjects. Criterion in this context is defined as each animal choosing the reward arm over the non-reward arm for nine out of ten trials for two consecutive days during behavioral testing. In the dentate lesion condition, we observe that three subjects took up to 170 trials (17 days) to reach criterion. In the control condition, we observe several litters of animals that

were run concurrently with KA induced animals. Despite being from separate litters, the distribution of these animals' performance across several behavioral groups is even meaning that they all performed in a relatively similar manner in the task. Among this distribution, the majority of animals took between 40 and 90 trials to learn the task. However, there are still some that took up to 120 trials to learn. Still, this maximum value of trials for a control to learn was less than the maximum value of trials for a dentate gyrus-lesioned animal to reach criterion. A Wilcoxon Ranksum Test was run for statistical analysis, which is a nonparametric two-sample t-test. Using this form of comparative analysis, we found that the performance between the control and dentate lesion groups on the adjacent condition of the 8 radial arm maze was statistically significant ($p = 0.01$). As previously mentioned, due to the findings by Yassa and Stark, 2012, these findings were congruent with our expectations that an intact dentate gyrus provides greater efficiency with rodent pattern separation. Lesion of the dentate gyrus would remove neuronal networks and circuitry underlying this function of memory, which would make spatial processing of nearby, overlapping stimuli increasingly more difficult to differentiate between. However, control animals, having no alteration of their dentate gyrus and minimal (if any) interruption of mossy fiber connectivity would be able to complete the task with uninterrupted use of pattern separation mechanisms.

The experimental group of this experiment is the KA induced condition. To reiterate our experimental question, we were curious whether observed pattern separation behavioral impairments were a precursor to, or a result of, epilepsy. Within the KA induced condition of Figure 3, there are two subcategories. The first subcategory is denoted by the data points with only an outline and no fill. These points represent rats

that were never confirmed to have chronic temporal lobe epilepsy via the Q-See 24-hour video monitoring system. In other words, these animals did not reach the chronic phase of seizure development and were most likely still in the latent phase of chronic epileptogenesis. At this stage of disease development, these rats mirror being at high risk for developing epilepsy. They represent the condition testing for whether the behavioral deficits seen on the pattern separation task are a precursor to epilepsy since the latent phase of chronic epileptogenesis precedes the final, chronic stage. The second subcategory is denoted with filled in data points on the plot and are listed in the legend as "Group A - Epileptic." These three animals had confirmed seizures from the 24-hour video monitoring procedures. Given that an experimenter witnessed at least two seizures from each of these animals during the time frame that they were being behaviorally tested on the apparatus, these animals were confirmed to be in the chronic phase of epileptogenesis. Thus, the animals in this subcategory of the KA induced condition served as a method of examining whether the impairments on the pattern separation behavioral task are an effect of already having epilepsy. The KA induced condition serves to mimic the severity of the results seen in the dentate lesion condition because the low-dose kainate model of inducing chronic temporal lobe epilepsy functions to damage the mossy fiber pathway of the dentate gyrus and hinder efficient pattern separation behavior. This method proves successful as we see no significant difference ($p = 0.9$) in the performance on the 8 radial arm maze when comparing the KA induced animals to the dentate lesion animals. However, when comparing KA induced animals to the controls, we see that there is a significant difference ($p = 0.007$) in their number of trials to learn, and thus, in their pattern separation capabilities. This demonstrates that the

dentate gyrus is vital in being able to discern heavily overlapping spatial stimuli that would be present in an adjacent condition of the 8 radial arm maze where the subject must distinguish between two spatially similar arms to determine which constant arm contains the reward. Interestingly, when running the statistical analysis after removing the three confirmed epilepsy animals from the data set, the significance between the control and KA induced conditions is lost. This means that these three animals are upholding the statistical significance and suggests that the behavioral impairments observed during this behavioral study is a product of already having developed chronic temporal lobe epilepsy. Due to this finding, a pattern separation task would not be a suitable prognosis tool used on patients who have experience severe head trauma or been diagnosed with chronic traumatic encephalopathy (CTE), febrile seizure, or other high risk factors for developing chronic TLE.

Of additional interest is that the three animals with confirmed epilepsy, two of them took the two greatest numbers of trials to reach criterion (Firgure 3). Again, this suggests that the neuronal reorganization that occurred secondary to the kainate induction altered the dentate gyrus' functionality and efficiency in pattern separation behavior.

The non-adjacent condition on an 8 radial arm maze is meant to create an environment where the stimuli establishing a choice for the animal do not overlap as much as in the adjacent condition. Therefore, the similarity between the spatial memories is decreased in this condition. Pattern separation is then no longer as strongly needed in order to achieve the same criterion (learning to pick the reward arm for nine out of ten trials on two consecutive days). Figure 2 outlines the apparatus setup for this condition and illustrates that the reward and non-reward arms are no longer directly next to each

other, but rather separated by a distance of 2 arms (120 degrees). In Figure 4, we compared the number of trials to learn between control and KA induced animals. There was no dentate lesion data donated for this behavioral condition. Among both the control and KA induced rats, those in Group C have a total of five (n = 5) rats that took up to 200 trials (20 days to reach criterion). These rats were unable to learn the task and were capped at 20 days of behavioral trials before being sacrificed. We believe this was mainly due to lack of motivation for obtaining the food reward (Coco Pebbles) and for the succeeding group, D, we changed the food reward to Coco Puffs, which the data above suggests increased motivation and decreased trials to learn since the Group D animals, in general, took less trials to learn. Whether or not the five Group C rats that were unable to meet criterion before day 20 were kept in or removed from the statistical analysis, the Wilcoxon Ranksum Test still measure no significant difference in the trials to learn when comparing control to KA induced. To maintain our analysis as a function of all the animals used in the study, Figure 4 displays the statistical analysis in the presence of these five animals that did not learn within 200 trials. Again, we observed no statistically significant difference ($p = 0.8$) when comparing the performance of all the animals on the non-adjacent task. Despite the five animals that reached 200 trials, there is fair dispersion of Group C, D, and F animals in both the control and KA induced conditions suggesting that the experimental protocols were well controlled across many different groups of animals.

Similar to the adjacent task, in the non-adjacent task, the non-filled data points represent animals still in the latent phase of chronic epileptogenesis whereas those with fill represent animals in the chronic phase. Yet, this distinction is not of particular

notability in the non-adjacent task because of the even distribution and mixture of performance of animals in both subcategories. In the non-adjacent condition, we see that within the KA induced animals, there were five (n = 5) that reached the chronic stage of chronic epileptogenesis and that had at least 2 confirmed seizures observed via 24-hour video monitoring. These five subjects fall in a median range of all KA induced subjects, which demonstrates that despite the late progression of their disease, learning the behavioral task does not take them the as long as other KA induced subjects who did not reach the chronic phase of chronic epileptogenesis.

After collecting behavioral data, it then became important to understand the underlying pathology that could be attributing to the impairments witnessed on the behavioral apparatus. I perfused animals from groups A, B, C, and D with a sulphide solution that reacts with silver to induce precipitation of metals found in the hippocampus. This technique allows for visualization of newly sprouted axons or axon terminals within the central nervous system. Visualization of these details provides evidence of where in the anatomy of the hippocampus synapses may have occurred. Since epilepsy is characterized as hyper-excitation of neuronal circuitry, evidence of new axon sprouting in regions that do not normally express such features would indicate pathology associated with the disease. On magnified Timm stain sections, one should be able to identify granules (if any), which are small, dark, circular impurities that usually rest on the midline border between the granule cell layer (GCL) and the inner molecular layer (IML) of the hippocampus.

Figure 7 displays select sections of one control and one KA induced animal with confirmed epilepsy. In the control animal's 10X images, we appreciate very minimal,

sparse dispersion of granules scattered throughout the GCL. Per Cavasos et al, this pathology is congruent with a sprouting score of 1, but is essentially negligible in its impact on pattern separation function. While we try to maintain consistency in scoring between different scorers, the Timm scoring is to some extent subjective. The difference in criteria between scores 0, 1 and 2 is fairly minimal, which could account for human error in determining these scores. Nonetheless, the sprouting observed in the control subject is blatantly less extensive than that of the KA induced subject in Figure 7. The KA induced 10X images exhibit prominent sprouting along the outer edge of the IML. At the apex and at the tails of these sections, note the dark laminar band that forms between the numerous granules. This KA induced subject is one of the confirmed seizure rats that took 170 trials to meet criterion in Figure 3. Here, we are able to appreciate a possible positive correlation between increased sprouting and increased impairment with pattern separation behavior on the adjacent condition of an 8 radial arm maze since this rat exhibited one of the highest Timm scores among the KA induced rats and was one of two epileptic rats to reach this value of trials to meet criterion.

In Figure 5, we quantified the number of animals that were given a certain score as a function of the mode. Note that the control animal in Figure 7, having received a score of 1, is among the majority of animals in this group who also received the same score. However, among the KA induced rats in Figure 5, only two received a score of 4, the highest score of all analyzed tissue from the adjacent condition. This, along with the significant difference in number of trials to reach criterion between control and KA induced animals (Figure 3), draws a promising positive correlation between increased mossy fiber sprouting and increased behavioral deficits on the adjacent condition.

Furthermore, the small range (0 to 2) of assigned Timm scores to the controls in Figure 7 provide an explanation of the consolidated dispersion of trials to meet criterion for controls in Figure 1. As previously mentioned, scores of 0, 1 and 2 for mossy fiber sprouting vary in minuscule attributes and most likely do not substantially alter pattern separation function or efficiency. We confirm this since the maximum number of trials for a control animal (120 trials) is at least 50 trials less than the maximum number of trials for a dentate lesion or KA induced animal to reach criterion. KA induced animal present a wide range spanning from 40 to approximately 180 trials to criterion in Figure 1. They also scored within a higher range (1 to 4) for mossy fiber sprouting as seen in Figure 5. These two results coupled together suggest a positive correlation between mossy fiber sprouting and performance on a pattern separation task on an adjacent condition of an 8 radial arm maze. To provide an example of this correlation, the right column of Figure 7 depicts select images from a KA induced animal with confirmed epilepsy from video monitoring. This animal received a score of 4 for mossy fiber sprouting (the highest score received by any animal in the adjacent condition). This subject is further represented as the animal that took 170 trials to criterion in Figure 1. As one of the highest scoring animals in terms of sprouting and in terms of trials to criterion, this again suggests that there is a positive correlation between these two parameters.

Figure 6 presents an illustration of the number of rats run on the non-adjacent condition that received each Timm score for mossy fiber sprouting on a scale of 0 to 5. The control rats in the non-adjacent condition, unlike those seen in the adjacent condition (Figure 5), had relatively equal score distribution from 1 to 4. Oddly, these scores were higher than would be expected for healthy animals. However, the behavioral results in

Figure 4 provide an explanation for this observation. We see no prominent clustering of animals that received a small range of differing trials to criterion. Rather, we witness a large range beyond what was seen in Figure 1. Granted, three control animals were capped at 200 trials for being unable to learn the task, which could have been attributed to non-motivating rewards on the reward arm. The sprouting scores for KA induced animals in Figure 6 show a similarly predictable distribution of scores as we saw in Figure 5; even more so, this is what we would expect for induced animals since these animals only received the highest three scores possible (3, 4, or 5). The images on the right column of Figure 8 exhibit sections of a KA induced animal with confirmed chronic TLE. This subject (one of five epileptic animals among groups C and D) is denoted within the cluster of data points between 90 and 110 trials to criterion. This subject also received a sprouting score of 5 for exhibiting a confluent, laminar band formed of granules with some of these granules seeping into the inner molecular layer of the hippocampus. Despite the visualization of this severe epilepsy-associated pathology, this induced animal reached criterion in less trials than other induced animals that had no recorded seizures from the video monitoring.

These results demonstrate that despite neuronal reorganization that occurs in the hippocampus of animals induced with chronic TLE, there is no significant difference between KA induced and control performance on the non-adjacent condition. The decrease in overlapping stimuli from the adjacent condition to the non-adjacent condition relieves the necessity for dentate gyrus-dependent pattern separation behavior to learn the task because a decrease in similar neuronal pattern inputs no longer requires the process to transform them into distinct outputs. Animals with restructured hippocampal circuitry

secondary to the low-dose kainate induction are still able to perform similarly to healthy animals (Figure 4).

Conclusion

In summary, this project provides additional evidence that spatial pattern separation is dentate gyrus-dependent. On the adjacent condition of an 8 radial arm maze, we were able to establish an environment of choice between two arms 40 degrees apart from one another. This condition tests for pattern separation since the spatial stimuli surrounding these two arms heavily overlaps. Our results provide evidence that dentate lesion animals and KA induced animals perform similarly, whereas both of the former conditions take significantly longer to reach criterion and learn the task than control animals.

We assessed whether these impairments on a pattern separation task were a precursor to epilepsy or an effect of it. With the establishment of a 24-hour video monitoring system, which is novel to this study, we could distinguish KA induced animals that successfully reached the chronic phase of temporal lobe epilepsy from those that did not exhibit spontaneous seizures during behavioral trials. The animals with confirmed epilepsy represented a sub-condition testing whether impairments on the pattern separation task were an effect of epilepsy, whereas induced animals with no observed seizures represented a condition testing if these impairments were a precursor to epilepsy. The results in this project provide evidence to support that in the adjacent condition, the behavioral impairments on the pattern separation task are a result of already having developed chronic TLE. In future studies, we hope to obtain additional behavioral data from more animals that reached the chronic phase of epileptogenesis. Should these animals also perform similarly to epileptic animals in the adjacent study and

take longer to reach criterion than control animals, this would strengthen our evidence portraying that pattern separation behavioral impairments are an effect of epilepsy.

These behavioral results were further analyzed in the context of histological analysis. Sectioned brain tissue from behavioral subjects was Timm stained and scored for mossy fiber sprouting. The mode of all scores for all sections of each animal was presented and we found there to be a positive correlation between pattern separation impairment and mossy fiber sprouting in the adjacent condition.

This project also consisted of behavioral trials of animals on a non-adjacent condition. The choice in arms presented in this behavioral apparatus is separated by a distance of two arms in order to minimize overlapping patterns of spatial stimuli. The absence of these stimuli decreases the necessity of pattern separation behavior to learn the task and is thus not dentate gyrus-dependent. Our results concur with this expectation as there was no significant difference between the number of trials it took for control and KA induced animals to reach criterion. The non-adjacent condition lacks data from a dentate lesion group, which we would like to add in the future.

Although a similar distribution of mossy fiber sprouting scores was observed in the non-adjacent condition, these scores showed no correlation with the behavioral results, which confirms that the non-adjacent task does not test pattern separation behavior. Despite strong evidence of sprouting in the magnified images presented, animals with this pathology were able to complete this task with similar efficiency as control animals.

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