UNIVERSITY OF CALIFORNIA SAN DIEGO

Pattern Separation Impairment, Mossy Fiber Sprouting and Somatostatin Interneuron Loss During Epileptogenesis in Kainate-Induced Adolescent Rats

A Thesis submitted in partial satisfaction of the requirements
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in

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by

Gabrielle Jessica Madera Suarez

Committee in Charge:

Jill Leutgeb, Chair
Stefan Leutgeb, Co-Chair
Jon Christopher Armour

2018

The Thesis of Gabrielle Jessica Madera Suarez 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

2018

DEDICATION

This thesis is dedicated to my parents, who have worked tirelessly and supported me unconditionally.

This thesis is dedicated to my brothers, who helped to shape the woman I am today.

This is thesis is dedicated to my friends, who challenge me to grow every day.

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LIST OF ABBREVIATIONS

IACUC	Institutional Animal Care and Use Committee
mTLE	Mesial Temporal Lobe Epilepsy
CNS	Central Nervous System
SE	Status Epilepticus
KA	Kainic Acid
SST	Somatostatin
RAM	Radial Arm Maze
PFA	Paraformaldehyde
PBS	Phosphate-Buffered Saline
DAB	3,3’-Diaminobenxidine
IgG	Immunoglobin G
dSST	Dorsal hilus Somatostatin

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ABSTRACT OF THE THESIS

Pattern Separation Impairment, Mossy Fiber Sprouting and Somatostatin Interneuron Loss During Epileptogenesis in Kainate-Induced Adolescent Rats

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

Mesial temporal lobe epilepsy is a common focal seizure disorder in adults. Unfortunately, the onset of spontaneous and chronic seizures is the only current method of diagnosis. However, previous research has demonstrated that mTLE originates in the hippocampus by altering neuron concentrations and synaptic connections through a

process called epileptogenesis. These alterations occur in the dentate gyrus of the hippocampus – a subregion that is also critical for pattern separation memory. As shown by previous rat and human studies, pattern separation allows the brain to differentiate very similar experiences from each other. Therefore, this study hypothesized that rats induced with mTLE would display impairments on a dentate-dependent task prior to the onset of spontaneous, recurrent seizures. This was tested by administering kainic acid to 40 day postnatal rats to cause status epilepticus, then training them using the adjacent arms of an 8-Radial Arm Maze. The kainate-induced rats were also monitored 24-hours, in order to distinguish between asymptomatic and chronically epileptic rats. After behavioral testing, the hippocampal tissue of control and kainate-induced rats were immunostained for hilar somatostatin interneurons and stained with Timm sulphide silver to visualize mossy fiber sprouting – two hallmark dentate alterations of epileptogenesis. We found a significant impairment in dentate-dependent behavior among the all kainate-induced rats (asymptomatic + chronically epileptic). However, a significant impairment was not found in the asymptomatic, kainate-induced rats alone, due to the age-dependent effects of kainate induction on behavior. Additionally, increased mossy fiber sprouting correlated with less hilar somatostatin interneurons of the dorsal dentate gyrus.

Introduction

Mesial temporal lobe epilepsy (mTLE) is a common seizure disorder in adults that is marked by the chronic hyper-excitation of neurons in the central nervous system (CNS). Most adult mTLE patients report prior brain injury before the onset of spontaneous recurrent seizures later in life (Sloviter, 2008). Different brain injuries that have been associated with adult mTLE include recurrent fever-induced seizures in early childhood, perinatal trauma and hypoxia, and an episode of status epilepticus or closely spaced seizures. mTLE seizures are hypothesized to originate in the mesial temporal lobe of the brain, primarily the hippocampus (Margerison and Corsellis, 1966). The importance of the hippocampus in mTLE has been demonstrated by the use of hippocampal sectioning as an effective treatment to eliminate or reduce the frequency of seizures in mTLE patients (Falconer and Taylor, 1968). Further studies have shown that the hippocampus experiences neuronal damage and synaptic reorganization after initial injury. However, the damaged hippocampus requires further epileptic-priming before spontaneous recurring seizures are observed – this gradual development of chronic temporal lobe epilepsy is known as epileptogenesis (Sloviter, 2008).

Despite the ethical and experimental limitations of human mTLE studies, a greater understanding of underlying mTLE mechanisms have been established through animal models. Current rat models of acquired epilepsies are a valuable tool in studying epileptogenesis as it allows researchers to gather large data sets and visualize similar molecular mechanisms seen in postmortem human mTLE studies (Nadler et al., 1980; Mathern et al., 1992; Buckmaster and Dudek, 1997a,b). One widely accepted method for

establishing epileptogenesis is by inducing status epilepticus in rats. Status epilepticus (SE) can be induced through intraperitoneal administration of kainic acid (KA) – a chemotoxin with potent excitatory effects on hippocampal neurons (Pisa et al., 1980; Nadler, 1981; Cavalheiro et al., 1982; Ben-Ari, 1985; Cronin and Dudek, 1988; Buckmaster and Dudek, 1997b; Hellier and Dudek, 2005). After inducing SE, the hippocampus of kainate-induced rats mirrors the hippocampal changes seen in human mTLE patients. These hippocampal changes are visualized specifically in dentate gyrus, a sub-region of the hippocampus. One mTLE-hallmark alteration is the creation of new synapses between granule cells in the dentate gyrus, known as mossy fiber sprouting. Granule cells normally synapse onto pyramidal neurons through the mossy fiber pathway; instead, granule cells synapse back onto themselves and create a recurrent circuit in the dentate gyrus (Sutula, 1990). This an important mechanism in epileptogenesis because it may give rise to the hyper-excitatory activity of mTLE (Cornin and Dudek, 1988; Cavazos and Sutula, 1990). Another mTLE-hallmark alteration is the neuronal death of various cell types in the hilus region of the dentate gyrus (Sloviter, 1989a; Gulyás et al., 1991; Tallent and Qui, 2007; Mazarati and Teledgy, 1992). The cell type most susceptible to neuronal death is the hilar somatostatin (SST)-expressing interneuron (Robbins et al., 1991); studies have suggested that this vulnerability is due to the lack of the calcium-binding proteins that buffer the neurons from experiencing excitotoxicity from calcium overload (Nitsch et al., 1990; Sloviter, 1989). Further studies regarding the synaptic connections of hilar SST-expressing interneurons have found that the axons of these cells may inhibit the granule cells through a feed-forward and feed-back mechanism (Leranth et al., 1990). It is suggested that with both the

calcium overload-vulnerability and the presynaptic inhibitory function of SST-expressing interneurons, the death of SST-expressing interneurons results in the further progression of epileptogenesis into chronic mTLE (Tallent and Qui, 2007). Epileptogenesis not only affects the neurons and synapses in the hippocampus, but we hypothesize that epileptogenesis results in memory impairment due to the disruption of the normal hippocampal structure.

A healthy hippocampus plays an important role in the consolidation of experiences from short-term memory into long-term memory. One method of consolidating experiences into long-term memory is through a process that allows the brain to differentiate very similar experiences from each other – this process is known as pattern separation (Madar et al., 2016). Pattern separation occurs through the recruitment of neurons in the dentate gyrus structure of the hippocampus (Leutgeb et al., 2007). Pattern separation functionality in rodents can be tested through spatial memory tasks that involve highly overlapping patterns. We chose to conduct our spatial memory task on an 8-radial arm maze (RAM) because the spatial background cues largely overlap for the adjacent arms of the RAM. Morris et al. (2012) found that dorsal dentate gyrus lesioned Long-Evans rats were significantly outperformed by healthy control rats on the adjacent arm task of the 8-RAM. Poor performance on the dentate-dependent RAM task demonstrates poor pattern separation functionality (Clelland et al., 2009; Yassa and Stark, 2011). We hypothesize that kainate-induced rats will have impaired pattern separation functionality due to the neuronal damage and synaptic reorganization of the dentate gyrus caused by epileptogenesis.

We were able to investigate the progress of epileptogenesis by distinguishing between chronically epileptic, kainate-induced rats and asymptomatic, kainate-induced rats. This was done by using a continuous monitoring system of all kainate-treated rats. Since the asymptomatic, kainate-induced rats remained in the latency period of epileptogenesis for the duration of the study, we used the sulphide silver Timm stain to visualize mossy fiber sprouting in the dentate gyrus of all rats to confirm if epileptogenesis had taken place in all kainate-induced rats. This allowed us to investigate the behavioral effects of epileptogenesis, regardless of chronic epilepsy and seizure activity.

One aim of this study was to correlate the amount of mossy fiber sprouting, neuronal death, and pattern separation functionality to the different stages of epileptogenesis. We gathered histological data of all subjects by Timm staining and somatostatin-immunostaining all hippocampal tissue. The Timm stain marked places of high zinc content, which was an effective method for visualizing mossy fiber sprouting due to the high zinc content in mossy fiber terminals. Somatostatin-immunostaining utilized a somatostatin antibody to visualize the somatostatin-expressing neurons of the dentate gyrus. Both Timm staining and somatostatin-immunostaining are well established methods of visualizing the hippocampal effects of epilepsy (Cronin and Dudek, 1988; Sloviter, 1989a; Cavazos and Sutula, 1990; Robbins et al., 1991; Gulyás et al., 1991; Mazarati and Teledgy, 1992; Tallent and Qui, 2007). We hypothesize that the dentate gyrus-dependent behavior of pattern separation, mossy fiber sprouting, and somatostatin-expressing neurons will demonstrate a gradient of effects according to their stage of epileptogenesis. By identifying stage-specific characteristics of epileptogenesis, future studies may be able to

develop prognostic or diagnostic tools evaluate patients following a traumatic brain injury. Studies have already identified dentate-dependent pattern separation tasks that test for both object pattern separation and spatial pattern separation functionality in humans (Bakker et al., 2008; Stark S.M. et al., 2010). Additionally, by deepening our understanding of epileptogenesis and mTLE, we progress closer to discovering a possible stage-specific intervention therapy to replace the anticonvulsant medications that only treat symptoms and not the disease.

Materials and Methods

Animal Model

The study's subjects were composed of 50 Long Evans male rats, which were housed in individual cages on a reverse light-dark schedule. Dark hours were 7:00 pm to 7:00 am while, light hours were 7:00 am to 7:00 pm. The protocols and procedures of the experiment were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego. Additionally, all experiments performed with these subjects were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

40 days after birth, male Long Evans rats from the same litter were induced (n=28) with mesial temporal lobe epilepsy using a low dose kainate model (Hellier and Dudek, 2005). 50 mg of kainic acid (Tocris Bioscience) was dissolved in 5 mL of sterile saline and placed on a high-speed shaker for 1 hour. Prior to injection, the rats were weighed and a 5 mg/kg dose of the kainate solution was prepared. The rats were administered the kainate solution by intraperitoneal injection once an hour until continuous stage IV or stage V seizures that lasted 2 hours was observed. This state is known as status epilepticus. The stage of seizure was determined according to a modified Racine scale (Racine, 1972; Ben Ari, 1985). After status epilepticus, an intraperitoneal injection of 35 mg/kg of Pentobarbital was administered to terminate the induction period. Most kainate-induced subjects remained in good health, except for one kainate-induced subject (n=1) died during the low dose kainate induction. The remaining male Long Evans rats from the same litter as the kainate-induced rats were not induced and placed in the control group (n=21).

Following induction and an incubation period of 140 days, the kainate-induced animals began being monitored on a 24-hour Q-See surveillance system in the vivarium. The 24-hour recorded video was used to observe, quantify, and score seizures of the kainate-induced rats. The observed seizures were scored according to the modified Racine scale (Racine, 1972). This scale included stages I-V, with I being the least severe and V being the most severe. Stage I seizures involved jaw and facial movements while, stage II progressed to also include head bobbing; however, these first two stages might have been overlooked because they were not apparent and visible in the recorded video. Stage III seizures involved forelimb clonus (i.e. a rapid repetitive forelimb movement). Stage IV seizures involved forelimb clonus and rearing onto back limbs. Stage V was a full motor seizure seen as rearing and falling. Once a kainate-induced rat was observed to have 2 or more seizures, they were characterized as chronically epileptic. From the recordings, 9 kainate-induced rats (n=9) became chronically epileptic. However, 2 of the chronically epileptic rats were removed from the behavioral paradigm because the severity of their seizures made them unable to handle and perform the behavioral task. The remaining kainate-induced rats (n=18) did not develop chronic epilepsy and were characterized as asymptomatic. The control rats (n=21) that were not induced were not recorded by the 24-hour monitoring system.

Behavioral Apparatus

In order to assess the subject's spatial pattern separation functionality, an 8-Radial Arm Maze (RAM) was utilized in a room without any obvious spatial cues. The RAM consists of 8 arms projecting from an octagonal stem and each adjacent arm is separated by

45º. Each arm is designated with number from 1-8, starting at the center arm and counting up counterclockwise – making arm 2 to the right of arm 1 and arm 8 to the right of arm 1 (Figure 1). The diameter of the RAM is approximately 188 centimeters while, its height is 36 centimeters tall. The 8-RAM also includes 6 clear Plexiglas walls that permanently block off arms 3-5; 1 additional removable clear Plexiglas wall is used to alternate blocking off arms 2 and 8. The clear Plexiglas is approximately 30 centimeters by 0.6 centimeters. At the end of each arm are identical, chrome, silver bowls taped in place. The bowls are used to place the food reward (e.g. Kellogg's Cocoa Puffs and Cocoa Pebbles).

Habituation

One week prior to habituation, the subjects were weighed and recorded as their baseline weight. They goal behavior weight for each rat was calculated to be 85% of baseline weight. In order to achieve this goal weight, the rats were placed on a food restricted diet, which included a limited amount of standard rat chow and a food reward every third day. Water was not restricted. The purpose limiting food and achieving goal behavior weight was to facilitate the rat's motivation to perform the behavioral task later.

The goal of habituation was to introduce each subject to the behavior room and to facilitate the association between the 8-RAM and food. Therefore, each rat was transported to the behavior room within the same strict 4-hour block each day – this strict time frame minimized the variability in behavioral performance.

At 6 months of age, habituation began using the 8-RAM with 6 clear Plexiglas walls blocking arms 3-5 and nothing blocking arms 1,2, and 8. Each animal's weight was measured and recorded daily, along with any health observations. The rat was then

individually placed on the center stem of the RAM and covered with an opaque bucket. The removal of this opaque bucket marked the start of each trial and the experimenter remained in one place in the room to record any observations. Prior to removing the bucket, the experimenter placed the food reward in specific places on the 3 open arms of the RAM. These locations of the food reward were determined by which phase of habituation the rat was on. Phase 1 of habituation used 10 food rewards: 1 on the stem, 1 midway down each of the 3 arms, 1 in front of the silver bowls on each of the 3 arms, and 1 inside the silver bowls on each of the 3 arms. The goal of this phase was to encourage the rat to explore throughout the 8-RAM and retrieve the food rewards for 10 minutes. After the 10 minutes, the rat was placed back into its home cage, fed an appropriate amount of rat chow to maintain/reach goal behavior weight, and returned to the vivarium – this occurred at the end of each habituation and behavior session. If the rat finished all 10 food rewards on the maze, he progressed to phase 2 of habituation on the following day. Phase 2 of habituation used 6 food rewards: 1 in front of and 1 inside the silver bowl on each of the 3 arms. If the rat finished the 6 food rewards within 10 minutes, he moved onto phase 3 of habituation the next day. Phase 3 of habituation involved 3 food rewards each placed in the bowls of the 3 arms. If the rat finished the 3 food rewards in 5 minutes, habituation was completed. The day following completion of habituation, the rat began day 1 of the behavioral trials.

Behavior: Adjacent Condition

The goal of the 8-RAM task using adjacent arms was to quantify each rat's ability to differentiate between the reward arm (arm 1) and an adjacent non-reward arm (arms 2 or

8). Behavioral sessions were conducted with control (n=21) and kainate-induced rats (n=26). Both groups of rats were handled in parallel by each experimenter. Each rat was tested within the same 4-hour time frame every day to ensure consistency. However, kainate-induced rats that experienced seizures were given 2 hours after the seizure before their behavioral session – this was to avoid the effects of post-seizure amnesia. The 8-RAM behavioral paradigm described below is based on that described in Morris et al. (2012) and McDonald and White (1995).

Prior to the beginning of each behavioral session, each rat's weight was measured and recorded daily, along with any health observations. The rat was then placed on the stem of the RAM and covered with the opaque bucket. While the rat was covered, the experimenter placed/removed the clear Plexiglas wall to block the adjacent non-reward arm that was not to be used during that trial. The adjacent non-reward arms 2 and 8 alternated pseudo-randomly throughout the behavioral session; this ensured that the rats did not use a simple left-right strategy for choosing between the reward and non-reward arms. The removal of the opaque bucket signaled the start of each trial within the behavioral session; at which point, the rat was allowed to choose between the reward arm (arm 1) and one adjacent non-reward arm. If the rat placed all four paws on the reward arm, he received the food reward; if the rat placed all four paws on the non-reward arm, he did not receive the food reward. Between each trial, the arms of the RAM were wiped with a damp water cloth to ensure that the rat did not use its sense of smell to guide its decision making. Each behavioral session involved 10 trials per day. After all 10 trials were completed, the rat was placed back into its home cage, fed its appropriate amount of rat

chow, returned to the vivarium and the 8-RAM was cleaned with 70% ethanol before the next rat's behavioral session. The behavioral sessions continued daily until the rat reached criterion, which was defined as 90% accuracy in the behavioral sessions for two consecutive days.

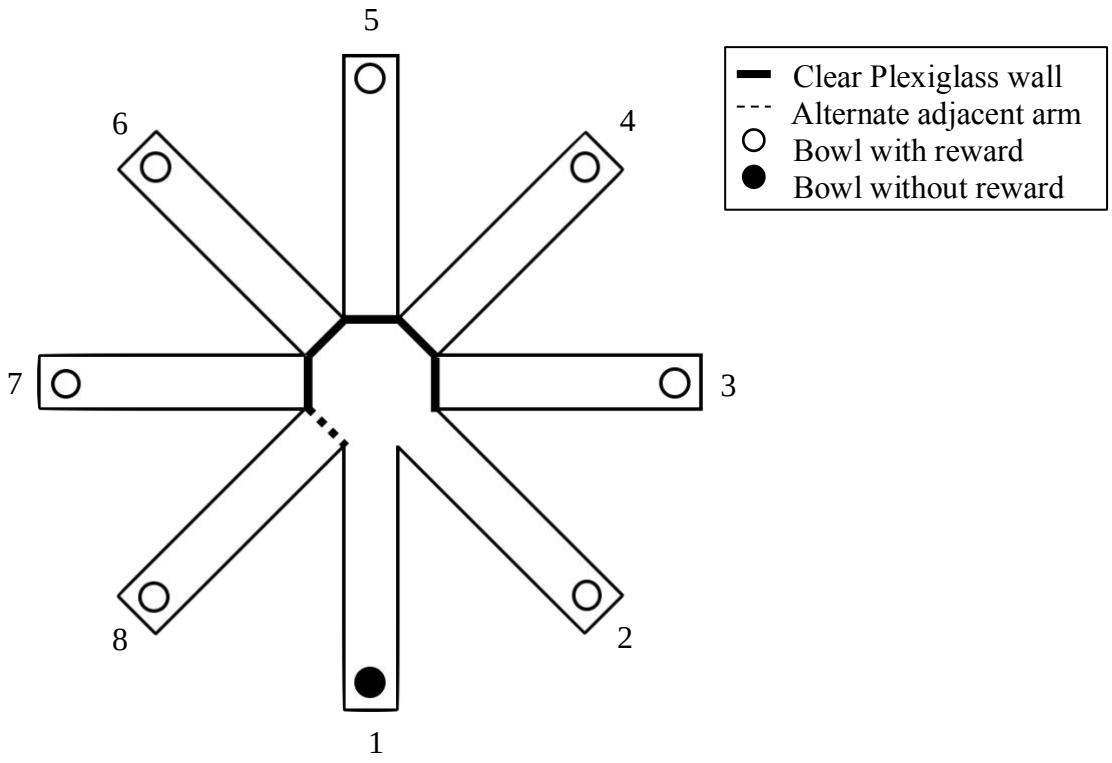

Figure 1. Schematic of the 8-RAM using adjacent arms for the dentate-dependent behavioral task.

Perfusion and Tissue Preparation

After all the rats reached criterion, subjects were anesthetized in a chamber filled with gaseous isoflurane. Subjects were then given a lethal intraperitoneal injection of Pentobarbital (800 mg-2000 mg) Perfusion began after the pain reflex was observed to be unresponsive to a pinch of the rat's feet. They were then systemically perfused with 0.3% sulphide solution for 5 minutes and 4% paraformaldehyde (PFA) for 10 minutes. When the

perfusion was complete, each brain was carefully extracted from the skull, stored in 4% PFA, and refrigerated for 24 hours. After 24 hours, each brain was transferred to 30% sucrose in 1X PBS solution and refrigerated.

The right hemisphere of the brains were individually sectioned and collected using a Leica microtome. This was done by freezing the entire hippocampus, coronally sectioning at 40 μm, and collecting every section in 0.02% Na-Azide until the sections were mounted on a glass slide.

Timm Stain and Scoring

Prior to Timm staining, every sixth coronal section of control (n=16) and kainate-induced groups (n=18) were mounted onto Fisherbrand Superfrost Plus Microscope Slides with a mounting solution, containing gelatin and PBS. After an overnight drying period, the slides were placed in distilled water for 3 minutes and incubated for 1 hour in the Timm developer solution in the dark. The developer solution was mixed again at the 30-minute mark. The developer solution contained 120 mL of Gum Arabic (100 g/200 mL), 10 mL of filtered citrate buffer (51g $C_6H_8O_7$/200 mL; 47g $C_6H_5Na_3$-O_7•$2H_2O$/200 mL), 60 mL of 1.7% hydroquinone solution, and 1 mL of 0.09% light-sensitive, silver nitrate solution. After incubation, the slides were washed twice in 50^oC distilled water and placed in room temperature distilled water for 15-30 minutes. The slides were then fixed in 5% sodium thiosulfate pentahydrate, dehydrated in various ethanol concentrations (70%, 80%, 90%, and 100% twice). Finally, the slides submerged in xylene, coverslipped with Permount, and allowed to dry completely.

Timm sulphide silver stain was performed on the hippocampal sections in order to visualize the zinc-concentrated mossy fiber axons in the dorsal dentate gyrus. The severity of mossy fiber sprouting was imaged on 10X magnification using a Leica CTR 6000 microscope. Each image was then blindly scored by two experimenters using the Cavazos et al. (1991) 0 to 5 scale. An image with the score of 0 indicated there were no granules; a score of 1 indicated there were minimal granules; 2 indicated there were several granules; 3 indicated that granules merged together to form patches; 4 indicated the presence of a dense laminar band of granules; and 5 indicated severe sprouting seen by thick, dense laminar band of granules that extend into the inner molecular layer.

Each rat was assigned a mean sprouting score after adding the individual scores of each section from that subject and dividing by the number of scored sections. From there, the assigned scores were separated according to their treatment condition and those are the values that were used for statistical analyses.

Somatostatin Immunohistochemistry and Stereology

The SST immunohistochemistry protocol described below is based on that described by Zambetti (2016). Sections adjacent to those chosen for Timm staining were also chosen for somatostatin immunohistochemistry and processed free floating (control, n=11; kainate-induced, n=14). Every sixth, 40 μm, coronal section was washed three times in 1X PBS for 5 minutes (3x5minutes) using either a 3D shaker. The free-floating sections were then blocked for 1 hour in blocking solution, which contained 10% horse serum (HS) and 0.3% Triton-X in PBS, on a 3D shaker at room temperature. After blocking, the sections were incubated in diluted 1:300 monoclonal mouse anti-somatostatin antibody

(GeneTex, code GTX71935, clone SOM-018) with 10% HS and 0.3% Triton-X in PBS over two nights at 4ºC on a 3D shaker. Following the two incubation nights, the primary antibody was washed from the free-floating sections three times with 1X PBS. The sections were then incubated for 1 hour at room temperature in diluted (1:200) biotinylated horse anti-mouse IgG antibody (Vector BA-2000) with 0.3% Triton-X in PBS. Then, the sections were washed three times once again and endogenous peroxide activity was blocked with fresh 0.3% H_2O_2 in diH_2O_2 for 30 minutes at room temperature. The sections were washed three times again; then, incubated with the avidin-biotin-peroxidase technique (Vectastain ABC Kit, Vector Labs, USA) for 30 minutes at room temperature. The complex was developed using 0.05% of 3,3'-diaminobenxidine (DAB) tetrahydrochloride, 0.3% $NiCl_2$•$6H_2O$ stock, and 0.05% hydrogen peroxide as a substrate for the peroxidase reaction. The sections were washed of all toxic DAB solution, mounted onto Fisherbrand Superfrost Plus Microscope Slides with mounting solution, containing gelatin and PBS, and allowed to dry overnight at room temperature. The following day, the slides were dehydrated in various ethanol concentrations: twice in 70% for 1 minute, once in 90% for 1 minute, twice in 95% for 5 minutes, and twice in 100% for 10 minutes. After dehydration, the slides were submerged in xylene, coverslipped with paramount, and allowed to dry until stereology was performed.

Stereology of the SST-immunostained tissue was performed using a Leica CTR 6000 microscope and the optical fractionator method and the Cavalieri principle (West et al. 1991) on Stereo Investigator 10 (MBF Bioscience). The counting frame was 300 μm × 300 μm and the counting grid was also 300 μm × 300 μm. After counting SST

interneurons in the dorsal, intermediate, and ventral hilus of each rat, the total SST interneuron population was estimated based on the thickness of the mounted sections (40 μm).

Results

Dentate-Dependent Behavior Results

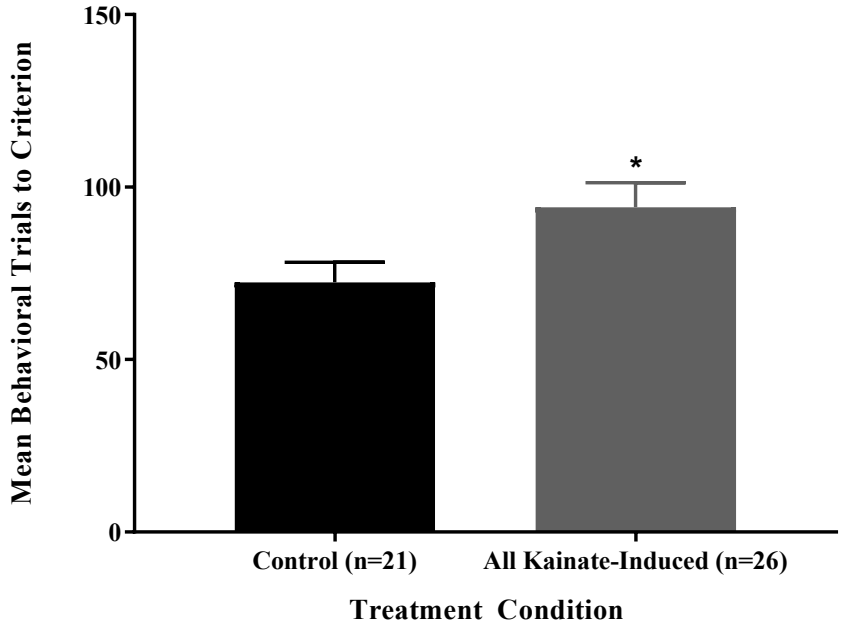

Figure 2. Increased mean number of trials to criterion on the dentate-dependent behavior in kainate-induced rats. The mean (±SEM) number of trials to learning criterion on the 8-RAM for both control and kainate-induced rats are depicted. Significance (*) was established by an unpaired two-tailed Welch's t-test, p=0.0223.

Figure 2 summarizes the mean (±SEM) number of trials required by the control and kainate-induced groups to reach the learning criterion on the dentate-dependent behavioral task. As stated in the *Materials and Methods*, criterion was defined as 90% accuracy in the daily behavioral sessions for two consecutive days. An unpaired two-tailed Welch's t-test was used to analyze the data, and the results revealed a significant increase in the number of trials required by the kainate-induced group, p=0.0223. Among the kainate-induced group, 7 were confirmed to be chronically epileptic; the number of trials to criterion

seizure frequency (trials:seizures)for individual chronically epileptic, kainate-induced rats were as follows: 184:2, 170:11, 110:15, 90:2, 90:17, 90:2, and 40:6.

Mossy Fiber Sprouting Results

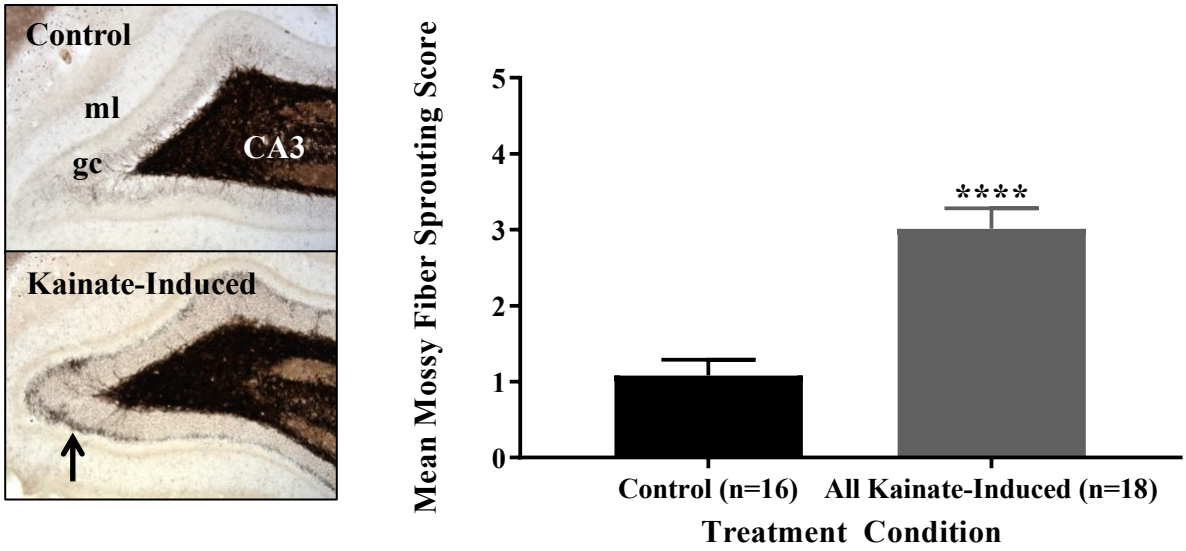

Figure 3. Confirmed epileptogenesis in all kainate-induced rats, indicated by increased mossy fiber sprouting scores. (left) The selected images depict Timm sulphide silver stain of both control and kainate-induced rats at 10X magnification. Mossy fiber sprouting of granule cells (gc) is indicated by the thick black band (arrow) in molecular layer (ml) of the dorsal region of the dentate gyrus. Significance (****) was established by an unpaired two-tailed Welch's t-test, $p<0.0001$.

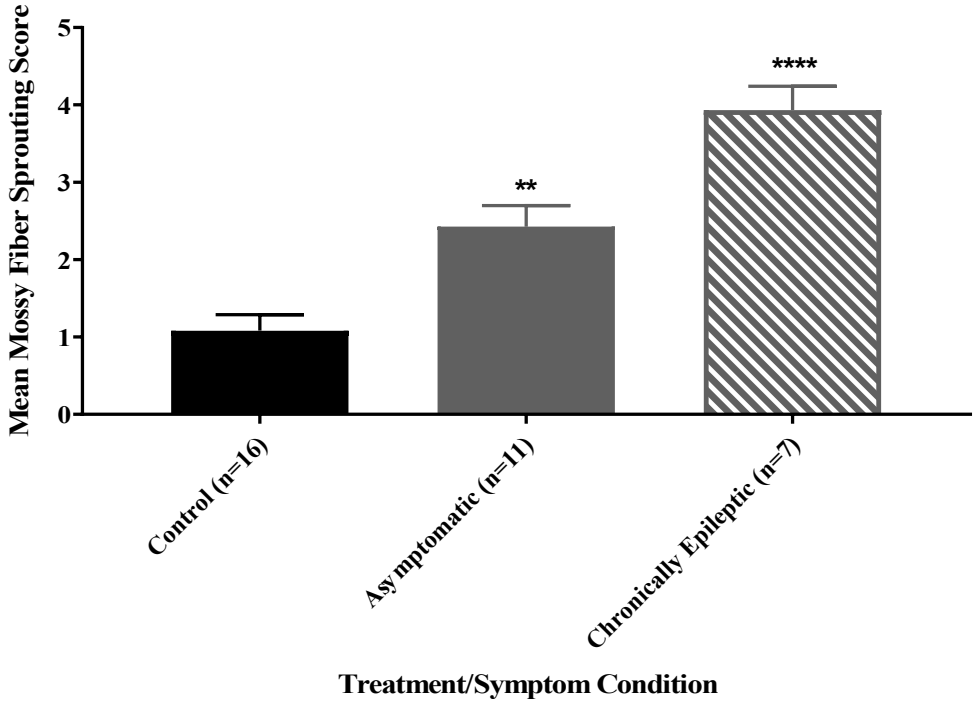

Figure 4. Among the two categories of the kainate-induced group, chronically epileptic rats had the most severe mossy fiber sprouting. The kainate-induced group was separated by symptom condition and their mean mossy fiber sprouting scores (±SEM) compared to control. Significance (** and ****) was established by an unpaired two-tailed Welch's t-test, $p=0.0008$ and $p<0.0001$ respectively.

Histology from kainate-induced rats indicated the presence of mossy fiber sprouting in the dentate gyrus (Figure 3, left). The selected images depict severe sprouting (score = 4) in the kainate-induced rat, but no sprouting in the control rat (score = 0). An unpaired two-tailed Welch's t-test of control and kainate-induced groups revealed significantly higher mossy fiber scores in the kainate-induced group with a mean (±SEM) of 3.013 (±0.27). The mean mossy fiber score among the control group was 1.083 (±0.20) as shown in Figure 3 (right). Investigating the condition of mossy fiber sprouting in the kainate-induced group was an essential element of our study because mossy fiber sprouting is indicative of epileptogenesis, regardless of seizure activity. Figure 4 illustrates a further

analysis of mossy fiber sprouting in the subcategories of the kainate-induced group. When separated according to seizure activity or lack of, the asymptomatic rats (n=11) still exhibited elevated levels of mossy fiber sprouting with a mean score of 2.427 (±0.27), again confirming that epileptogenesis had taken place in the kainate-induced rats that were asymptomatic. Among the confirmed chronically epileptic, kainate-induced rats (n=7) the mossy fiber sprouting scores were the most severe with a mean score of 3.933 (±0.31). However, whether the severity of mossy fiber sprouting in the chronically epileptic was a trigger, or a result of seizure activity is not known.

Somatostatin Interneuron Population Results

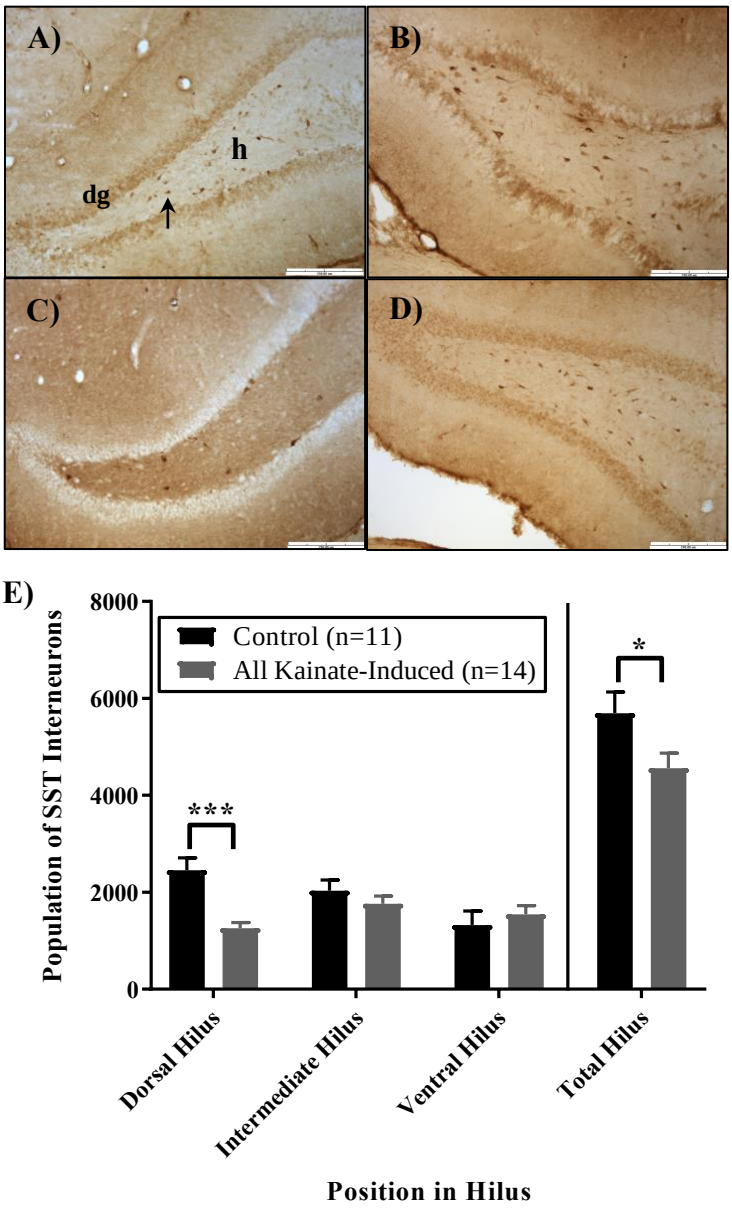

Figure 5. Selective loss of SST interneurons in the dorsal hilus of kainate-induced rats. Control (A,B) and kainate-induced (C,D) hippocampal tissue were immunostained for SST-positive interneurons. Significantly less SST interneurons were found in the dorsal hilus of kainate-induced rats (C) in comparison to the dorsal hilus of control rats (A). The same trend was not found in the intermediate (C,D) or ventral hilus (not pictured), as summarized in the graph (E). SST interneurons (arrow) of interest are visualized as brown dots in the hilus (h) of the dentate gyrus (dg) in the immunostained tissue. Significance (*** and *) established by unpaired two-tailed Welch's t-tests, p=0.0007 and 0.0477 respectively.

After immunostaining hippocampal tissue for SST-positive interneurons, population of SST interneurons were quantified using stereology. The mean (±SEM) SST interneuron population according to hilar position and treatment groups are summarized in Figure 5E. An unpaired two-tailed Welch's t-test comparing the average SST interneuron populations of control and kainate-induced rats revealed an average 49% loss of SST interneurons in the dorsal hilus of kainate-induced rats, p=0.0007. However, significant changes in SST interneuron populations were not found in the intermediate or ventral hilus. Furthermore, the selective loss of dorsal SST interneurons translated into a 20% loss of total hilar SST interneuron populations among kainate-induced rats, p=0.0477.

Correlation Analysis of Measured Values

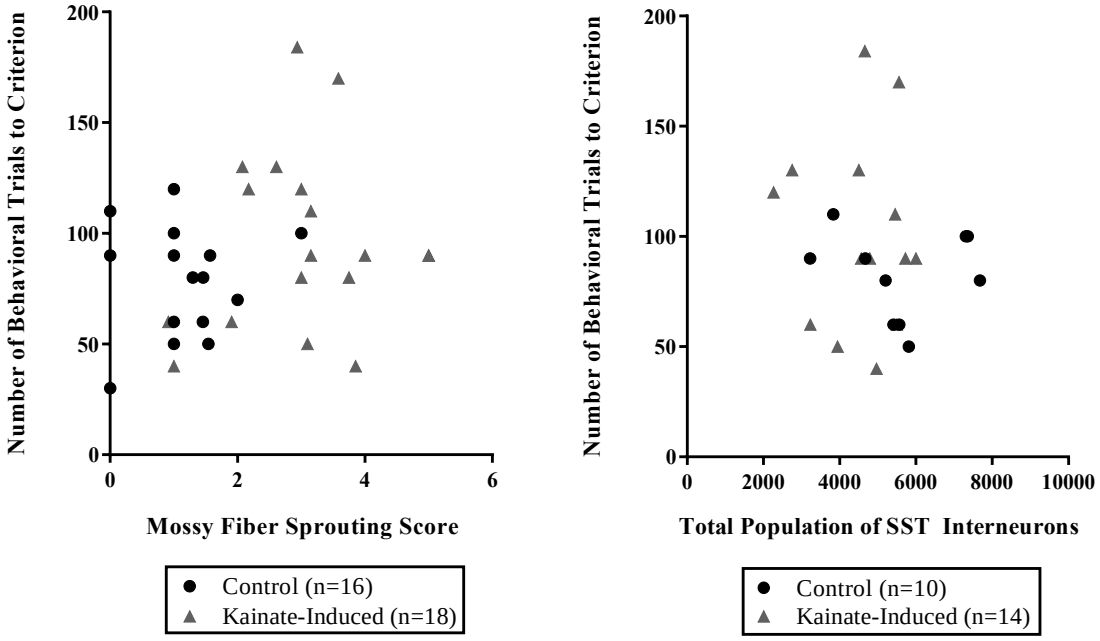

Figure 6. No linear relationship between dentate-dependent behavior performance and mossy fiber sprouting scores or total SST interneuron populations. (left) Each point represents the assigned average mossy fiber sprouting score and number of trials required for learning criterion of one subject. (right) Each point represents the total SST interneuron populations and number of trials required for learning criterion of one subject.

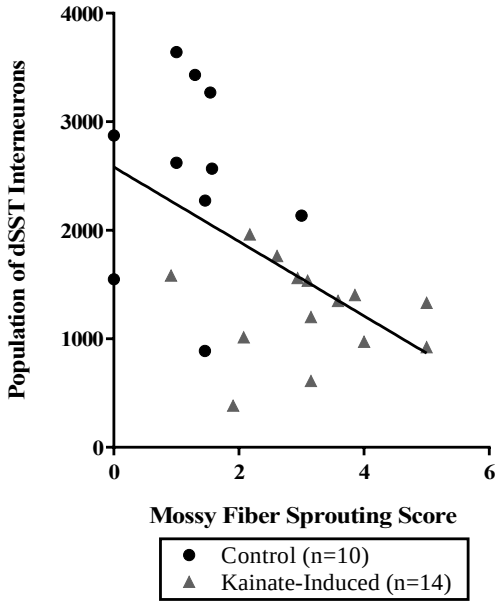

Figure 7. Increased mossy fiber sprouting moderately correlated with lower populations of dorsal SST interneurons. Each point represents the dSST interneuron population and mossy fiber sprouting score of an individual rat. Pearson's correlation was performed between mossy fiber sprouting scores and dSST interneuron populations ($r = -0.5303$; $p=0.0077$).

Linear relationships were not found between behavioral performance and mossy fiber sprouting scores (Figure 6, left) or SST interneuron populations (Figure 6, right) among control or kainate-induced rats. However, looking at both molecular dentate changes together, higher mossy fiber sprouting scores correlate with lower dSST populations.

Discussion

Mesial temporal lobe epilepsy is the most common focal seizure disorder in adults, yet it is still poorly understood due to the gradual process of epileptogenesis. This gradual development of chronic epilepsy is what contributes to the seizure-free, latency period that makes mTLE difficult for physicians to diagnose before the full onset of seizures (Sloviter, 2008). One hallmark of human and rodent mTLE patients is synaptic reorganization, specifically mossy fiber sprouting (Margerison and Corsellis, 1966; Cornin and Dudek, 1988; Cavazos and Sutula, 1990; Buckmaster and Dudek, 1997a,b). The increased mean mossy fiber sprouting scores among kainate-induced rats revealed that induction with kainic acid established the expected neuronal imbalance necessary for epileptogenesis (Figure 3). Additionally, the results from the kainate-induced rats on the dentate-dependent behavior task supports our hypothesis that epileptogenesis is accompanied by a spatial pattern separation impairment. The behavioral findings of this study are consistent with previous research that found dentate gyrus lesioned rats to be less capable of learning to distinguish between adjacent arms of the 8-RAM (McDonald and White, 1995; Morris et al., 2012).

Through the Q-See 24-hour monitoring, we were able to distinguish two groups among the kainate-induced group: asymptomatic and chronically epileptic. The asymptomatic, kainate-induced rats did not have any observable seizures and served to mirror high risk individuals before the onset of spontaneous, recurrent seizures. The chronically epileptic rats were those that had 2 or more observable seizures (e.g. stage III-V according to the modified Racine scale (Racine, 1972)). These chronically, epileptic rats

served to demonstrate the effects of fully progressed, chronic epilepsy. Therefore, it was necessary to account for these two groups when considering if pattern separation functionality could be used for a prognostic or diagnostic tool. After removing the chronically epileptic rats from the statistical test between control and kainate-induced rats (i.e. only asymptomatic rats), the statistical significance was lost ($p=0.082$) – meaning that pattern separation impairment was not observed in pre-epileptic rats.

Although our results did not support the hypothesis that pattern separation tasks could be used as a prognostic or diagnostic tool for mTLE due to dentate alterations of epileptogenesis, our results did validate previous age-dependent epilepsy research in rats. Strafstrom et al. (1993) found that acquisition of place learning in the Morris water maze was only impaired in kainate-induced rats that were induced during adulthood (i.e. kainate-induction at postnatal 60 days). Rats that were induced postnatal 5, 10, 20, and 30 days performed similar to control rats of the same age. Rats that were induced postnatal 20 and 30 days only showed a memory impairment when tested for spatial bias in a platform-absent Morris water maze. Postnatal 5-10 days are considered neonatal while 20-40 days postnatal are considered pre-pubescent – with adolescence occurring in male rats at postnatal 45-48 days and adulthood occurring at approximately postnatal 60 days (Sengupta, 2013). Our rats were considered pre-pubescent (postnatal 40 days) when they underwent kainate induction, which may have resulted in the observed no effect on dentate-dependent pattern separation from the asymptomatic rats. Kainate-induction in adult rats may cause a pattern separation deficit prior to the onset of spontaneous recurrent seizures, but it would be difficult to study since adult inductions are associated with a

higher occurrence of epileptogenesis progressing to chronic epilepsy (Strafstrom et al. 1993; Sun et al. 2007).

Numerous behavioral studies have demonstrated the strong association between the dentate gyrus and pattern separation. However, studies involving electrophysiological recordings have demonstrated that both in response to highly overlapping stimuli, the downstream structure, CA3, is recruited through the mossy fiber pathway (Vazdarjanova and Guzowski, 2004; Leutgeb et al., 2007). The normal mossy fiber pathway that connects the dentate gyrus to the CA3 is the same pathway that is altered by mossy fiber sprouting during epileptogenesis. This pathway is normally unidirectional, where granule cells of the dentate gyrus receive input from the entorhinal cortex through perforant path projections, then granule cell axons (i.e. mossy fibers) synapse onto pyramidal neurons in the CA3 (Sutula, 1990). The sprouting of granule cell mossy fibers to synapse onto other dentate granule cells or onto their own dendrites during epileptogenesis may lend to the unregulated epileptic activity. However, a correlation analysis between mossy fiber sprouting and behavioral performance on the dentate dependent task was incoherent – reinforcing the idea that epileptogenesis involves numerous dentate alterations (i.e. not only mossy fiber sprouting) and that behavioral effects of kainate-inductions are age-dependent (Lothman, 1994; Parent and Kron, 2010).

Our investigation into the hilar SST interneuron populations associated with mTLE and epileptogenesis confirmed previous research that hilar SST interneurons are highly vulnerable to cell death (Sloviter, 1989a; Gulyás et al., 1991; Robbins et al., 1991; Tallent and Qui, 2007; Mazarati and Teledgy, 1992). The pattern of decreased SST interneuron

populations in the dorsal hilus was also found to validate the findings from Buckmaster and Dudek (1997b). While the functional role of hilar SST interneurons is still uncertain, these interneurons have been found to project axons in the outer molecular layer, where those axons synapse with distal dendrites of granule cells (Leranth et al., 1990). This suggests that SST interneurons are involved in the excitatory chain that activates the dentate gyrus and may exert inhibitory action on dentate granule cells (Leranth et al., 1990). Therefore, the loss or decrease in SST interneurons due to epileptogenesis may decrease dentate resistance to hyperexcitation. However, alike to mossy fiber sprouting scores, our results expressed no correlation between total SST interneuron populations and behavioral performance on the dentate-dependent task (Figure 6, right).

Despite behavioral performance showing no correlation with either molecular changes that were measured, higher mossy fiber sprouting did correlate with lower dorsal somatostatin interneuron populations (Figure 7). The molecular results agree with previous research demonstrating that age is a factor in seizure-induced hippocampal damage (Sperber et al., 1991). By the age of 3 weeks, kainic acid receptor expression and the mossy fiber system reach adult maturity in rats (Amaral and Dent, 1981; Ben-Ari et al. 1984; Miller et al., 1990). Our rats were induced at postnatal 40 days, so it was expected to observe adult patterns of mossy fiber sprouting and decreased dorsal SST interneuron populations.

Conclusion

Our study found that chronic epilepsy may cause pattern separation impairments, but not prior to the onset of spontaneous recurrent seizures. Therefore, pattern separation tasks may not be an appropriate diagnostic or prognostic tool for mTLE. However, it is important to note that age of brain injury or status epilepticus plays an important role in evaluating the behavioral or molecular effects of experimental epileptogenesis.

Kainate-induction at postnatal 40 days was too early to observe a significant impairment in pattern separation functionality among asymptomatic, kainate-induced rats. However, the hallmark dentate gyrus alterations of epileptogenesis (i.e. mossy fiber sprouting and decreased dorsal SST interneuron populations) were observed since maturity of epileptogenesis-associated structures occurs at approximately 3 weeks of age. Given that neither mossy fiber sprouting, nor dorsal SST interneuron populations were able to correlate to the pattern separation-dependent behavioral performance, the alterations may exert a combined effect on memory.

There are two interesting future directions that may further our understanding of epileptogenesis. First, collect additional behavioral data after performing kainate inductions during rat adulthood rather than adolescence. This may confirm if age was a factor in observing no significant impairment in asymptomatic, kainate-induced rats. Second, gather more behavioral data from chronically epileptic rats using the same protocol. This would confirm if the deficit in pattern separation-dependent behavior is a significant effect of epilepsy.

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