FINAL SCIENTIFIC/TECHNICAL REPORT SEPTEMBER 2011

Specific Aim 1

We started to use the first animal model of provoked status epilepticus to test the hypothesis that acute seizures induced by osmotic disruption of the blood-brain barrier result in delayed epileptogenesis. These initial experiments were aimed at perfecting the technique used. One of the problems with the approach used in the past is the fact that intrarterial injections are performed across an open incision, which does not allow survival. We have therefore changed the surgical approach as detailed below (Figure 1).

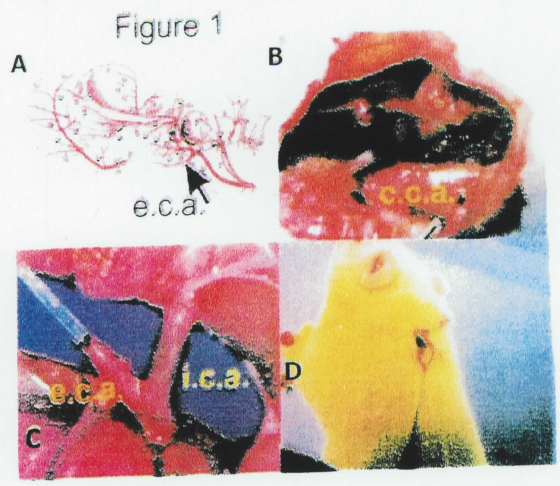

Fig 1: Panel A points to the schematic representation of the External Carotid Artery (ECA). Panel B shows the Common Carotid Artery (CCA) as seen during surgery. Panel C shows the insertion of polyethylene catheter (PE-2P) into the ECA and its relationship to the Internal Carotid Artery (ICA). Panel D shows the surgical and methodological approach for osmotic Blood Brain Barrier Disruption (BBBD) in rat. Note the externalized catheter.

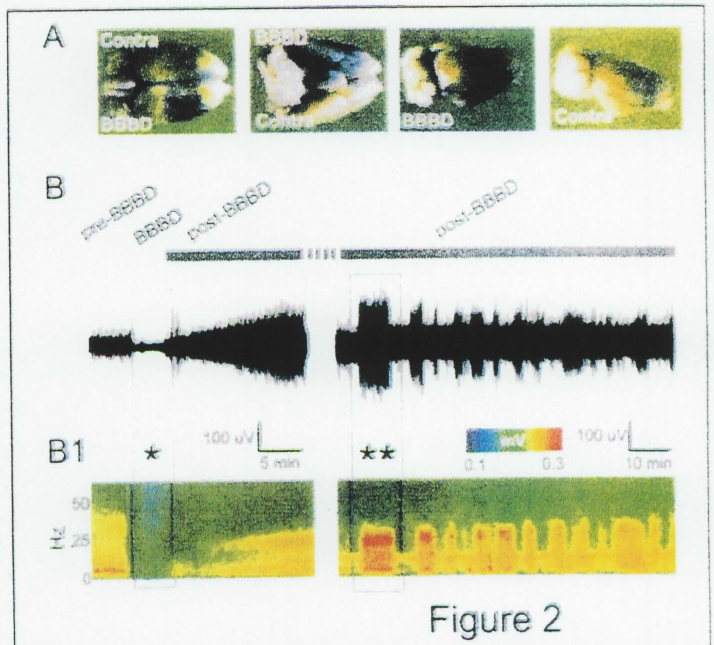

Figure 2

Fig 2A: Gross anatomical analysis of Evans Blue (EB) leakage across the Blood Brain Barrier (BBB). Note the pronounced extravasation of dye and the hemispheric specificity of the BBBD procedure.
Fig 2B: Timeline of the procedures. BBBD is an important player in development of seizures.
Fig B1: Note the EEG changes after mannitol injection. We used the Fast Fourier Transformation to examine in the frequency domain a measurement signal that is located in the time domain. An obvious caveat of this approach is that the values in the time channel must be strictly monotonic increasing and equidistant. Thus, EEG artifacts are averaged together with real signals. This was not however an issue due to the brief duration of these events. Furthermore, DIAdem calculates the FFT to powers with a base of two and therefore might not use all the measurement data. For example, if a time signal has 340 values, DIAdem only uses the first 256 (2^8) values for the FFT. We solved this issue by equalizing the sample size for all EEG records in the average.

Methods

Rats (male Sprague-Dawley 350–450 g) were anesthetized with ketamine and xylazine. After anesthesia, a 2–3 cm vertical incision was made from the suprasternal notch to below the chin. After exposure of the sternothyroid muscle, the common carotid artery was exposed and was separated from the posteriorly placed vagal nerve. After exposure of the bifurcation of internal and external carotid arteries, a silk tie (3-0, Ethicon, 24", Ethicon, Sommersville, NJ, U.S.A.) was looped around the external branch of the external carotid artery (ECA), while a micro-clamp was positioned ready at the common carotid artery (CCA). In temporal sequences the following steps were then performed within 2–3 min: (1) clamp the CCA; (2) ligate the distal ECA, leaving approximately 5 mm stump proximally; (3) insert the polyethylene catheter (PE-2P) into the external carotid artery retrogradely

toward the bifurcation with the common carotid; (4) tighten the silk tie around the catheter; release the clamp at the CCA.

The novel approach consists of the fact that the cannula was externalized subcutaneously and sutured in a fashion that allows sterility and repetitive injection of mannitol. The injection of a hyperosmolar mannitol solution (1.4 m bolus in saline, 0.1 ml/s, 3 ml) lead to a stereotyped set of events (Figure 2B) consisting of seizures. It has long been held that BBB damage observed in the seizure/epilepsy patients is caused by seizures. Recently our lab and others have demonstrated that BBBD may be an important etiology for the development of seizures. In order to further investigate the cause-effect relationship between BBBD and development of seizures, we used hypertonic mannitol injection into the internal carotid artery as a BBBD model. After injection of hypertonic mannitol, we recorded EEG changes on the rats. Our data demonstrated that about 25% rats developed either behavioral or EEG seizures after mannitol injection. The remaining 75% rats did not show signs of behavioral seizures or characteristic EEG seizures. One feature of EEG change was observed in the rats with seizures after mannitol injection as shown in Figure 2 B1. First the EEG demonstrated a shift towards high frequency (20 - 25 Hz). This is a change that has also been reported in human epileptic patients. On the contrary, 75% rats did not show seizure symptoms or EEG changes. **An additional novel technological development that was achieved is shown in the bottom portion of the figure.** By using a customized software routine running on the Diadem (National Instruments) platform, we were able to visually identify EEG changes as joint time frequency events. By using the **frequency domain we are now able to obtain meaningful averages of EEG signals derived from different animals Figure 2 B1.**

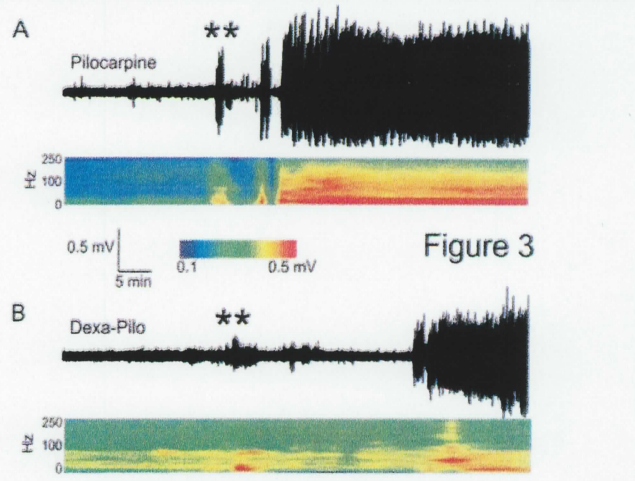

Fig 3 A: EEG correlation of acute seizure. Note the widespread increase in BBB permeability associated with seizure development (fig 4). Fig 3B: Also note the effect of Dexamethasone(given before Pilocarpine). This effect was seen in 20% of animals. In the remaining animals no seizures or BBBD were seen.

Methods

Digital EEG recordings (5 channels per rat) were performed using Pinnacle Technologies Model 8206 EEG-Video recording apparatus. The system consists of a recording workstation and a review system connected to a 21-inch monitor. EEG data were sampled a rate of 200 Hz. Spectrum and spike amplitude analysis was performed after the experiments. All data are transferred via a USB connection to a PC. The USB connection also provides power to the 8206 and preamplifier. Origin FFT software is used in conjunction to the acquisition system for data analysis.

One of the main goals of our proposal is to understand why blood-brain barrier disruption leads to seizures. A recent paper in a high profile journal (Nature Medicine, (1)) has initiated a flurry of experiments to prove or disprove that leukocyte extravasation is a necessary feature of BBB-induced epileptogenesis. We used another model of epilepsy to evaluate the role of BBB damage and leukocyte extravasation in contributing to seizures activity. We have injected rats with the pro-convulsant agent pilocarpine which leads to full blown status epileptics of characteristic EEG appearance (Figure 3A). **Note**

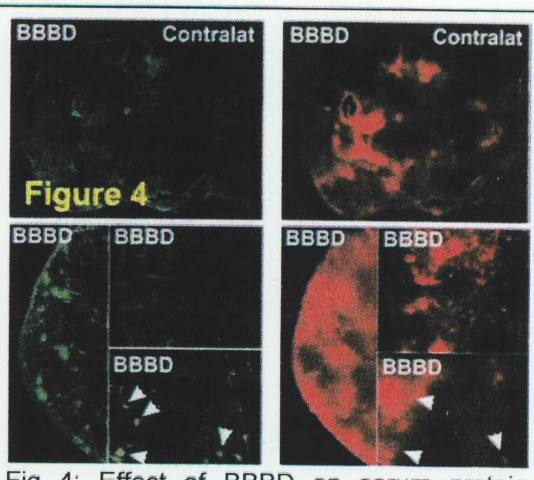

Fig 4: Effect of BBBD on serum protein extravasation. Micrographs describing of EB or FITC labeled albumin. In this specific experiment seizures were induced with BBBD. Red EB fluorescence was detected in the brain parenchyma around the vessels in the injected brain hemisphere in seizure rats. Minimal tracing of EB was seen in brain parenchyma in the contralateral hemisphere.

the similarity between seizures and BBB damage as induced using the two methods (Figures 2B and 3).

We performed several immunocytochemical experiments coupled with a microangiographic approach and a variation of the same to investigate the consequences of BBBD or Pilocarpine-induced seizure (Figures 5-7). The latter is a **new technological advancement that was enabled by the funding provided by DOE for our research.** We used a trans- illumination UV chamber to visualize the extent of Evans blue leakage in the whole brain (Figure 7A). The image obtained was then manipulated by a newly developed routine (Diadem) to quantify in a realistic dimension (micrograms albumin/mg tissue) the extent of blood-brain barrier disruption. We noted a remarkable similarity between the focal appearance of leakage in rat brain treated with pilocarpine **and prior to *status epilepticus*** and the appearance of FLAIR MRI scans taken from epileptic patients (Figure 7B). These results were obtained in collaboration with Dr. Tiziana Granata, a pediatric epileptologist at the Besta Institute in Milan. This is an important finding, because we were then **able to study the outcome of drug treatment in patients to the rat model used** (e.g., dexamethasone Figure 3B).

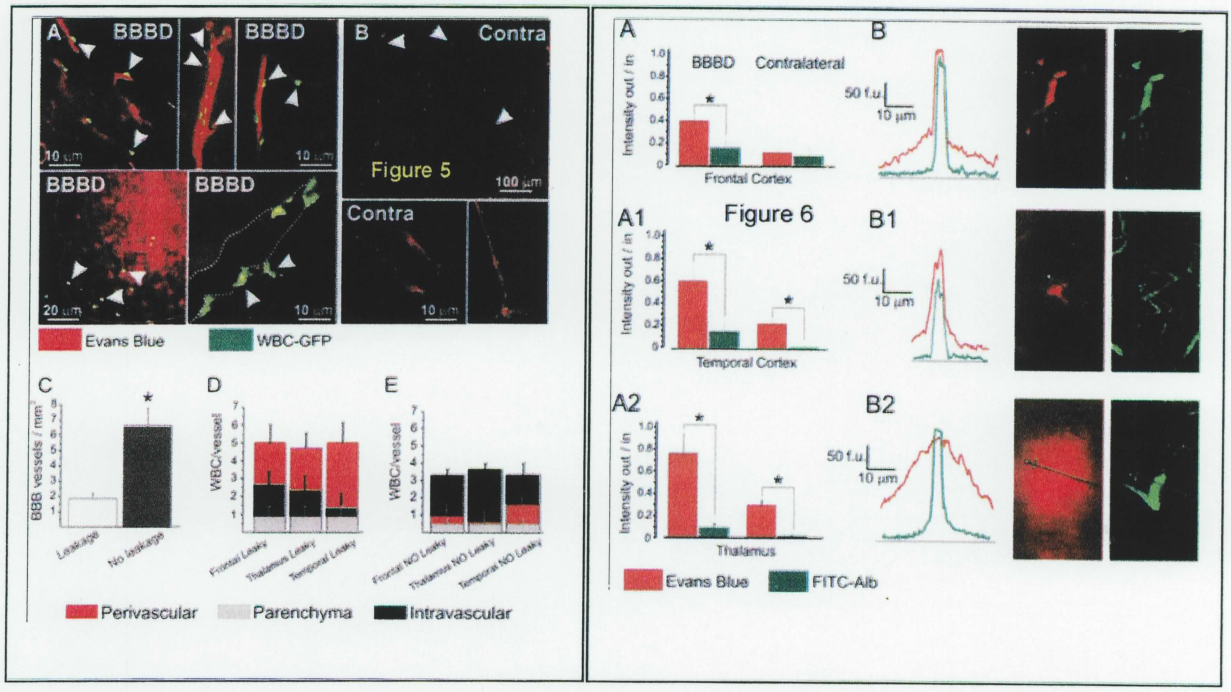

Fig 5A&B: GFP-WBCs were injected into the ICA after mannitol. Our data demonstrated that in seizure rats GFP-WBC were observed only in the perivascular space in both hemispheres in seizure rats. Fig 5 (C.D. &E): No Parenchymal WBCs were observed. Legend follows next page.
Fig 6: Comparison of extravasation of FITC albumin and EB in seizure rat.

Methods

For these experiments, seizures were induced with the cholinergic agent pilocarpine as shown by us and others (1-4). The animals (pilocarpine or mannitol-treated) were injected with the BBB integrity tracer Evans Blue (EB) (5-8) or FITC-labeled albumin (9-12). Evans blue is a low molecular weight maker that binds to serum albumin after administering into the blood forming EB-albumin complexes. Red EB fluorescence could be detected in the brain parenchyma around the vessels in the injection brain hemisphere in BBBD seizure rats (Figure 4). Minimal tracing of EB was seen in the brain parenchyma outside the vessels in the contralateral hemisphere of seizure rats. We concluded that the minimal extravasation of the EB on the contralateral hemisphere is due to the collateral circulation through the circle of Willis.

Although the lack of red fluorescence in the contralateral hemisphere of BBBD seizure rats or the brain parenchyma of non-seizure rats helped us rule out the possibility that free EB leakage into the brain parenchyma through the intact BBB was responsible for the red fluorescent signal, we designed a FITC-albumin study to further rule out this as a possibility. In this study albumin labeled with fluorescent dye FITC was injected after mannitol injection. As shown in Figure 4, in seizure rats, there was abundant green fluorescence around the vessels during seizures. Although the lack of red fluorescence in the contralateral hemisphere of seizure rats or the brain parenchyma of non-seizure rats helped us rule out the possibility that free EB leakage into the brain parenchyma through the intact BBB was responsible for the red fluorescent signal, we designed a FITC-albumin study to further rule out this as a possibility. In this study albumin labeled with fluorescent dye FITC was injected after mannitol injection. As shown in Figure 2A and 4, in seizure rats, there was abundant green fluorescence around the vessels in mannitol injection hemisphere. After superimposing the EB and FITC images as shown (Figure 6), we found a very good correlation between EB and FITC fluorescence in the hemisphere of seizure rats. In addition, we also quantified the intensity of the fluorescence signal in three brain regions: frontal cortex, temporal cortex and thalamus in seizure and non-seizure rats. We compared the ratio between the fluorescent signal intensity of outside and inside of the vessels in these 3 regions. There was a statistically significant difference between inside fluorescent density and outside fluorescence in all 3 regions including frontal cortex, temporal cortex and thalamus on the BBBD hemisphere in seizure rats. We also detected a statistically significant difference between inside and outside fluorescent signal density in the thalamus and temporal cortex on the non-BBBD hemisphere in seizure rats. There was however no statistically significant difference between inside and outside fluorescent signal density in frontal cortex on the non-BBBD hemisphere of seizure rats.

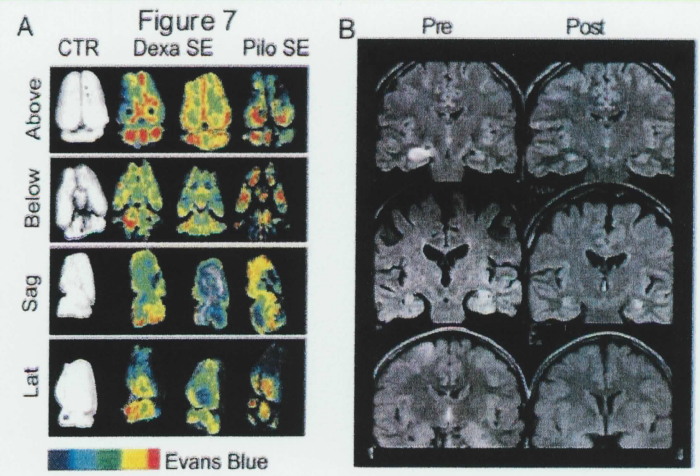

Fig 7A: EB leakage in the whole brain. The images obtained were then manipulated to quantify in a realistic dimension the extent of BBBD.

Fig 7B: FLAIR MRI scans taken from an epileptic patient before and after treatment with steroids. Note that the hyperintense signal was decreased in a manner comparable to the effect of Dexamethasone on rat brain.

Previous studies have demonstrated that after BBBD, blood constituents such as albumin and potassium leaked out to the brain parenchyma were responsible for the development of seizures. Recent data by Fabene et al (2008) demonstrated that inhibition of leukocyte-vascular interaction and depletion of neutrophil decreased both acute seizures and chronic seizures. Although we speculated that the next logical step after leukocyte adhesion is extravasation, no study has ever demonstrated whether leukocytes were able to extravasate into the brain parenchyma after BBBD. If leukocytes are extravasated after BBBD, another question we wanted to ask is whether they could trigger the development of seizures. In order to answer these questions, we labeled rat WBCs with GFP (GFP-
WBCs), after lentiviral vector infection. GFP-WBCs were then injected into the internal carotid artery after mannitol injection. Our data demonstrated that in seizure rats GFP-WBCs could be seen in the perivascular space in both brain hemispheres in the seizure rats (Figure 5A).

Methods

Rats and pigs were killed by anesthetic overdose per veterinarian's supervision. The brains were immersion fixed for 48 h in 4% paraformaldehyde pH 7.4, and then cryoprotected overnight in a 20% sucrose solution, frozen in isopentane, and stored at -80^{O}C. Coronal sections were cut on a cryostat. Sections were then washed with saline and mounted in a Mowiol-based mounting medium containing 0.1% para-phenylenediamine hydrochloride and DAPI staining for nuclei. Sections were examined using a Leica confocal laser-scanning microscope.

The final and most exciting new results obtained with the funding provided for our studies is the first demonstration of:

1. **Antiepileptic action of a commonly prescribed anti-inflammatory agent (dexamethasone) in rats.**
2. **The mechanism of action of dexamethasone on pilocarpine-induced seizure**
3. **Preliminary results from human studies showing a remarkable efficacy of steroids in the treatment of multiple drug resistance in epileptic children.**

The data are described below

Clinical Methods

Inclusion Criteria: *We enrolled pediatric patients who were treated at the Besta Institute in Milan with steroidal therapy or ACTH to alleviate drug-resistant epileptic seizures, including status epilepticus. Patients were included in the study only when therapy was administered on an in-patient basis; in 12 patients, therapy was initiated because of >50% increase in seizure frequency in 7 patients because of development of epileptic encephalopathy, and in 18 patients because of status epilepticus, and in 8 patients due to partial epilepsy. These patients had a seizure onset between one day post-natal and 13 years of age (2 years and 8 months on average). Treatment was initiated at 6 months to 21 years of age. Breakdown of the pathologies is shown in Figure 8B.* ***Exclusion criteria*** *were: a) demonstrated epileptic pathology due to West Syndrome, Lennox-Gastaut, Landau-Kleffner Syndrome, or Rasmussen's encephalitis and b) development of the effects. Patients were evaluated by a team of neurologists, physiologists, and received specialized medical care and routine exams. Steroid treatment had a significant effect on a variety of parameters; those directly related to epilepsy are described in the result section. In addition, we monitored cognitive, behavioral, and motor function; in all experiments quality of life was assessed. Steroidal treatment had on average a significant (>50%) on cognitive function and (pL0.05; 750%) on behavior. Motor function was improved on average by 30% while quality of life improved by >65% in all patients (pL0.05). Steroidal treatment was, when successful, repeated when necessary. For this reason, the number of treatments reported in the results is greater than the number of patients enrolled in the study. When the first treatment with steroids was deemed to be non-successful (no improvement at all in seizure frequency or EEG evaluation) the treatment was not repeated. The most common side effect of the treatment was increased body weight followed by anxiety and insomnia. Other effects were most commonly marginal and did not require cessation of therapy. However, when treatment induced changes in coagulation, altered electrolytes, or glycemia, the treatment was suspended. This occurred in 5% of the patients; the data collected from these patients are not reported in the results.*

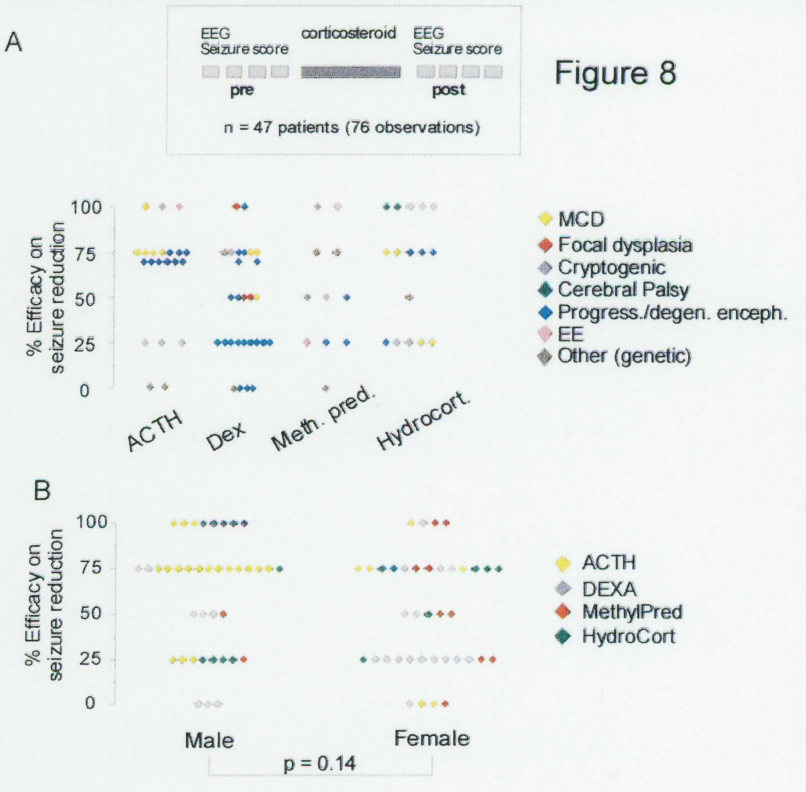

Figure 8

Fig 8A: Timeline between administrations of corticosteroids and patient evaluation. Note the efficacy of corticosteroids against seizures. Fig 8B: Note the lack of effect of gender on outcomes.

We investigated the efficacy of anti-inflammatory corticosteroid or ACTH treatment in 47 patients affected by drug-resistant epileptic seizures. These patients were admitted in the hospital and enrolled in the study after fulfilling the admission criteria listed in the Methods section. The drugs used, as well as the epileptic pathology of the subjects enrolled in the study are shown in

Figure 8B. We found no correlation between the efficacy and etiology of seizures and seizure reduction after treatment with ACTH corticosteroids. Overall, the response was variable ranging from complete reduction of seizures in 12 subjects, to no benefit from the treatment at all in 7 patients. We found no correlation between seizure history and response to treatment.

The effect of corticosteroids on circulating white blood cells (WBC) consists of a reduction of expression of adhesion molecules resulting in mobilization of leukocytes from the bone marrow and a decreased propensity for WBC extravasation. In our study, both effects were evident in subjects who responded to the treatment. Subjects were divided in two groups: "responders" where the seizure reduction was >50% and "non-respondents". A statistically significant difference was found in the number of WBC, which increased in the respondent population; these effects were paralleled by a statistically significant increase in lymphocytes. These data suggests that the lack of effect on seizures was due to an apparent "resistance" to corticosteroids; this phenomenon is referred to as steroid resistance.

In conclusion, we have developed a set of useful techniques to study epileptogenesis in an animal model. We were also able to extend some of our findings to clinical practice to show an unexpected effect of steroids on patients affected by multiple drug resistant seizures.

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Study 2: ELECTRICAL STIMULATION TO TREAT BRAIN TUMORS

Introduction: Each year there are over 45,000 new cases of brain tumors diagnosed in the United States (Central Brain Tumor Registry of the United States, Primary Brain Tumors in the United States). This accounts for approximately 1.4 percent of all cancers, 2.4 percent of all cancer deaths, and 20–25 percent of pediatric cancers. Ultimately, it is estimated that there are 13,000 deaths per year in the United States alone as a result of brain tumors.

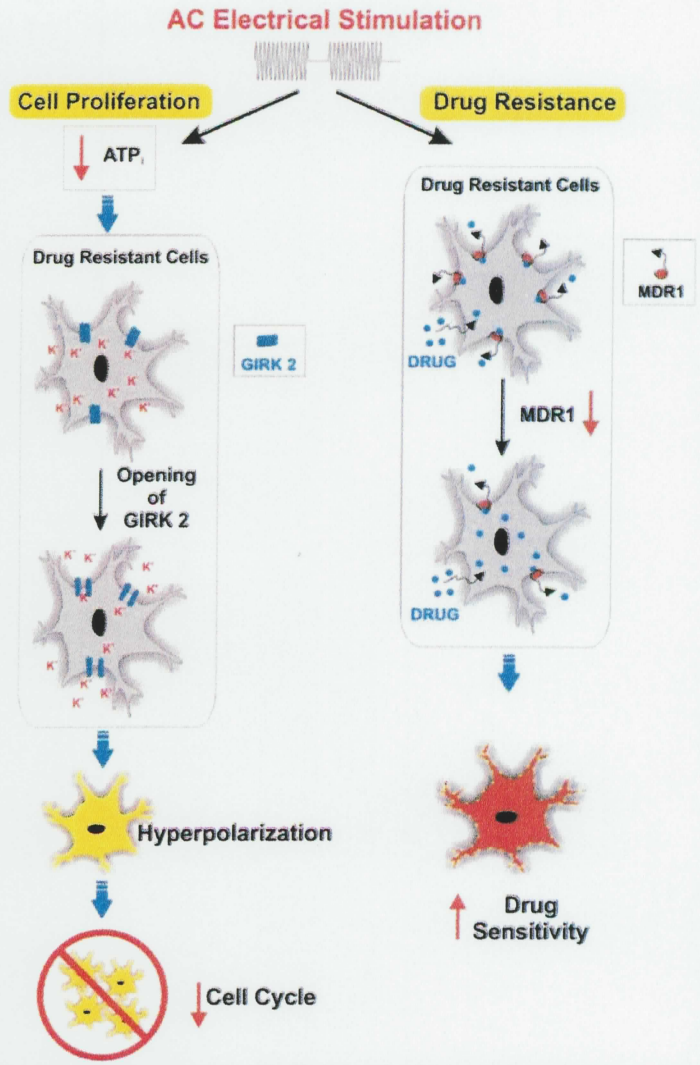

Figure 1: Summary of effects of AC stimulation on tumor cell proliferation and drug uptake *in vitro*.

Despite of improved pharmacotherapy protocols, modern imaging and advanced surgical techniques, the prognosis for a variety of malignant cancers is still bleak. It is thus not surprising that a number of approaches have been deployed to arrest cell cycle in neoplastic cells while sparing surrounding and presumably normal cells.

Our previous work has shown that *in vitro* proliferation of different tumor cell lines was significantly reduced by controlled low frequency, very low intensity alternating current (AC) electrical stimulation (ES; 7.5 μA, 50 Hz AC pulses) and that the efficacy of this treatment required a permissive role for GIRK2 (or $K_{IR}3.2$) potassium channels (1). Furthermore AC stimulation synergistically increased the cellular uptake of the chemotherapic agent doxorubicin in spite of robust MDR1 expression (2) (see **Figure 1**). Taken together, these findings suggested a potential application of low intensity AC in the treatment of tumor growth by synergistically reducing neoplastic cell division and tumor drug resistance.

This is the first **antitumor approach** that utilizes specifically modulated alternating currents to bypass the drug resistance of neoplastic cells. If the technology is proved safe and effective, it may provide a unique technological advancement with high impact in biomedical research and the possibility for wide therapeutic and clinical applications. Therefore, building on these encouraging results we wanted to test and validate this innovative approach *in vivo*.

The study was originally divided in two parallel phases. The first one was aimed at evaluating the feasibility of the stimulation protocol in vivo in Sprague-Dawley rats and the absence of neurological side effects such as electrographic seizures, spontaneous seizures or seizure-like events leading to freezing or falling; twitching of cranial or other muscles, behavioral changes.

The second phase aimed at evaluating the efficacy of AC stimulation. Differently from phase one, Sprague-Dawley rats received an intraparenchymal injection of rat glioma C6 cells.

Specific Aim 1: To test the safety of AC ES *in vivo*

Methods: 6 Male Sprague-Dawley rats (3 controls and 3 tests) weighing between 225 and 250 g were used so far to assess the safety of AC stimulation in vivo. For the experiment rats were anesthetized with isofluorane and maintained under deep anesthesia for the duration of the surgery. The hair along the site of incision at the convergence of the head midline and intercanthal line were shaved and the skin underneath cleaned with a disinfecting iodine and alcohol tampon. A skin incision of about 2 cm in length was then made. The cranial bone was exposed and 4 skull holes were stereotactically drilled (using the Kopf stereotactic frame and the stereotactic atlas of the rat brain) to accommodate the *electroencephalogram* (EEG) recording headmount and the stimulating electrodes. The drilled holes had a depth of 1 mm to 1.5 mm, and a diameter thread 2.15 mm, to avoid penetrating the dura. This is a well-established procedure (3-6). Specifically, four stainless steel screws (MX-0090-2, Small Parts Inc., Miami, Florida) were placed bilaterally on the dura mater of the fronto-parietal cortex. A prefabricated Pinnacle pre-amplifier was connected to the screws. The system has three bio-potential channels - 2 EEG and 1 EMG. Prefabricated head implants (Pinnacle Inc., USA) ensured accurate electrode positioning and reliable, robust contacts. Cable artifacts were eliminated by pre-amplification of the EEG and EMG waveforms at the animal's head. The model used (Pinnacle 8206) does not require any additional acquisition cards or amplifiers.

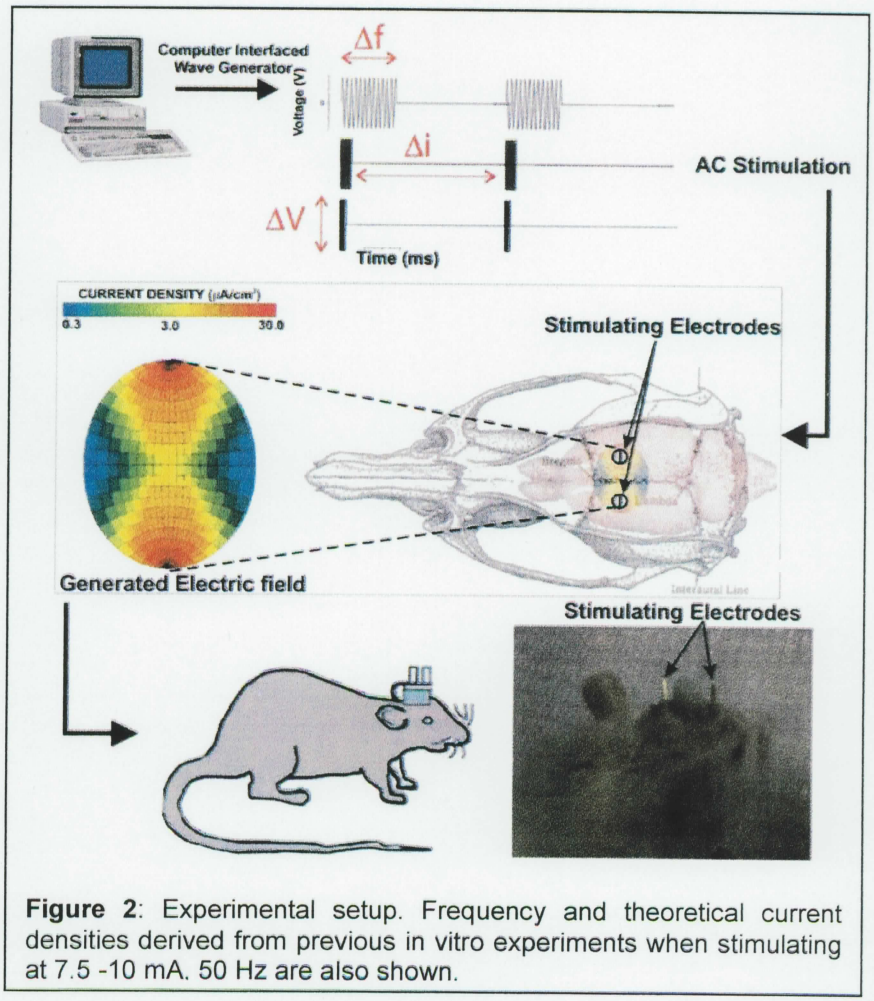

Figure 2: Experimental setup. Frequency and theoretical current densities derived from previous in vitro experiments when stimulating at 7.5 -10 mA. 50 Hz are also shown.

A pair of stimulating electrodes was positioned bilaterally around the back screws of the headmount in contact with the dura and then connected to a grounded computer-controlled waveform generator capable of producing sine wave outputs and to select frequency, intensity, interstimulus interval and length of the stimulation period (see **Figure 2**).

Rats were injected intramuscularly with 0.1 mg/kg buprenorphine right after the surgery and 8-12 hours later to alleviate any pain. Each rat was kept unrestrained (for 2-3 days) in a separate cage under 12-hours dark-light cycles with free access to food and water to recover from surgery before initiating the stimulation protocols. Parallel unstimulated animals similarly implanted with EEG headmount and electrodes were used as controls. Test animals were then exposed to Low frequency (50Hz,) very low intensity (7.5 – 15 μA) electrical stimulation cyclically applied in short burst (1 second every 9) for a period of 5 days. EEG data were sampled at a rate of 200 Hz. All data were transferred via a USB connection to a parallel PC.

Origin® Microcal 8.0 (OriginLab, Northampton, MA) software was used in conjunction to the acquisition system for data analysis. Spike detection, was performed using pClamp 9.2. A customized software routine running on the Diadem (National Instruments) platform was used to assess whether AC stimulation caused EEG changes by time-joint frequency analysis. **Results:** During the experiment, the EEG signaling was acquired and analyzed by pClamp 9.2. **Figure 3 A** shows 1.2 second sample of EEG recording during AC stimulation in comparison to that of an unstimulated control. Our observations show that AC stimulation does not cause spontaneous seizures or seizure like events including electrographic seizures (see **Figure 3B**). No major neurological side effects or behavioural alteration including freezing or falling were observed during the experiment in test animals. Note that AC stimulation was contacted for 5 consecutive days. Even though AC does not cause seizure like disorders it affect EEG reading when compared to unstimulated controls (see **Figure 4** top panel). However, as demonstrated by joint time frequency analysis, these alterations are solely linked to AC stimulation and disappear when the stimulation is interrupted (see **Figure 4** lower panel).

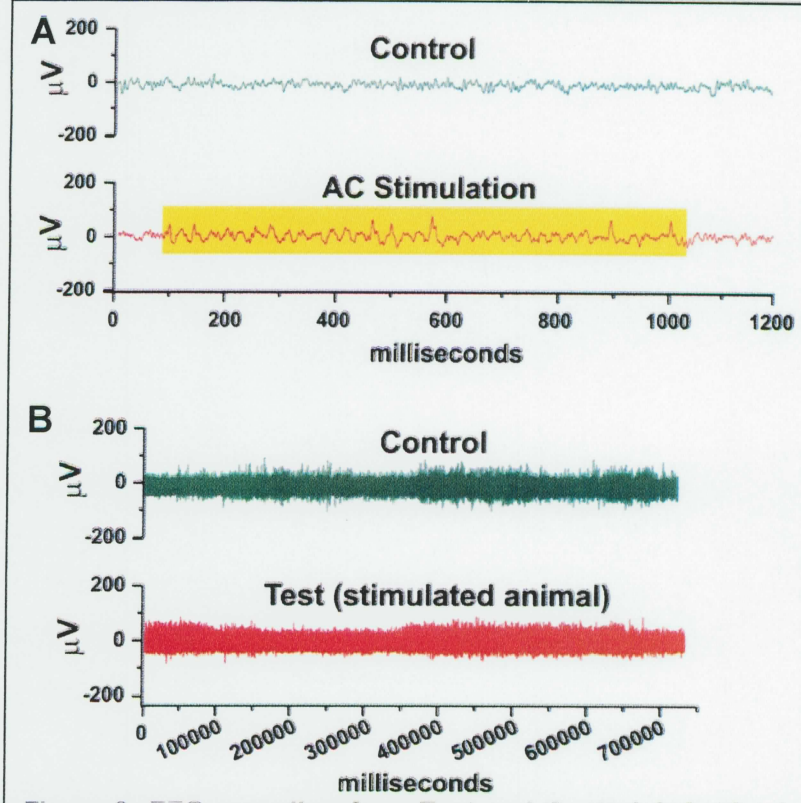

Figure 3: EEG recording from Test and Control Animals. A sample of EEG recording during AC stimulation and unstimulated control is shown in panel **A.** Note that AC stimulation does not cause electrographic seizures (panel **B**).

In summary, our findings suggest that low frequency; low intensity AC stimulation is well tolerated and does not cause significant neurological alterations. Additional study however, are necessary to assess whether our finding stand true for much longer period of exposure to AC protocol (e.g., 4-8 weeks).

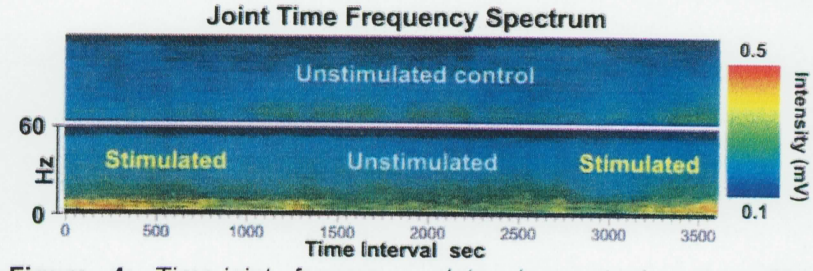

Figure 4: Time-joint frequency plots demonstrating that EEG alterations caused by AC stimulation are reversible.

Experimental Antiproliferative effect of ES

The second phase of this project was designed to evaluate the efficacy of AC stimulation *in vivo* to reduce neoplastic cell division. For this experiment we used 6 Male Sprague-Dawley rats (3 controls and 3 tests) which received an intraparenchymal injection of C6 rat glioma cells tagged with green florescent protein (GFP) to facilitate the identification of the tumor cells in the brain tissue. The rat brain tumor inoculation method has been shown to be appropriate for the study of antitumor efficacy of various agents under physiologically relevant conditions. Rat brain tumor is a good model for such studies in these regards.

Methods: Male Sprague-Dawley rats (4 controls and 4 tests) weighing between 225 and 250 g were used so far for stereotaxic tumor cell injection and concomitant AC electrode and EEG head mounting. The experimental procedure is similar to that described for phase 1 with the exception that prior electrode and head mount implantation for EEG recording; animals were stereotactically injected with rat glioma C6 in right brain. The hole through the skull was drilled 0.3 mm behind the point of intersection of the coronal and sagittal suture, and 3 mm to the right of the midline. Before implantation, C6 cells cultured to confluence into 25 cm^2 flasks were briefly trypsinized, harvested by centrifugation and suspended in double RPMI-1640 containing 10 g/L agarose. Cells were counted in a Burke's chamber to adjust the concentration of the cell suspension to 1×10^5 C6 cell /µL. $5x10^5$ tumor cells were stereotactically lowered into the hole in the scull and delivered into the brain at a depth of 3.2 mm below the dura (bregma -0.3, Lat 3, DV 3.2) distant from sensory-motor cortex (see **Figure 5**).

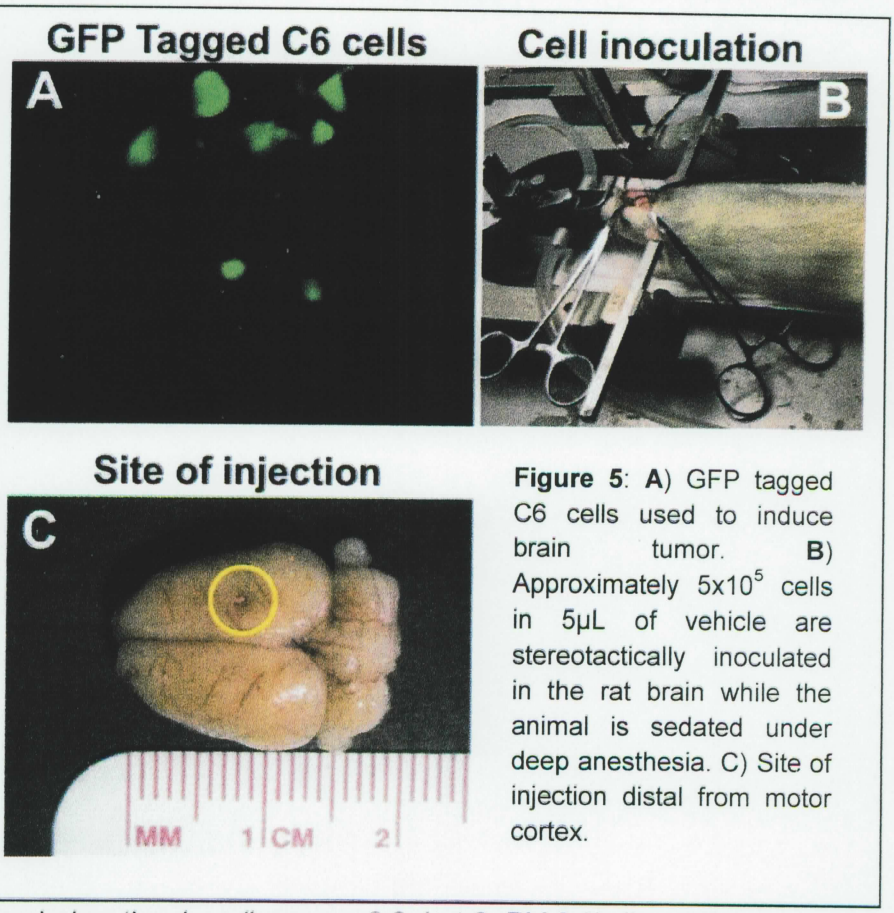

Figure 5: **A**) GFP tagged C6 cells used to induce brain tumor. **B**) Approximately $5x10^5$ cells in 5µL of vehicle are stereotactically inoculated in the rat brain while the animal is sedated under deep anesthesia. C) Site of injection distal from motor cortex.

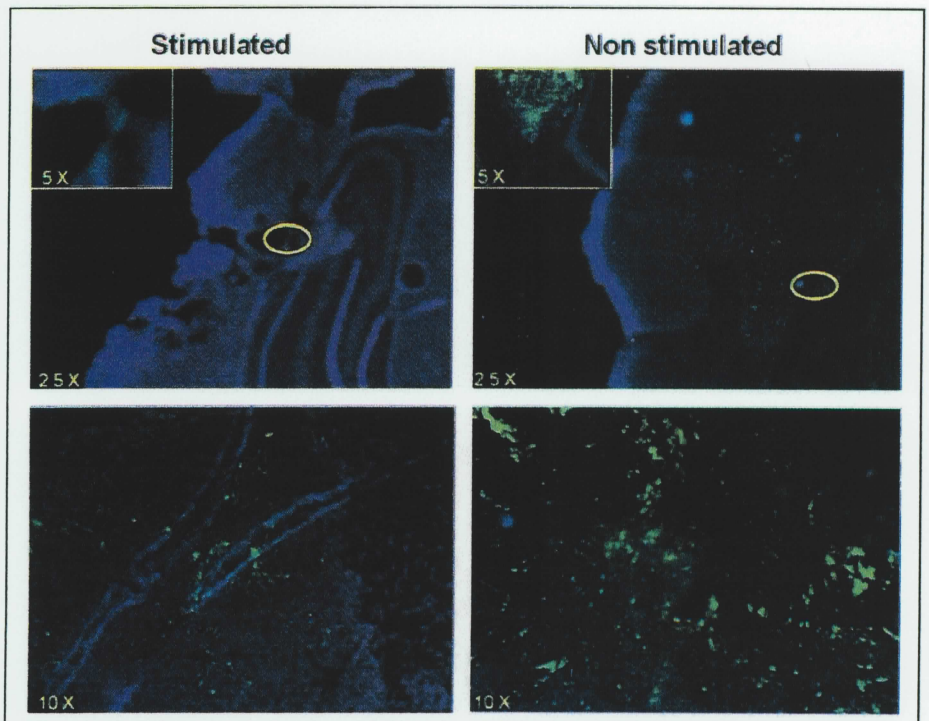

Figure 6: **Coronal section (30µm in thickness) of rat brains harvested from stimulated and un-stimulated rats implanted with GFP tagged C6 rat glioma cells.**

Rats were then provided with food and water ad libitum and left unrestrained for 2-3 days to recover from surgery before proceeding with the stimulation protocols. Parallel tumor bearing animals left unstimulated were used as control to assess the efficacy of AC protocol to decrease neoplastic cell proliferation.

Test animals were then exposed to continuous cycles (1 second every 10) of electrical stimulation for 5 days. At the end of the 5th test and control animals were sacrificed by decapitation under deep anesthesia, the brains were removed and immersed in 10% formalin and cryopreserved in 30% sucrose solution. Coronal sections (≈ 30 µM thickness) of the regions proximal to the injection site of the tumor cells were stained with DAPI (4',6-diamidino-2-phenylindole) and reviewed by fluorescent light

microscopy to assess the progression/regression in tumor growth. Parallel unstimulated animals underwent the same procedure. Our preliminary results indicate that AC stimulation effectively reduced C6 proliferation thus negatively affecting the progression of tumor growth (see **Figure 6**).

To assess whether AC stimulation improves chemotherapic efficacy, in parallel experiments stimulated and non-stimulated animals that developed brain tumors were exposed to known concentrations of the chemotherapic agent doxorubicin (DOX). Specifically Male Sprague-Dawley rats (controls and tests) weighing approximately 300 g underwent stereotaxic tumor cell injection and concomitant AC electrode and EEG head mounting as previously described. Five days after tumor implantation, animals started receiving a daily injection of DOX (2mg/kg) via tail vein. In addition to DOX, test animals started receiving AC stimulation. After 5 days of treatment both test and control animals were sacrificed and the brains removed. Coronal sections (≈ 30 μM thickness) of the regions proximal to the tumor injection site were stained with DAPI and reviewed by fluorescent light microscopy to assess whether AC stimulation reduces drug resistance and facilitate the uptake of DOX. Our results show that intracellular incorporation of DOX in unstimulated animals is risible (see **Figure 7**). Note how DOX (red) accumulate in the space surrounding the tumor (green) without entering the cells. By contrast, in AC stimulated animals (see **Figure 8**) tumor cell penetration of doxorubicin is significantly enhanced (yellow-orange). In summary, our preliminary data in vivo seems to

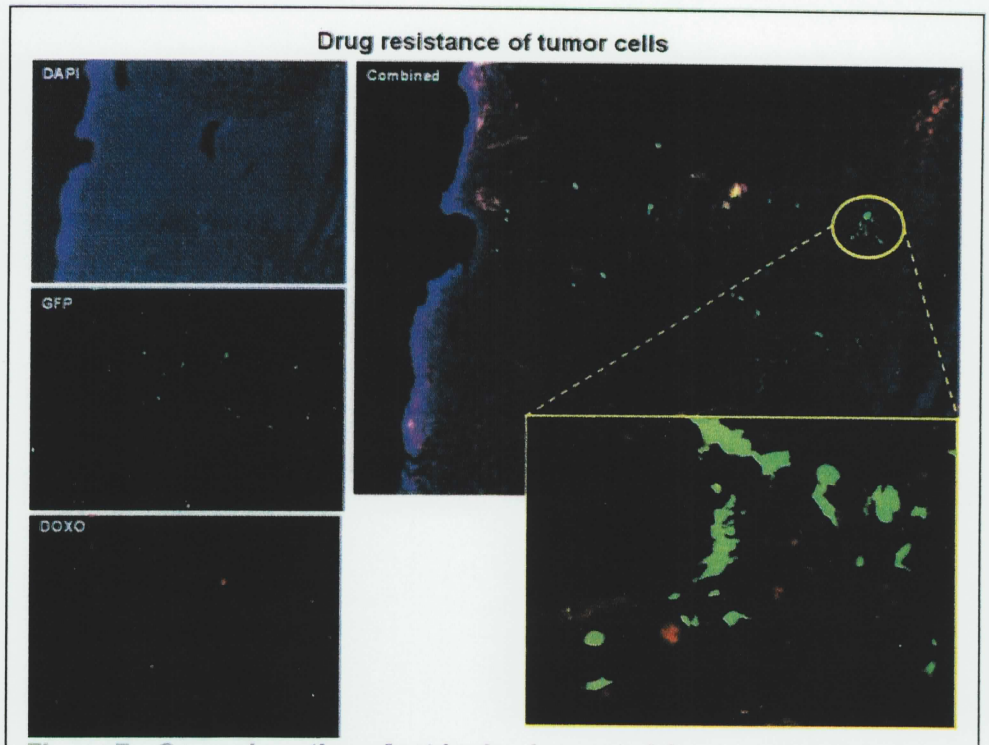

Figure 7: **Coronal section of rat brains harvested from un-stimulated rats implanted with GFP tagged C6 rat glioma cells and treated with DOX.** Note the absence of intracellular co-localization of DOX (red) with GFP (green) which would produce a yellow-orange color. This demonstrates the absence of drug

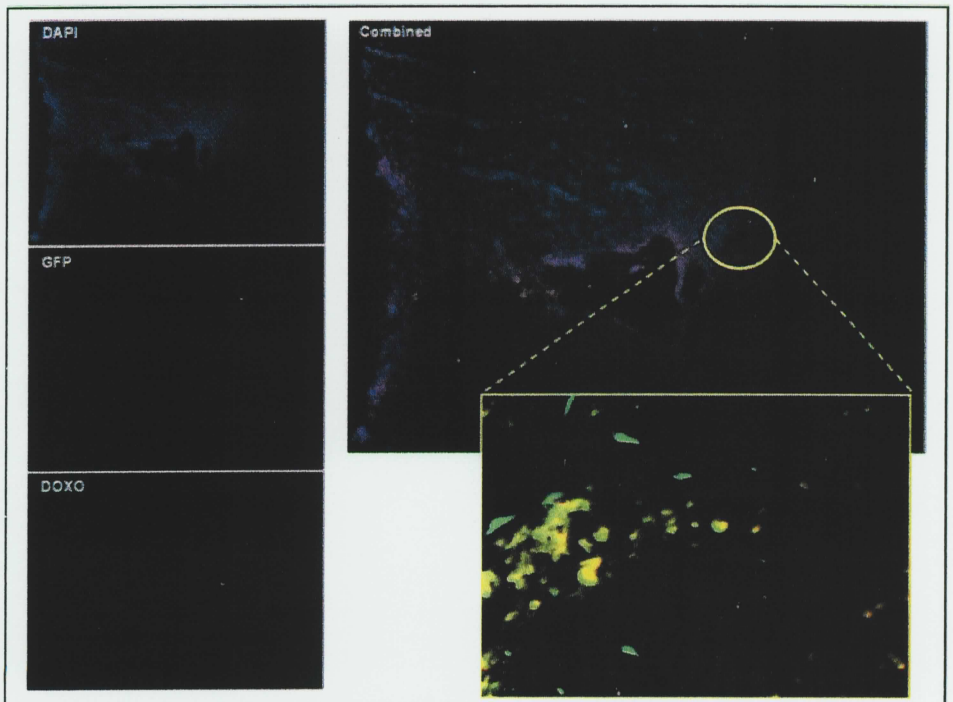

Figure 8: **Coronal section of rat brains harvested from stimulated rats implanted with GFP tagged C6 rat glioma cells and treated with DOX.** Note that the yellow-orange color is caused by intracellular co-localization of DOX (red) with GFP (green) inside the C6. This indicates that AC stimulation enables doxorubicin penetration into the tumor cells.

confirm our previous finding where controlled low frequency, low intensity AC stimulation can be used to synergistically reduce neoplastic cell division and enhance chemotherapy efficacy by facilitating its penetration into the tumor targets.

In view of the widespread use of DBS stimulators and stimulating electrodes for the treatment of a variety of other diseases, coupling electrical stimulation to current chemotherapy protocols hold the promise to improve the efficacy of our therapeutic approach to neoplasms. However, additional experiments are needed to assess the long term efficacy of AC protocol and whether this novel approach significantly increases the survival rate.

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