Photodynamic Therapy for Epilepsy

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ABSTRACT

Epilepsy is surgically curable if the seizure focus can be localized and does not include areas of eloquent cortex. Because epileptic cells are indistinct from surrounding brain, resection typically includes normal tissue. Using the rat kindling model of epilepsy, we evaluated Photodynamic Therapy (PDT) as a super-selective lesioning technique. We present a series of pilot studies to evaluate: 1) Protoporphyrin IX (PpIX) fluorescence, 2) the efficacy of PDT to raise seizure thresholds, 3) the safety of PDT using behavioral studies, and 4) histologic results. Bipolar electrodes were chronically implanted into the cortex and animals received successive low-level stimulation generating seizures of increasing severity. Following 5-aminolevulinic acid (ALA) administration, fully kindled rats received electrical stimulation to induce a generalized seizure. Animals were irradiated with laser light focused onto a temporal craniectomy. Our results show: 1) an increase in PpIX fluorescence in the seizure group, 2) PDT treated animals failed to demonstrate seizure activity following repeat stimulation, 3) no statistically significant difference between treated and control animals were observed on behavioral tests, 4) histology showed pyknotic hippocampal pyramidal cells in the CA3 region without areas of obvious necrosis. In conclusion, this is the first report of heightened PpIX-mediated fluorescence in epileptic brain. The selective accumulation of PpIX with laser PDT may provide a less invasive and more precise technique for obliteration of epileptic foci. PDT warrants additional research to determine if this technique may augment or replace existing procedures for the surgical management of epilepsy.

Keywords: Epilepsy, Photodynamic Therapy (PDT), rat kindling, seizure, 5-aminolevulinic acid (ALA), protoporphyrin IX (PpIX)

1. INTRODUCTION

For more than two million people with epilepsy, the daily life challenges are well known; decreased school and work performance, medication side effects, difficulty or inability to obtain a driver's license, psychosocial problems and fear of injury or sudden death. Epilepsy is characterized by recurrent, unprovoked seizures, the outward sign of abnormal brain electrical activity as measured by electroencephalography (EEG). While a single seizure may have many causes, such as fever, meningitis, oxygen depravation, and drug or toxin exposure, the diagnosis of epilepsy is made only when repeated seizures occur without these known antecedent factors. These patients require a thorough medical evaluation including a detailed history and physical examination, laboratory blood and often spinal fluid analysis, EEG and Magnetic Resonance Imaging (MRI) [1]. Once a patient is diagnosed with epilepsy, antiepileptic drug (AED) therapy is initiated. Approximately 25% of patients continue to have seizures or intolerable side effects to AEDs. These patients, deemed medically refractory epileptics, are considered for epilepsy surgery.

Today, resective surgery is the only known "cure" for patients with epilepsy. Surgery is considered as a treatment for patients with difficult to control epilepsy when seizures are localized to a single brain region, and the focal point of the seizure can be surgically removed without damaging other important functions [2]. Each epileptic patient being evaluated for surgery must undergo extensive tests to determine the exact location of the epileptic focus responsible for the seizures. Additionally, the brain may be mapped to locate important functional areas such as speech, memory, motor

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function and vision. Once the pre-operative evaluation is complete, the neurosurgeon selects the best method for removing or disconnecting the epileptic cellular focus while preserving brain function and quality of life for the patients. Different types of epilepsy surgeries include:

- Lobectomy and Cortical Resection – most common form involving removal of all or part of the region containing the epileptic focus
- Hemispherectomy – removal or disconnection of one cerebral hemisphere
- Corpus Callosotomy – sectioning or separating the physical connection between the two sides of the brain to confine the spread of epileptic seizure to one hemisphere. This method is commonly used for patients when the epileptic focus of the seizure cannot be localized, to reduce loss of consciousness and drop attacks in severe epileptic patients
- Multiple Sub-pial Transection – used to control spread of the epileptic seizure by dividing the parallel connections between cells in the affected area. This method is commonly used when the seizure focus is located in a vital area of the brain that cannot be removed.

Epilepsy is surgically curable if the seizure focus can be localized and the resection site does not include areas of eloquent cortex. Because epileptic cells are visually indistinct from surrounding brain, resection typically includes normal tissue. Using the rat kindling model of epilepsy, we evaluated Photodynamic Therapy (PDT) as a super-selective lesioning technique with translational application for patients with refractory epilepsy. PDT, approved for use in brain tumors [3, 4, 5] and other systemic diseases, is a two-part process that may add both sensitivity and specificity to the surgical treatment of epilepsy [6]. The first part of the process involves selective uptake of a photoactive compound into "epileptic" neurons (i.e., cells that contribute to seizure generation) within the brain region of interest. The second step targets laser light to that specific brain region activating the photosensitizing agent and initiating a cell death process [7].

We selected the fluorescent and photoactive substrate protoporphyrin IX (PpIX) for this series of pilot studies because of its known efficacy in human brain tumors [8, 9]. Work by Brian Wilson, PhD [10, 11], Stuart Bisland, PhD [12], and others has demonstrated in brain tumors that PpIX accumulation increases with 5-aminolevulinic acid (ALA) administration as a function of pH, metabolism and breakdown of the blood-brain barrier and to an even greater extent in epilepsy. The process of PpIX accumulation is dependant on the heme biosynthesis pathway (Figure 1), a naturally occurring chemical reaction found in human cells [13]. 5-Aminolevulinic acid is converted to the photoactive molecule PpIX during the production of intercellular heme. As the rate limiting step in the synthesis of PpIX, ALA synthetase is strongly regulated by the concentration of free heme [14]. Averting the normal feedback mechanism, excess ALA bypasses the rate-limiting step of the heme pathway and leads to increased intracellular accumulation of PpIX.

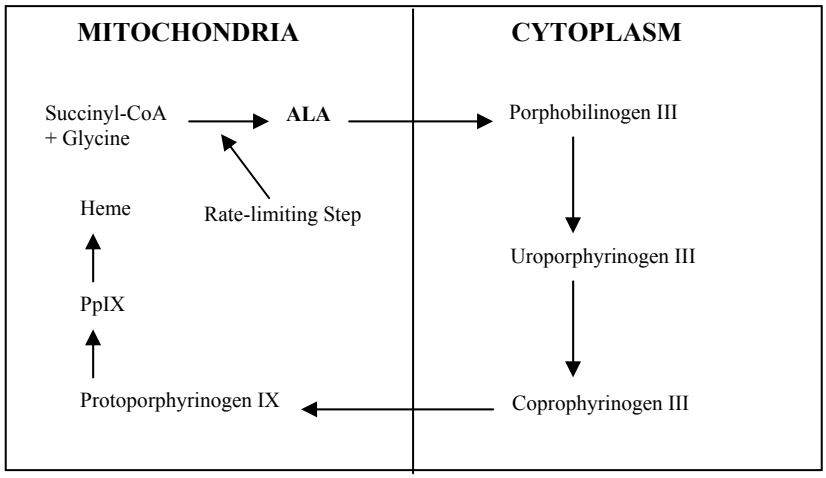

Figure 1. Intracellular heme biosynthesis pathway

Conjugated PpIX molecules are excited by violet light (400 nm) and emit red light (635 nm) that can be visualized by fluorescent microscopy. In vivo, PpIX can be activated by exposure to laser energy producing cytotoxic photoproducts, mainly singlet oxygen species that lead to tissue apoptosis and necrosis. The wavelength of light utilized to activate the

fluorescent mitochondrial label, is largely distinct from the wavelength of light utilized to activate the photodynamic therapeutic effect [15].

We hypothesize that epileptic tissue, like brain tumor tissue, will show an increased selectivity for ALA uptake and subsequent PpIX fluorescence. While fluorescent labeling of epileptic cells is of value to improve surgical targeting as an endpoint in itself, coupling this function with PDT may provide a minimally invasive and super-selective technique to cure epilepsy with preservation of normal brain. This paper describes a series of pilot studies to evaluate: 1) PpIX fluorescence in epilepsy, 2) the efficacy of PDT to raise seizure thresholds, 3) the safety of PDT as determined by behavioral studies, and 4) histologic results following PDT.

2. STUDY DESIGN

Preliminary results of these four pilot studies are presented:

***1) PpIX Fluorescence in Epilepsy*:** Protoporphyrin IX fluorescence was compared in three groups of Sprague-Dawley rats. The groups were divided and treated as shown in Figure 2. In each group, animals were sacrificed four hours after ALA injection. In one group (Group C), kindled animals were stimulated to induce a generalized seizure prior to sacrifice.

Group	Number in Group	Kindled	Given 5-ALA	Seizure
A	6	No	Yes	No
B	6	Yes	Yes	No
C	6	Yes	Yes	Yes

Figure 2. Animal study groups for PpIX fluorescence in epilepsy

***2) Efficacy of PDT to Raise Seizure Threshold*:** After bounding the parameters for PDT in this novel setting (5 minute duration of PDT showed no effect; animals treated for 20 minutes did not survive) a small group of kindled animals with stimulated seizures (n=3) underwent PDT (Methodology 3.5). These animals were then returned to the kindling paradigm receiving daily stimulations over a 7 day period. EEG was recorded and clinical behavior was monitored for seizure activity.

3) Safety of PDT: The purpose of this study was to determine if PDT caused cortical or hippocampal functional impairment in a rat model of epilepsy – a necessary assessment for research with translational potential to humans. Thirty-one Sprague-Dawley rats were placed into four groups (Figure 3) to measure the behavioral impact of PDT. All animals underwent the same behavioral paradigms; Inclined Plane was used to screen for hemiparesis, a primarily cortical function, and Morris Water Maze was selected to identify problems with spatial learning, a hippocampal function [16].

Group	Number in Group	Kindled Stage	Given 5-ALA	Craniotomy	PDT
A	8	N/A	No	No	No
B	8	N/A	No	Yes	No
C	8	5	Yes	Yes	No
D	7	5	Yes	Yes	Yes

Figure 3. Animal study groups for safety and histological comparison following PDT

4) Histological Results Following PDT: The purpose of this study was to evaluate targeting selectivity and extent of cell loss following PDT. Thirty-one Sprague-Dawley rats used in the behavioral analysis above were placed into four groups (Figure 3) to test the effect of PDT treatment parameters: kindling, administration of 5-ALA, craniotomy and laser application.

3. METHODOLOGY

3.1 Subjects

Adult male Sprague-Dawley rats weighing 275 g at the time of surgery were individually housed with food and water available ad libitum. Each animal was handled daily. Experiments were conducted in the light portion of the 12:12 hour light/dark cycle.

3.2 Surgical perforant path electrode implantation

Electrode implantation into the perforant path is one way to induce seizure activity in the rat model of epilepsy. Perforant path kindling allows for electrical stimulation to reach the hippocampus without physically damaging the hippocampal areas of interest. Bipolar electrodes are implanted into the entorhinal cortex, a neuronal pathway that synapses onto neurons in the dentate gyrus of the hippocampus. Granule cells in the dentate then project to the CA3 region of the hippocampus.

Animals received excellent care consistent with the UC Davis animal care standards and the methods and procedures followed an approved animal use protocol. All rats were intubated and surgically anesthetized with isoflurane gas mixed with gaseous oxygen (2 parts) and nitrogen (1 part). Bipolar stimulating electrodes were sterotactically implanted through a 1.5 mm diameter craniotomy at 7.4 mm posterior to Bregma, 4.1 mm lateral to midline, and 3.3 mm ventral from cranial surface. The electrodes were constructed from two twisted strands of teflon-coated nichrome wire, attached to a female connector pins [17]. A 2 mm diameter silver ball was placed deep to the skull and served as the ground/reference electrode. The electrodes were fixed to the skull using three stainless steel skull screws and dental acrylic.

3.3 Kindling

Kindling is a reliable and well-characterized animal model of epileptogenesis, the process by which cells become epileptic. In kindling, brief, low-intensity stimulation induces an epileptic progression from focal, to complex, to fully generalized tonic-clonic seizures in rats. This seizure propagation parallels the seizure propagation seen in humans with secondarily generalized partial epilepsy. Once fully kindled, animals retain their seizure threshold and remain epileptic indefinitely. Continued stimulation can yield spontaneous seizures, making kindling an excellent experimental model.

Kindling began after a 7-day post-surgical recovery period following electrode implantation. Afterdischarge thresholds (ADTs) were determined by delivering electrical stimulation consisting of a 1-s train of constant current, symmetrical, biphasic square-wave pulses (1 ms duration, 100 Hz) through the chronically implanted bipolar electrodes. These pulses were delivered at an initial intensity of 10 μA and increased to higher intensities by increments of 10 μA at 30 sec intervals until at least a 5-10 sec epileptiform afterdischarge (AD) was evoked. The afterdischarge threshold (ADT) was therefore defined as the stimulation intensity that first evokes an AD, a brief focal seizure recorded by the electroencephalogram (EEG). A specific ADT was determined for each rat and was used throughout the kindling process. Repeated stimulations (1 per day, 5 times per week) gradually results in the development of epileptic convulsions and increased durations of epileptic spiking on the EEG (Figure 4).

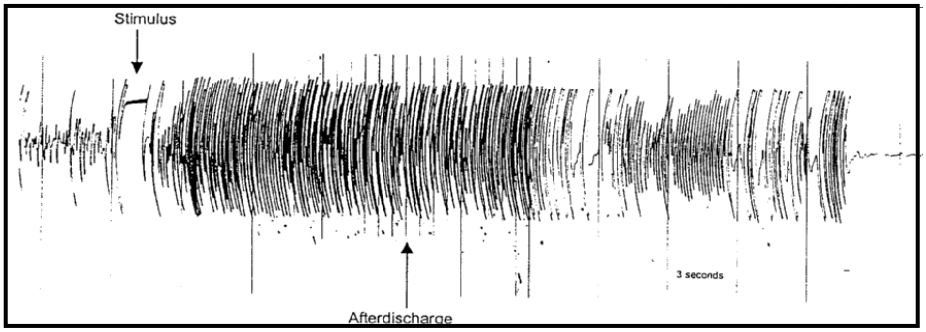

Figure 4. Electroencephalogram (EEG) recording of an afterdischarge (AD) from an implanted animal having a stage five seizure

Using observable behavioral criteria, seizures were classified into progressive stages [18, 19]: behavior progresses from short episodes of epileptic spiking without behavioral elements (stage 0), episodes of blinking (stage 1), and episodes of chewing/nodding (stage 2), to longer AD episodes that include forelimb clonus (stage 3), bilateral forelimb clonus and rearing (stage 4), and fully generalized bilateral tonic-clonic convulsions with rearing and falling (stage 5). Rats were considered "kindled" when they experienced three consecutive stage 5 seizures.

3.4 Drug administration and perfusion

Once fully kindled, animals were surgically equipped with a femoral vein catheter. ALA was prepared at 300 mg/ml with sterile phosphate buffer and pH adjusted to 6.5 with 6N NaOH. For all groups, 400 mg ALA / Kg body weight was injected intravenously through the femoral vein catheter.

3.5 Photodynamic Therapy

To prepare animals for laser treatment to activate the photosensitive metabolite of 5-ALA, PpIX, rats were first anesthetized then placed in the stereotaxic frame. A craniotomy was made in the left temporal region, just dorsal to external auditory meatus, ventral to the temporalis muscle insertion, and posterior to the zygomatic arch. The dura was left intact. A black plastic shield with 4mm aperture was attached to the skull around the craniotomy. The Spectraphysics Laser Model 2500 Argon-pumped tunable dye laser of 635 nanometers wavelength and powered density of 200 milliwatts per centimeter squared was focused on the 4mm aperture for a total of 10 minutes. Following laser therapy, the plastic shield was removed, scalp sutured, and animals were kept warm and hydrated until fully recovered from the anesthesia.

3.6 Sacrifice and tissue collection

All animals were euthanized with excess euthasol and perfused in subdued light with 300 ml cold, phosphate buffered saline. The whole brain was extracted and immediately flash-frozen in cooled isopentane for 30 seconds, then stored at −70°C in an ultra-cold freezer until sectioned on the cryostat.

3.7 Cryostat sectioning, tissue collection and Cresyl Violet staining

All cryostat sectioning and tissue collection occurred in subdued light in order to reduce photobleaching. Overhead laboratory lights were turned off and the working area was illuminated minimally using a non-fluorescent light source. Reduced illumination procedures were used as a precaution although our previous experiments showed no significant reduction of fluorescence (light-induced bleaching) throughout a continuous 60 min exposure to ambient laboratory lighting. The cryostat was maintained between –15 and –20 degrees Celsius throughout the tissue preparation and sectioning. Beginning at the anterior hippocampal regions corresponding to 1.6 mm posterior to Bregma and ending at the posterior aspect of the hippocampus (6.3 mm posterior to Bregma) brains were sectioned at 20 micron. Every third section was mounted, 3 per subbed slide, and will soon be analyzed with fluorescent microscopy, as well as H /E and Nissl staining. Cryostat-prepared brain sections are slide-mounted, air-dried overnight, rinsed in distilled water for 10 seconds and then immersed in Cresyl Violet solution (12 ml of 1% stock solution in 100 ml water) for 30 minutes. Slides are rinsed, dehydrated through alcohols and xylene and coverslipped.

3.8 Behavioral tests

The Morris Water Maze (MWM) has been used extensively to assess and compare memory and learning in rodents. The target of our therapy, the hippocampus, is believed to be responsible for spatial learning and memory. We selected this task to determine if excessive functional damage occurred as a result of PDT. The MWM requires no pre-training period, can be accomplished in a short amount of time, and performance can be compared between and within groups. The Incline Plane test (IP) can evaluate bilateral grip strength. This test was used to screen for any motor and/or coordination deficiencies resulting from the surgical craniotomy or laser effect on the cortex. Any sign of hemiparesis may indicate undesired cortical injury.

After a 10 – 14 day recovery period from laser application animals underwent a behavioral battery that consisted of the MWM and IP. On days 1 – 4 animals were first evaluated on the IP. The IP involves the initial placement of the animal at a 65° angle to the horizontal. The flat plane can move up or down in intervals of 5°, until they are at an angle where they can hold their position without sliding down. Then the opposite side it tested. All animals participated in the MWM test conducted over 5 consecutive days. The MWM consisted of a small pool (180 cm diameter) painted white

and filled with water (26°C ± 2). Visual markers throughout the room helped the animals navigate to a submerged platform. Each day the animal is placed in the pool at four randomly assigned compass points and taken out when they find the platform or when 120 seconds has elapsed. A video camera is connected to a computer that automatically calculates an average speed from these two variables and this data is recorded for each trial.

4. RESULTS

4.1 PpIX fluorescence in Epilepsy

Preliminary data with a small number of animals suggests that fully kindled rats with elicited seizures show greater ALA uptake in hippocampal cells compared with both fully kindled rats without elicited seizures and the implanted controls. The results for the PpIX fluorescence in epilepsy are displayed graphically in Figure 5. The pictures of the PpIX fluorescence for each group are shown in Figure 6.

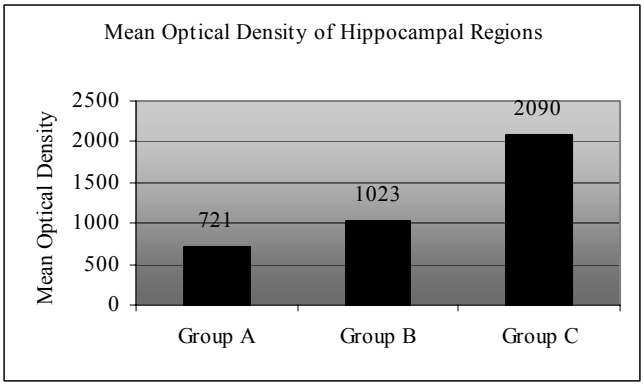

Figure 5. Mean optical density (amount of Fluorescence) in hippocampal regions calculated using gray scale conversion (Image Pro software) of Texas Red imaging

Figure 6. PpIX fluorescence in ALA control, kindled with ALA and no stimulated seizure and kindled with ALA and stimulated generalized seizure. Photos were captured at 2 second using a 10x objective and a Texas Red filter

4.2 Efficacy of PDT to raise seizure threshold

PDT treated animals failed to demonstrate seizure activity by either clinical observation or EEG recordings over a period of seven days of repeat stimulation using the kindling protocol.

4.3 Safety of PDT

The behavioral analysis results for the IP are displayed in Figure 7 and the MWM in Figure 8.

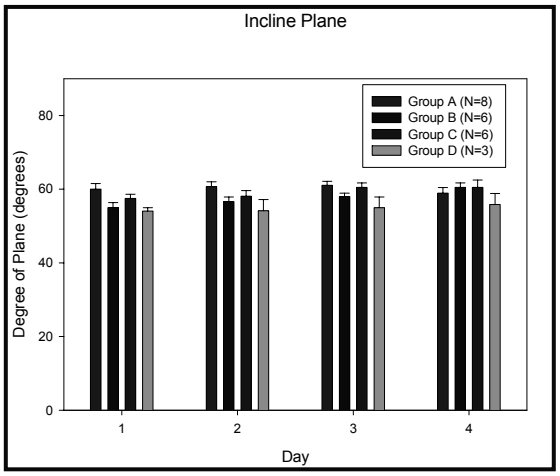

Figure 7. Incline Plane behavioral test results

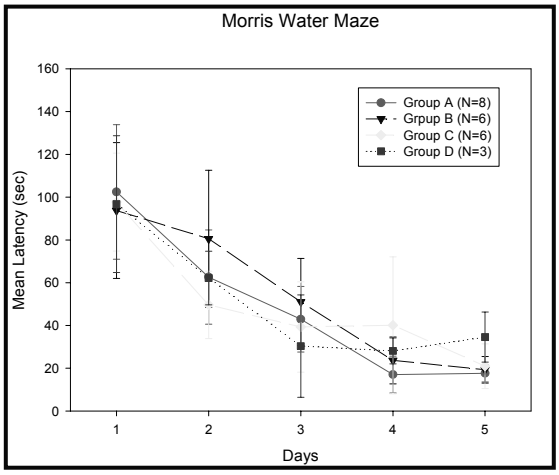

Figure 8. Morris Water Maze behavioral test results

There was no difference between groups on the Incline Plane assessment [F(3,27) = 1.616, p = .209, between groups] which demonstrates that the treatment did not influence motor performance (Figure 7). Statistical analysis of performance on the MWM task indicated that there were no significant differences between animals that received laser treatment and control animals [F(3,27) = .920, p = .445, between groups] (Figure 8). The time to find the platform decreased across days [F(4,27) = 238.745, p < .001] indicating that the rats did learn to located the hidden platform. Moreover there was no significant day by group interaction [F(3,27) = 1.470, p = .245] indicating that the rate of learning did not differ across groups.

No statistically significant difference between PDT treated animals and control groups were observed on Incline Plane or Morris Water Maze behavioral tests.

4.4 Histological results following PDT

The following two pictures show histological images of the hippocampus without and with PDT, Figures 9 and 10, respectively.

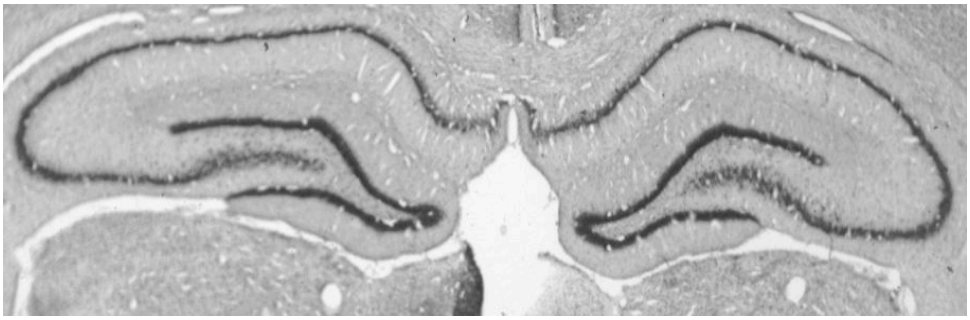

Figure 9. Image of the hippocampus after all procedures without PDT (Group C – Figure 3)

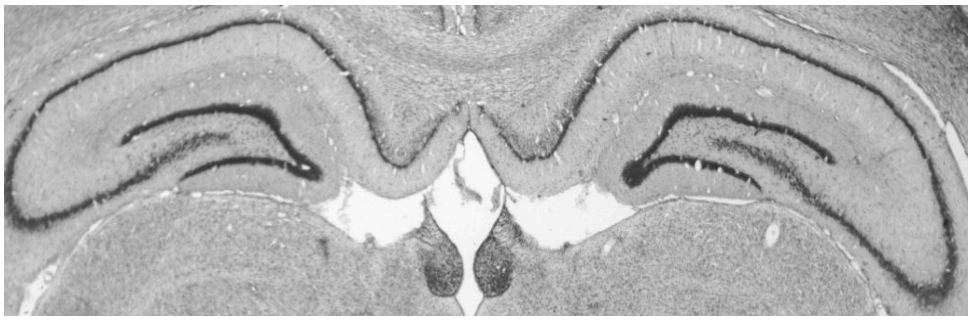

Figure 10. Image of the hippocampus after all procedures with PDT (Group D – Figure 3)

When Figure 10 was viewed under 40X magnification there were numerous shrunken pyramidal cells, particularly in the CA3 region and occasionally in the CA 1 region restricted to the region of laser treatment that was not observed in Figure 9. Histology showed pyknotic hippocampal pyramidal cells in the CA3 region without areas of obvious necrosis, which represents a significant improvement in selectivity of lesioning compared to existing surgical techniques.

5. CONCLUSIONS

In conclusion, this is the first report of heightened PpIX-mediated fluorescence in epileptic brain. The results of the four pilot studies show that 1) "epileptic cells" demonstrate increased PpIX fluorescence, 2) PDT may raise seizure thresholds, 3) PDT shows no alteration in normal brain function according to behavioral tests and 4) therapeutic effect occurs without obvious tissue necrosis. The selective accumulation of PpIX combined with laser PDT may provide a less invasive and more precise technique for obliteration of epileptic foci. PDT warrants additional research to determine if this technique may augment or replace existing resective procedures for the surgical management of epilepsy.

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