CREATION, VALIDATION, AND ANALYSIS OF A NOVEL MODEL FOR THE STUDY OF NONADHERENCE IN NEWLY DIAGNOSED EPILEPTIC RATS

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

One in 26 people in America will develop epilepsy at some point in their life. Approximately one-third of patients with epilepsy do not have full control of their seizures on their current antiseizure drug (ASD) regimen. In the past 20 years, 16 new ASDs have been made available to the patient with epilepsy. However, the percentage of pharmacoresistant patients has remained relatively constant. Thus, it is necessary to explore alternative avenues to explain the lack of seizure control in these patients. It is unknown how the practice of nonadherence contributes to the percentage of refractory patients. An animal model of nonadherence would permit the study of nonadherence and its consequences on seizure control. To model nonadherence, a computer-automated system was created to dose animals with antiseizure drugs (ASDs) using a drug-in-food protocol. Two studies were conducted and serve as a first-pass characterization. In the first study, newly-diagnosed epileptic rats were administered carbamazepine (CBZ) at varying levels of adherence. Two groups, one adherent (100%), and one nonadherent (50%), were compared to placebo control (0%). The 50% group displayed similar seizure frequency and severity to the placebo controls, while the 100% adherent group were observed to have fewer seizures overall. This suggesting that taking medication 50% of the time had no measurable effect on seizure control. In the second study, a group of newly diagnosed epileptic rats was subjected to a two-week on (100%), two-week off (0%) and two-week on CBZ (100%) paradigm. Despite numerous seizures after discontinuation of CBZ

treatment, animals regained the same level of seizure control when CBZ treatment was reinstituted. Finally, nonadherence can lead to status epilepticus (SE), a severe seizure lasting several minutes. In an effort to aid drug discovery, a new and simple method which results in an unbiased method to assess the duration and severity of the electrographic component of SE was created. This method has been used to quantify the response of electrographic SE to novel investigational compounds. Together, this dissertation aims to aid in the treatment of epilepsy by providing etiologically relevant models of nonadherence and analysis of electrographic SE.

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CHAPTER 1

INTRODUCTION

One in ten people will experience a seizure resulting from a brief period of abnormal synchronized brain activity at some point in their life (Hauser, Annegers et al. 1996). Three to 4% of Americans will, experience multiple unprovoked seizures during their life, a condition known as epilepsy (Hauser, Annegers et al. 1996; Hesdorffer, Logroscino et al. 2011; England, Liverman et al. 2012). Epilepsy is a worldwide issue, affecting approximately 65 million people (Leonardi and Ustun 2002; England, Liverman et al. 2012). Epilepsy often leads to debilitating effects on quality of life, including the loss of independence, fear of the next unprovoked seizure, embarrassment due to the stigma of epilepsy, and concern over being able to maintain gainful employment (Fisher, Vickrey et al. 2000a). Patients report that their feelings of lost independence come from the lack of their ability to drive (Fisher, Vickrey et al. 2000a). The stigma of having epilepsy is both "enacted" stigma, in the form of discrimination, as well as "felt" stigma, i.e., the feeling of shame that comes from the incorporation of epilepsy into personal identity (Scambler and Hopkins 1986). These and other stigmas result in increased loneliness, a decreased likelihood of marriage, and increased levels of stress and depression (Morrell 2002). Patients who were otherwise capable of holding a job were more likely to report being unemployed or underemployed (Fisher, Vickrey et al. 2000b). Patients with epilepsy are

also economically burdened with a significant cost-of-illness. The majority of costs are associated with hospital visits and the purchase of pharmaceutical interventions (Strzelczyk, Reese et al. 2008). Given the summation of the negative factors that seizures have on quality of life for patients with epilepsy, finding an appropriate and effective treatment for the prevention of seizures is of paramount importance.

History of Epilepsy and Pharmaceutical Interventions

The word epilepsy comes from the Greek word *epilambanem,* which means "to lay hold of" (2005). Throughout history, epilepsy has been given other names, such as the "Sacred disease" or the "falling sickness" (Shakespeare, 1899). The first recorded discussion of epilepsy and its treatment was the book "On the Sacred Disease," attributed the Greek physician Hippocrates, dating back to 400 B.C. (Temkin 1971). At the time, epilepsy was widely considered to be of magical origin or demonic possession, hence the name "the Sacred Disease." Hippocrates disputed this notion, and stated that epilepsy was instead of physical origins. He was also the first to state that epilepsy was treatable by diet and drugs, as opposed to exorcism, spirit wards, or other ethereal-based methods. Unfortunately, the concept of epilepsy being regarded as having supernatural origins would persist well into the 15th century, and pharmaceutical interventions for epilepsy would not begin until the 19th century.

The majority of treatments for epilepsy rely on the use of antiseizure drugs (ASDs). The history of ASDs starts with potassium bromide, which is widely considered to be the first effective ASD to be used in regular practice, introduced by Sir Charles Locock in 1857 (Pearce 2002). Potassium bromide is a salt, which has side effects of sedation and impotence (Ryan and Baumann 1999). It is, however, still a first-line treatment today for

seizures in dogs (Dowling 1994). Potassium bromide was the main therapeutic for epilepsy until the discovery of phenobarbital, a barbiturate, by Alfred Hauptmann in 1912. These two ASDs, as well as other nonbarbiturates including phenytoin, trimethadione and ethosuximide comprise the first generation of ASDs (Shorvon 2009a).

The second generation of anticonvulsants include valproic acid, carbamazepine, and a variety of benzodiazepines. These drugs were considered to have higher tolerability and efficacy over the first generation ASDs; however, they too were found to possess substantial adverse effects and a high propensity for drug-drug interactions. Moreover, tolerance to their antiseizure effect develops rapidly and this limits their long-term utility for the treatment of epilepsy. The most successful second generation anticonvulsant is still considered to be carbamazepine (CBZ). CBZ, discovered in 1953 by Walter Schindler (Schindler and Häfliger 1954), was made available in the United Kingdom and Switzerland for the treatment of epilepsy in 1963 (Dalby 1971). Carbamazepine was the most widely-prescribed ASD until the introduction of levetiracetam (LEV), a third generation ASD.

In 1975, the Anticonvulsant Screening Program was founded by the National Institute of Neural Disorders and Stroke, and established at the University of Utah (Kupferberg 1989). This program was put in place to test the efficacy of novel compounds for the treatment of epilepsy in known acute seizure models. Since that time, there have been sixteen new "third-generation" compounds made available for the treatment of epilepsy (Loscher and Schmidt 2011). The history of ASD development is illustrated in Figure 1.1.

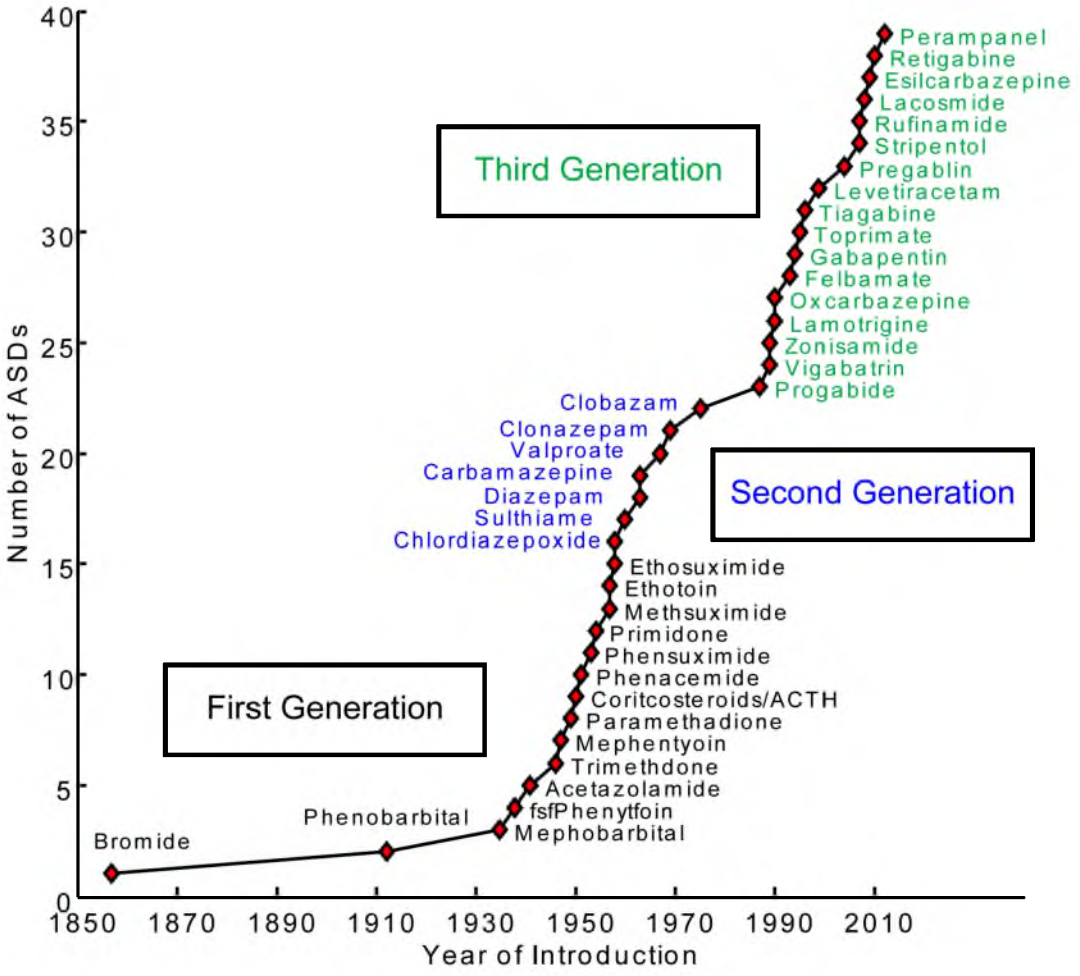

Figure 1.1 List of mainline ASDs from 1853 to 2014, based on their entry into the American market. Figure is adapted and updated from (Loscher and Schmidt 2011), with data from (Shorvon 2009a; Shorvon 2009b).

The Current Treatment of Epilepsy

There are currently 39 mainline pharmaceutical interventions available for the clinical management of epilepsy (Shorvon 2009b). These drugs are prescribed to a patient, following a seizure, when the risk of subsequent seizures is considered high. As previously mentioned, one in ten people will have a seizure at some point in their lives, and a single unprovoked seizure is generally not considered sufficient for the diagnosis of epilepsy. Following an initial unprovoked seizure event, the general risk of a subsequent seizure is 40-52% (Berg and Shinnar 1991). After two unprovoked seizures greater than 24 hours apart, the risk of another seizure increases to 73-87% (Hauser, Rich et al. 1998). Thus, the typical practice in clinical care is to begin treatment with an ASD following a second unprovoked seizure (Fisher, Acevedo et al. 2014). When an ASD fails to prevent subsequent seizures, a patient may be switched to another ASD, or additional ASDs may be added to the patient's treatment plan. Approximately three out of every four refractory patients are on a polytherapy regimen for the treatment of their epilepsy (Berg, Vickrey et al. 2003). The principle mechanism of action of ASDs falls into one of four categories: modulating voltage-gated ion channels, increasing gamma-aminobutyric acid (GABA)-ergic neurotransmission, decreasing glutamatergic neurotransmission, or other putative targets (Kwan, Sills et al. 2001). However, many ASDs have other therapeutic targets in addition to their main mechanism of action. For example, CBZ's main mechanism of action is presumed to be suppression of voltage-gated sodium channel activation, however, it also potentiates GABA-mediated inhibition, and acts as an N-Methyl-D-aspartate (NMDA) antagonist (Shorvon 2004).

When ASDs fail to control seizures, other options may be available to the patient

with epilepsy, such as the ketogenic diet, resective surgery, and implantable devices. The ketogenic diet consists of high fat, moderate protein, and minimal carbohydrates. This reduces the amount of glucose available in the body, and simulates the effect of continuous fasting by inducing a state of ketosis (Peterman 1925). The ketogenic diet has been shown to reduce the occurrence of seizures (Selter, Turner et al. 2014). Resective surgery consists of localizing the seizure focus, i.e., the location from which seizures are generated in the brain, and surgically removing this tissue. Patients are considered candidates for surgery if their current medication regimen is ineffective at controlling their seizures, their seizures are localizable to limited foci, and the area of resection is considered nonessential cortex. Approximately 74% of patients evaluated for surgery are considered possible candidates (Engel 1998). Resective surgery results in full seizure control for a minimum of one-year in approximately one-half to two-thirds of patients, depending on the age of the patient and etiology of their epilepsy (Spencer and Huh 2008). A third nonpharmaceutical option for patients is an implantable device, such as the vagus nerve stimulator or the responsive neural stimulator. The vagus nerve stimulator is a pacemaker-like device which sends pulses into the vagus nerve to prevent seizures. After one year of implantation, the typical patient experiences approximately 45% fewer seizures (Schachter 2002). The responsive neural stimulator (RNS) is a recently approved device by Neuropace Inc. that works to interrupt seizures by use of electrical stimulation proximal to the seizure focus or foci. It works as a closed loop system, in that it detects seizures, and delivers stimulation as required (Sun and Morrell 2014). Results from randomized clinical trials demonstrated that RNS results in a median 53% reduction in seizures, and increases the quality of life for patients (Heck, King-Stephens et al. 2014).

Despite these advances in pharmacotherapy and alternative treatments for epilepsy, approximately 38% of patients with epilepsy do not attain adequate control of their seizures, i.e., they are refractory to their current ASD regimen (Mohanraj and Brodie 2005). This statistic has remained largely unchanged over the past 60 years (Loscher and Schmidt 2011; Loscher, Klitgaard et al. 2013). Thus, it is necessary to continue to find new approaches and new models for testing the efficacy of investigational compounds for the treatment of epilepsy, as well as considering other explanations for the lack of seizure control in the population of patients with epilepsy.

The Problems of Nonadherence

Patient nonadherence to doctor prescribed dosing regimens is a significant problem in modern medicine. It is estimated that the health care costs attributable to nonadherence is $290 billion per year in the United States (McCarthy 1998). Nonadherence is also attributed to over 125,000 avoidable deaths annually (Cutler and Everett 2010). In the clinical quantification of nonadherence, a threshold of 80% consumption or greater is considered adherent, while below that threshold is considered nonadherent (Hughes, Bagust et al. 2001). This threshold is broadly used to categorize patients as adherent/nonadherent in the majority of clinical studies, regardless of the disease category or pharmacokinetics of the drug in question.

Nonadherence is a problem in nearly all disease states. In the clinical care of HIV/AIDs, nonadherence can result in the emergence of treatment-resistant HIV strains (Ho 1996). Despite this potentially life-threatening outcome, approximately 50% to 70% of patients treated with antiretroviral therapy are nonadherent (Chesney 2000). In diabetes, nonadherence can lead to increased complications (Stratton, Adler et al. 2000), and yet up

to 64% of diabetic patients practice imperfect adherence (Cramer 2004). Antibiotic nonadherence leads to the possibility of resurgence of infection for patients. Further, it has negative consequences that affect the continued effectiveness of antibiotics due to the emergence of antibiotic-resistant bacterial strains (Austin, Kristinsson et al. 1999). Despite both personal and public health consequences, approximately 20 to 60% of patients are noncompliant to their antibiotic treatment (Sclar, Tartaglione et al. 1994).

When it comes to the practice of nonadherence, epilepsy is not immune to the problems associated with clinical nonadherence. In the treatment of epilepsy, approximately 26 to 53% of patients taking prescribed ASDs do not meet the clinical threshold for adherence (McAuley, McFadden et al. 2008; Ettinger, Manjunath et al. 2009; Manjunath, Davis et al. 2009; Zeber, Copeland et al. 2010; Modi, Rausch et al. 2011; Faught 2012). Nonadherence has been associated with an increase in the risk of seizures (Stanaway, Lambie et al. 1985; Manjunath, Davis et al. 2009). Clinical observations suggest that near perfect adherence may reduce the number of refractory patients by nearly two-thirds (Modi, Rausch et al. 2014). ASD withdrawal is a leading cause of status epilepticus (SE), a prolonged seizure which is considered a medical emergency requiring immediate care (Trinka, Hofler et al. 2012). Patients with epilepsy have a two to three times higher mortality rate than the normal population (Gaitatzis and Sander 2004), and this increased mortality rate is exacerbated by nonadherence (Faught, Duh et al. 2008). Nonadherent patients also have an increased number of hospital visits, which is estimated to be caused by an increased number of seizures, falls, and accidents (Faught, Duh et al. 2008). An increase in hospital visits results in increased spending on health care for nonadherent patients (Faught, Weiner et al. 2009). Finally, nonadherence can result in an

increase in the comorbidities of epilepsy, including increased anxiety and depression (Dias, Bateman et al. 2010; Ettinger, Good et al. 2014).

Given the risks associated with nonadherence, patient compliance is an important factor to consider in the treatment of epilepsy. However, past clinical observations have been problematic. When relying on self-reported adherence, patients overestimate their adherence rates (Buelow and Smith 2004; Modi, Guilfoyle et al. 2011). Another method to keep track of adherence is the medication-possession ratio, which is a calculation based on the time period to a refill, and the number of pills left at that time (Steiner and Prochazka 1997). This measure is problematic, however, as patients will take multiple doses to make up for a missed dose (Buelow and Smith 2004), or create their own patterns of dosing (McAuley, McFadden et al. 2008). The lack of proper medication record keeping has been addressed with MEMS track caps, which are medicine bottle caps that can record the timestamp of openings (Cramer 1995). This technology has become the "gold standard" in adherence tracking. It has been used to create a correction factor for patient reporting of adherence in epilepsy (Modi, Guilfoyle et al. 2011). The resolution provided by this real-time tracking has allowed for the adherence rates of children with newly-diagnosed epilepsy to be tracked and plotted over the course of six months (Figure 1.2). This work showed that they were able to distinguish five different adherence rates that occurred in newly diagnosed pediatric epilepsy patients during the first six months following diagnosis and treatment with their first prescribed ASD (Modi, Rausch et al. 2011). These data have provided a first look into the trends and behaviors of newly diagnosed pediatric epilepsy patients that practice imperfect adherence. However, there is still the problem of patients reporting seizure events, as patients may keep incomplete seizure journals (Neugebauer

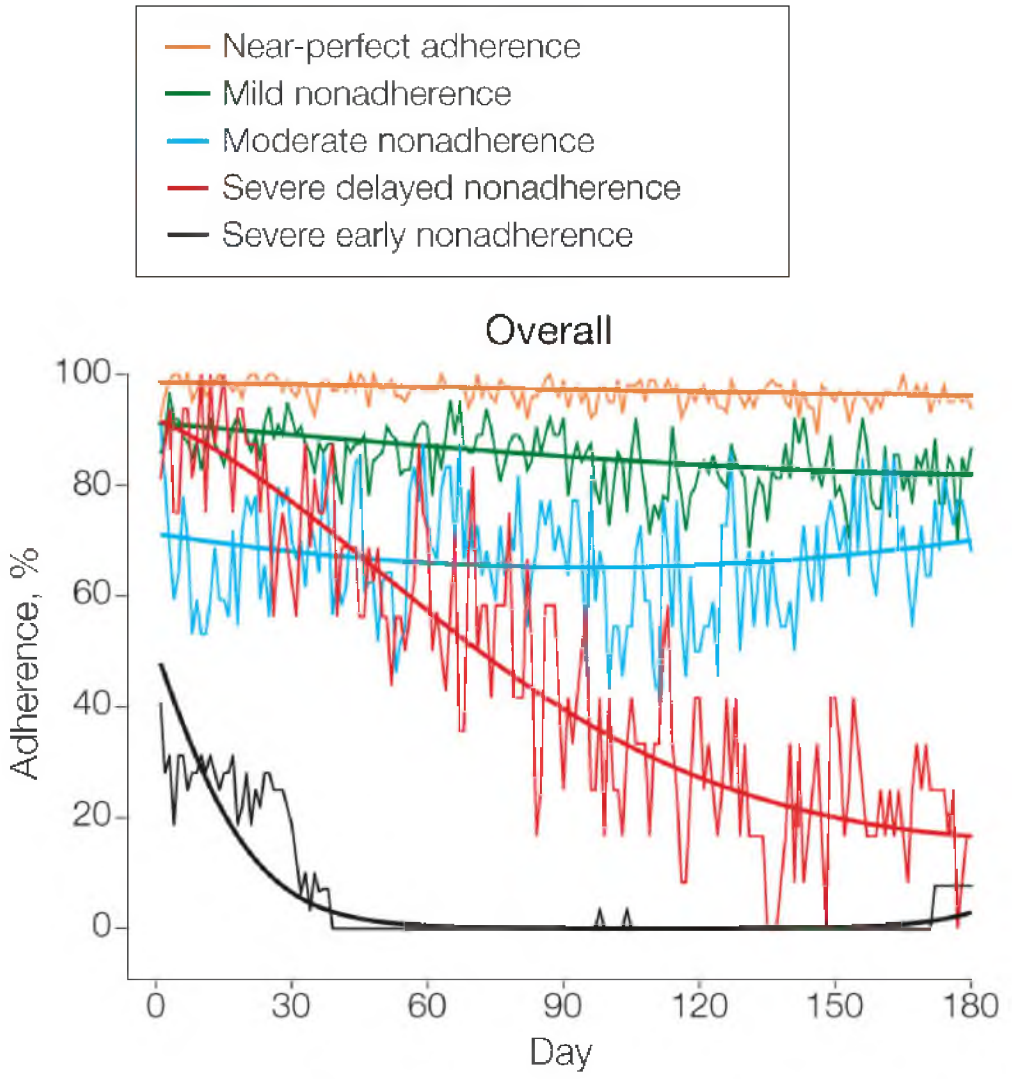

Figure 1.2 Patterns of nonadherence practiced by newly-diagnosed children with epilepsy. Figure taken from (Modi, Rausch et al. 2011)

1989), and are often unaware of nonconvulsive seizure events (Hoppe, Poepel et al. 2007).

It is notable that all literature cited herein that analyzes nonadherence has been from clinical observations. To date, no method for testing the effects of nonadherence in animal models has been proposed or executed. The problems of tracking adherence in the clinical setting, as well as the poor reporting of seizure events, demonstrates the need for a novel experimental model of nonadherence.

Models of Epilepsy

Epilepsy is a complex disease, consisting of both symptomatic events, seizures, and the underlying process that contributes to the development of epilepsy, called epileptogenesis (Walker, White et al. 2002). Both acute models of seizures and chronic models of epilepsy are used to ask pertinent questions about the disease, the process of epileptogenesis, as well as evaluating preclinical efficacy of investigational drugs. The holy grail of epilepsy research is a model in which investigational results obtained from animals can be directly translated to the patient in the clinic, informing pharmaceutical researchers, clinicians, patients, and their caregivers about the most effective methods for the treatment of epilepsy.

The first approach to modeling epilepsy is the use of acute models of seizures. These models use some form of stimulus to induce a seizure in a naïve animal, and are regularly used to test investigational compounds for efficacy in preventing the associated seizure phenotypes. The most commonly employed acute models include the maximal electroshock seizure (MES) test, subcutaneous pentyleneterazol test (scPTZ), and the 6 Hz psychomotor seizure test, as these models are used as a first-pass screen of potential investigational compounds for the treatment of epilepsy (White, Johnson et al. 1995;

Loscher and Schmidt 2011). The MES delivers an electrical shock at 50Hz into the cornea of a rat or mouse and measures if the investigational compound prevents tonic extension immediately following stimulation (Swinyard 1949). The scPentylenetetrazol (PTZ) test involves the subcutaneous administration of the chemoconulsant PTZ to mice and rats, and determines whether or not an investigation compound possesses the ability to block the phenotypic seizure that is induced by scPTZ (Swinyard, Brown et al. 1952). The 6Hz test measures the ability of an investigational drug to block the phenotypic 6 Hz seizure at 100%, 150%, and 200% of the CC97, i.e., the current intensity at which 97% of naïve animals have a seizure. (Toman 1951; Brown, Schiffman et al. 1953; Barton, Klein et al. 2001). The seizure induced in the 6Hz test reflects complex partial seizures in humans, in that they exhibit minimal clonic and automatistic behaviors, i.e., jaw-chomping and whisker twitching. The 6Hz test was added as a screening tool at the University of Utah ADD Program after LEV was found to be effective in this model after failing to be detected by either the MES or scPTZ tests (Loscher and Honack 1993). Previous work has shown that the 6Hz test acts as an acute model of pharmacoresistant epilepsy, in that its activity is localized to limbic brain regions (Barton, Klein et al. 2001). Collectively, these models have contributed to the identification of several new ASDs that possess better tolerability and safety (Bialer, Johannessen et al. 2007). These models are low-cost and have a high-throughput when screening investigational compounds (Loscher and Schmidt 2011). However, a criticism that still exists is that the compounds that are identified through this approach are likely to be "me-too" drugs (Loscher 2011). The use of more chronic models of epilepsy may identify compounds that may be more effective for the refractory patient population.

Two often used chronic models of epilepsy and seizures include kindling, i.e., repeated electrical stimulation of a limbic brain region such as the amygdala or hippocampus, or epilepsy that results from the induction of SE by chemoconvulsants. Kindling is a chronic model of epilepsy, as it causes rewiring of neural circuits to make them hyperexcitable, which can, with long-term repeated stimulations, lead to spontaneous seizures (McNamara 1984). Previous work has demonstrated that treatment with LTG and CBZ could lead to pharmacoresistance in the amygdala kindling model of epilepsy (Srivastava, Alex et al. 2013). Another model of chronic epilepsy uses chemoconvulsants to induce SE. Two such chemoconvulsant models are the pilocarpine model (Curia, Longo et al. 2008) and low-dose kainate (KA) model (Hellier, Patrylo et al. 1998). In these models, the induction of SE leads to a host of neuronal changes due to excitotoxicity. After a variable amount of time, called the latent period, animals begin to display spontaneous seizures (Walker, White et al. 2002). Following the initiation of spontaneous seizures, the animal's epilepsy increases in severity and frequency until the seizure frequency plateaus (Williams, White et al. 2009). These models reflect the condition of human epilepsy; i.e., spontaneous seizures resulting from a brain insult. For example, the low-dose KA model of chronic epilepsy results in the loss of hilar cells in the dentate gyrus (Buckmaster and Dudek 1997); a phenomena that has been linked to the pathology of temporal lobe epilepsy (Kobayashi and Buckmaster 2003). An accurate model of TLE is especially important, as patients with TLE make up the majority of the refractory patient population. The results described in Chapters 3 and 4 were obtained from the use of the low-dose KA and pilocarpine models, respectively.

Long-term efficacy studies designed to mimic treatment of human epilepsy require

not only a model of chronic epilepsy, but also a means by which ASDs can be administered chronically. A method for chronic delivery of drugs to animals is the use of a drug-in-food protocol. A fixed concentration of drug is placed in the food, so that the animals' consumption of food matches the desired dose. Using this method of drug delivery, it has been demonstrated that CBZ is acutely effective in reducing seizures in chronically epileptic animals (Grabenstatter, Clark et al. 2007), and achieves blood levels comparable to those obtained following systemic intraperitoneal (i.p.) injections (Ali, Dua et al. 2012). This previous work has demonstrated that CBZ-in-food is an effective method of delivering drugs in an etiologically relevant animal model of SE-induced acquired epilepsy. By using this drug-in-food approach, we were able to chronically deliver drugs to animals; however, as described in Chapter 2, we used a novel computer-controlled automated feeder so that food pellets could be delivered to epileptic rats without the stress associated with repeated systemic injections.

Additionally, to increase the translatability of the study to the newly diagnosed patient, treatment of post-SE rats began immediately following their first observed spontaneous seizure. This approach is essentially mimicking the clinical situation by initiating treatment at the time of diagnosis. Most often, treatment is initiated well after epilepsy onset once the animal's seizure rate has plateaued (Williams, White et al. 2009). While waiting for a consistent seizure frequency makes interanimal comparisons easier, it does not represent the standard management of epilepsy care in the clinical setting. A further discussion of the benefits and consequences of this approach to treatment is provided in Chapter 5.

As previously mentioned, sudden withdrawal of an ASD treatment can lead to SE,

which is a life-threatening condition, requiring immediate medical care. There are currently two major treatments for electrographic status epilepticus (ESE), a benzodiazepine, such as lorazepam or diazepam, and propofol. However, benzodiazepines become ineffective after approximately 60 minutes (Walton and Treiman 1988), and propofol causes respiratory depression (Mackenzie, Kapadia et al. 1990), making it problematic to use in an emergency situation. Luckily, there are already acute models of ESE; one such ESE model uses pilocarpine, and has previously been used to screen new possible therapies for ESE (Prasad, Williamson et al. 2002). The current method for scoring ESE data is to employ human evaluation methods (Treiman, Walton et al. 1990). However, there is not a computational method to quantify the results of this model, making drug discovery problematic in this area. Our quantifiable method, covered in Chapter 4, increases the quality of this model by providing an unbiased analysis of the effect of an investigational compound on ESE.

Dissertation Overview

The previous sections have outlined the need to deliver chronic ASD treatment to animals, both for modeling nonadherence, and for chronic models to be utilized in drug discovery. Chapter 2 provides technical instructions on how to build a system that allows for chronic and stress-free drug delivery. This method combines video, EEG and electronic feeders to create an automated system for the delivery of drug-in-food to chronically epileptic rats. This system is capable of simulating nonadherence by providing individually-housed animals with two automatically controlled feeders, one with drug-in-food, i.e, medicated pellets, and one with placebo control food, i.e., unmedicated pellets. To simulate taking a dose, an entire serving of food consists of medicated pellets; whereas

missing a dose is simulated by delivering only unmedicated pellets for one serving. We used this system to answer clinically relevant questions regarding the impact of nonadherence on seizure control and to assess whether abrupt nonadherence results in an increased rate of pharmacoresistance to the commonly prescribed ASD, CBZ.

Characterization of a novel model of nonadherence was performed by running two different studies utilizing this novel drug delivery system; the results of which are described in Chapter 3. In both studies, chronic epilepsy was induced using the low-dose KA paradigm (Hellier, Patrylo et al. 1998). In the first study, newly-diagnosed epileptic rats were treated with CBZ at different adherence levels, 100% and 50% adherent, and compared to those of a placebo control group. We expected, based on previous observations using CBZ-in-food that animals treated at 100% adherence would exhibit full seizure control (Grabenstatter, Clark et al. 2007). While we did see a significant reduction in seizures in the 100% adherent treatment group, when compared to 50% adherent and placebo control groups, only one of eleven animals exhibited full seizure control in this study. Additionally, our initial hypothesis was that animals practicing nonadherence at a rate of 50% would have a larger burden of seizures when compared to placebo control, due to random cycles of treatment and withdrawal inherent in the practice of nonadherence. However, we observed no significant difference in seizure control between animals in the placebo group and animals in the 50% adherent group. This suggests that being nonadherence at a rate of 50% was ineffective at treating the animal's epilepsy, but importantly did not result in a worsening of seizure control.

In the second study, we sought to determine if chronic treatment and withdrawal of CBZ would lead to changes in pharmacosensitivity when CBZ treatment was restored. The

initial hypothesis was that animals would exhibit full control during their first full (100%) treatment with CBZ, and would subsequently lose control during the second treatment, similar to that previously reported in the lamotrigine (LTG)-resistant kindled rat model (Srivastava, Alex et al. 2013). However, despite the many seizures that occurred during the period without CBZ treatment, animals regained comparable levels of seizure control during their second course of treatment with CBZ.

Chapter 4 describes the development of a simple mathematical method for quantification of power of the EEG observed during pilocarpine-induced ESE. This method uses EEG power in the gamma band (20-70Hz) as the input into an algorithm that creates an approximation of the resulting power signal over the course of ten hours, using an eighth order polynomial to fit the curve of the resulting power. The algorithm was validated by using known compounds and comparing the results to human scoring methods. The practicality of this approach is covered in Chapter 4, and in Lehmkuhle, Thomson et al., (2009). This approach serves as a simple method for assessing the effect of a treatment on the severity and duration of ESE.

Given that adherence is a problem in epilepsy, there is a fundamental need to understand the impact of imperfect adherence on seizure severity and frequency. The results described herein provide both a quantification of an existing model of acute ESE, as well as the creation of a novel animal model of nonadherence.

The path to understanding the impact of treatment on seizure control may lay with an animal model in which chronically epileptic animals can be chronically exposed to an ASD in a clinically meaningful manner. The ability to dose chronically using a non-stressful approach and to modify the dosing patterns to mimic various levels of

nonadherence may directly translate to a greater understanding of epileptogenesis and pharmacoresistance. The methods and results described in this dissertation move the field of epilepsy research towards that ultimate goal.

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CHAPTER 2

A NOVEL OPEN-SOURCE DRUG-DELIVERY SYSTEM THAT ALLOWS FOR FIRST-OF-KIND SIMULATION OF NONADHERENCE TO PHARMACOLOGICAL INTERVENTIONS IN ANIMAL DISEASE MODELS

Abstract

Nonadherence to a physician-prescribed therapeutic intervention is a costly, dangerous, and sometimes fatal concern in health care. To date, the study of nonadherence has been constrained to clinical studies. The novel approach described herein allows for the preclinical study of nonadherence in etiologically relevant disease animal model systems.

The method herein describes a novel automated approach which permits variable dosing of pharmacological compounds using a drug-in-food protocol. This computer-automated pellet delivery system allows for tight experimenter control of dosing to study nonadherence. Food-restricted animals receive either medicated or unmedicated pellets, using an experimenter-determined schedule designed to mimic "taking" or "missing" a drug dose.

The robust system described here will permit the reliable distribution of medicated or unmedicated food pellets on an experimenter-defined feeding schedule. The flexibility

of this system permits the delivery of drug according to the known pharmacokinetics of investigational drugs. The state of art for adherence research relies heavily on medication-event monitoring system (MEMS) tracking caps. MEMS caps allow the prescribing clinician to directly monitor patient adherence; however, correlating the effects of nonadherence on efficacy and tolerability still rely heavily on patient reporting. This means results still rely heavily on the veracity of the patient journals.

Modeling nonadherence in animal models requires an investigator to have tight control over the delivery of the investigational therapeutic agent. With a mechanism that can provide the necessary control, the investigator can tightly control the delivery of medicated food and is free to ask pertinent questions regarding the impact of nonadherence in an etiologically relevant animal model; an experiment that would otherwise not be feasible at the clinical level and unethical in patients. While the system described herein was designed to measure the impact of nonadherence on seizure control in a population of epileptic rats, it is clear that the utility this system extends beyond epilepsy to include other disease states. Importantly, it can also be utilized for chronic pharmacokinetic and pharmacodynamic studies.

Introduction

Patient nonadherence to a doctor-prescribed therapeutic regimen is a widespread problem across all prescribed pharmaceutical treatments and is often associated with expensive and sometimes fatal consequences. It is estimated that one-half to one-third of patients practice imperfect adherence across almost all disease categories (Osterberg and Blaschke 2005). In the United States alone, it is estimated that nonadherence is responsible for as many as 125,000 preventable deaths per year (McCarthy 1998) and $290 billion in

preventable health care spending per year (Cutler and Everett 2010). This makes nonadherence a major disease category on its own.

Previously, the study of nonadherence has been limited to clinical research. However, these studies are confounded by poor patient reporting of adherence (Modi, Guilfoyle et al. 2011). Electronic technologies, such as electronic Medication Event Monitoring System (MEMS) caps (Cramer, Mattson et al. 1989), have increased the fidelity of patient adherence data. However, there are still issues with the quality of patient reporting of symptoms and adverse events (Buelow and Smith 2004; Hoppe, Poepel et al. 2007). The reliance on patient reporting of adherence, state of disease control and drug adverse events increases the error when attempting to correlate nonadherence to specific disease state outcomes; e.g., efficacy and adverse events (Faught 2012). A novel system that can directly measure the impact of nonadherence in an etiologically relevant animal disease model would begin to address these concerns.

Simulating daily patterns of nonadherence is possible without use of an automated system. However, it would require a substantial commitment of resources 24/7 in order to deliver drugs on a fixed schedule; thus, it is not feasible for a long-term chronic experiment. To make the study of nonadherence feasible requires an automated system that can reliably deliver a fixed dose of drug for a protracted period of time. One method to deliver drugs to animals is through the use of food pellets that are formulated with a specific quantity of a given drug (Grabenstatter, Clark et al. 2007). Thus, adherence can be simulated by administering medicated pellets for a single meal to simulate "taking" a dose, and administering unmedicated placebo control food pellets to simulate "missing" a dose.

Having the drug-in-food pellets available still requires a means to deliver them to

the animal at a schedule that meets the needs of the individual investigator. This need led to the design and creation of the system described herein; i.e., an automated pellet delivery system capable of delivering medicated or unmedicated food pellets 24/7 according to an experimenter defined feeding schedule. The proof-of-principle experiments that were conducted to demonstrate the feasibility of the approach employed epileptic rats housed individually in a cage that was equipped with two feeders, thereby, allowing each animal to have independent levels of experimenter-defined nonadherence. The methods discussed herein describe the design and implementation of this system. While the system discussed below is integrated with video-EEG so that the delivery and consumption of antiseizure drugs could be correlated with seizure control, the implementation of this system without video-EEG would certainly permit the study of nonadherence in a variety of animal disease models. Moreover, it would also allow for chronic delivery of a drug for the purpose of conducting pharmacokinetic and pharmacodynamic studies.

Methods

System Architecture Overview

The system described herein is able to administer medicated and nonmedicated pellets to up to twelve individual epileptic rats in an effort to simulate clinically relevant patterns of nonadherence (Modi, Rausch et al. 2011). Additionally, the system coordinates the recording of EEG and video so that the resulting impact of nonadherence in an animal model of epilepsy can be assessed. The overall system design contains 24 automated feeders (Figure 2.1A), a feeder control system, a BioPac MP150 EEG recording system, and a computer running custom videoEEG software (Figure 2.1B). The feeder control system consists of 6 breakout boards and a main control board (Figure 2.1C). The

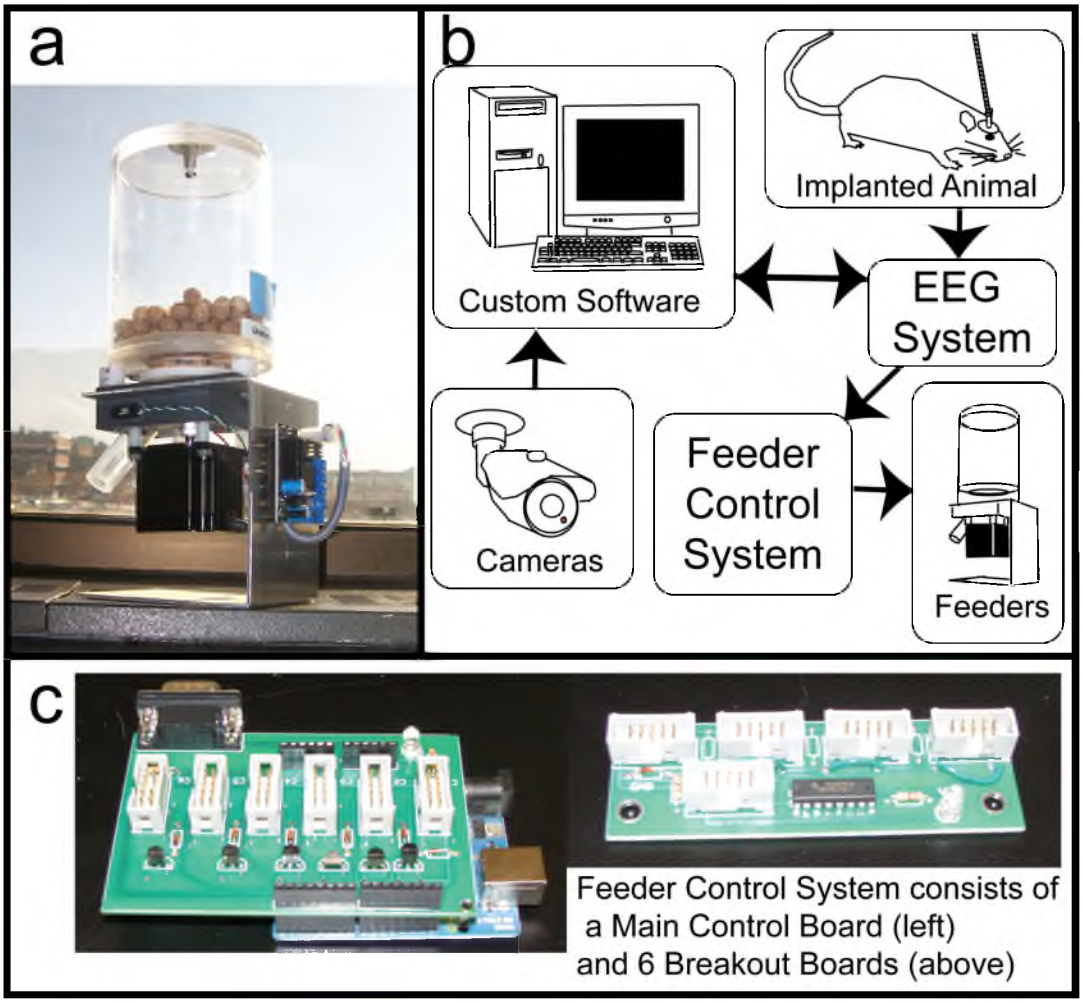

Figure 2.1 Overall system design of the novel nonadherence system. (a) A single modified feeder. (b) System schematic. (c) Populated PCBs that make up the feeder control system.

breakout board is a custom printed circuit board (PCB) that controls up to four feeders. The main control board consists of an open-source Arduino Uno microcontroller and a custom PCB that works as an Arduino shield, i.e., an expansion PCB which is designed to fit into the standardized Arduino pin-headers. Custom PCBs were designed using the freely available ExpressPCB software, and fabricated by ExpressPCB (www.expresspcb.com).

The system works by generating commands in the custom software, and using the feeder control system to distribute the commands to the individual feeders. The methods that follow discuss how commands are executed, starting at the software, and ending with the confirmation that a pellet was delivered. Figure 2.2 summarizes the layout of the entire system.

Simulating Nonadherence via Daily Feedings

Because patient nonadherence is dynamic (Glass, Battegay et al. 2010), the simulation of nonadherence requires the ability to simulate randomized patterns of "missed" and "taken" doses. For the purpose of this study, nonadherence is simulated by giving an animal randomized patterns of medicated and unmedicated feedings. To perform this randomization, the determination of whether the animal receives a medicated or unmedicated meal is based on a set integer percentage specified by the user. In the case of a fractional number of meals, the number of medicated meals are rounded up. At the beginning of a new week (Sunday night), or when the medication % value is entered in for a new animal, the software creates an array for all the remaining meals in the week. Then, values are sequentially removed from the array, until the medication % has been reached. This way, the percentage of meals that are medicated is fixed, while the

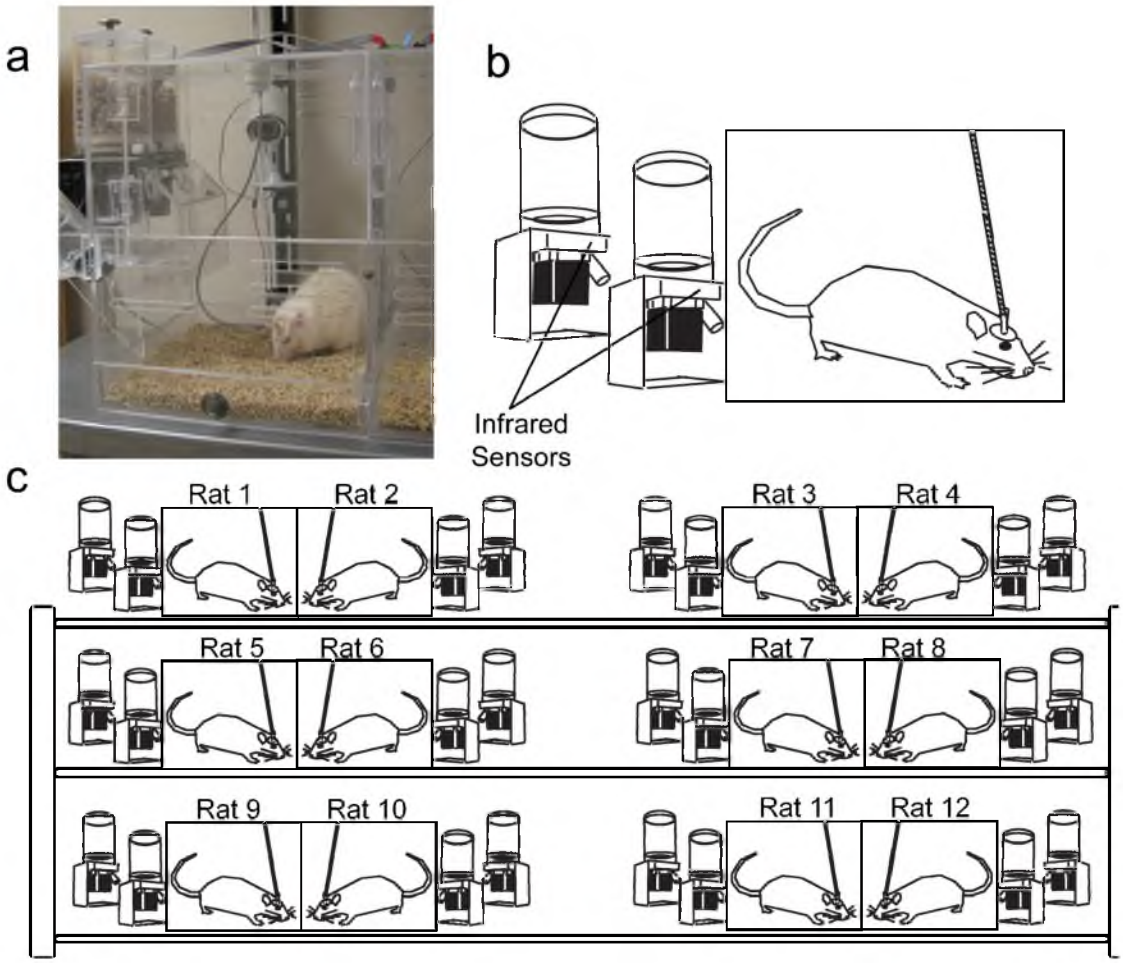

Figure 2.2. Layout of the feeding cages and video-EEG room. (a) A photo showing a tethered rat in one of the cage blocks with two feeders. (b) Schematic of single, dual feeder cage block. (c) Schematic layout of a set of 12 cage blocks. Each pair of singly housed animals has four feeders (two per cage); feeders are controlled by one of the -- breakout boards.

actual patterns of nonadherence are randomized in an independent manner. Figure 2.3 shows how all of these options are set in motion by the software. Up to six feedings can be delivered per day in our current configuration, and a meal can be disabled by entering a time of 0:00:00. Thus, no meals can occur at midnight. Increasing the number of daily feedings beyond six would require only trivial changes to the C# code.

Command Creation and Execution

The flow of commands is shown in Figure 2.4. The custom software, written in Visual C# (Microsoft, Redmond WA), synchronizes the recording of the BioPac MP150 EEG recording system (BioPac Systems Inc., Goleta, CA) with video recordings streamed from EZWatch Pro Security Cameras (EZWatch, Louisville, KY) and captured by a DVP7020BE MPEG4 Video card (Advantech, Milpitas, CA) and the delivery of non-medicated and medicated food pellets. A C# software timer checks if the current time matches the time required for a feeding, and creates the commands based on the procedure described previously. When the user-specified time arrives, the software enqueues all of the commands by calculating the number of pellets required for a feeding based on the weight of each animal.

Command Cycles

To continuously stream EEG data, the software sends a request to the MP150 for 250ms of EEG data from 1 to 16 channels of EEG recording. At the receipt of these data requests, the software writes the newly-acquired data to disk and checks for any commands that need to be sent to the feeding system. The MP150 sends these commands as an

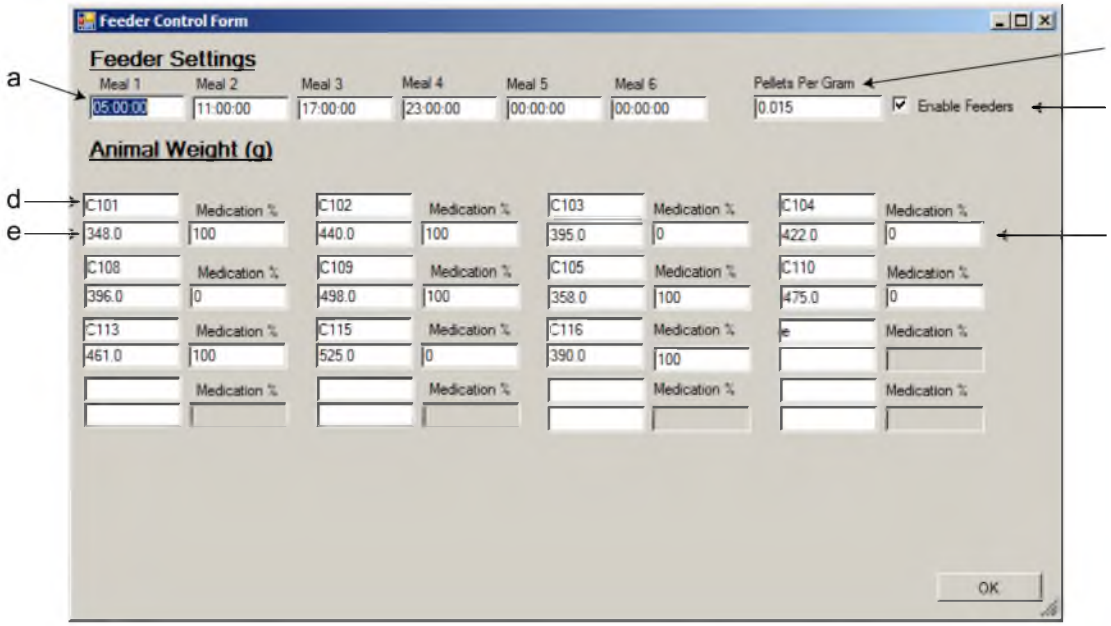

Figure 2.3. Feeder control form. This screenshot shows the system control form containing all the information needed to control daily feedings. (a) Feedings, up to six per day, are controlled by defining the time (military time), at which the feedings should be executed. A time of 0:00:00 disables that meal. (b) The investigator defines the ratio of pellets to body weight (in grams) per meal. (c) This box enables/disables scheduled feedings. (d) Animal ID#. This is recorded in the software, which allows for unique identification of the animals. Locations match the setup of the room, as illustrated in Figure 2.2c. (e) Animal weight, used to calculate how many pellets are given per meal, in conjunction with (b). (f) Medication %, which determines what percentage of all meals are medicated per week.

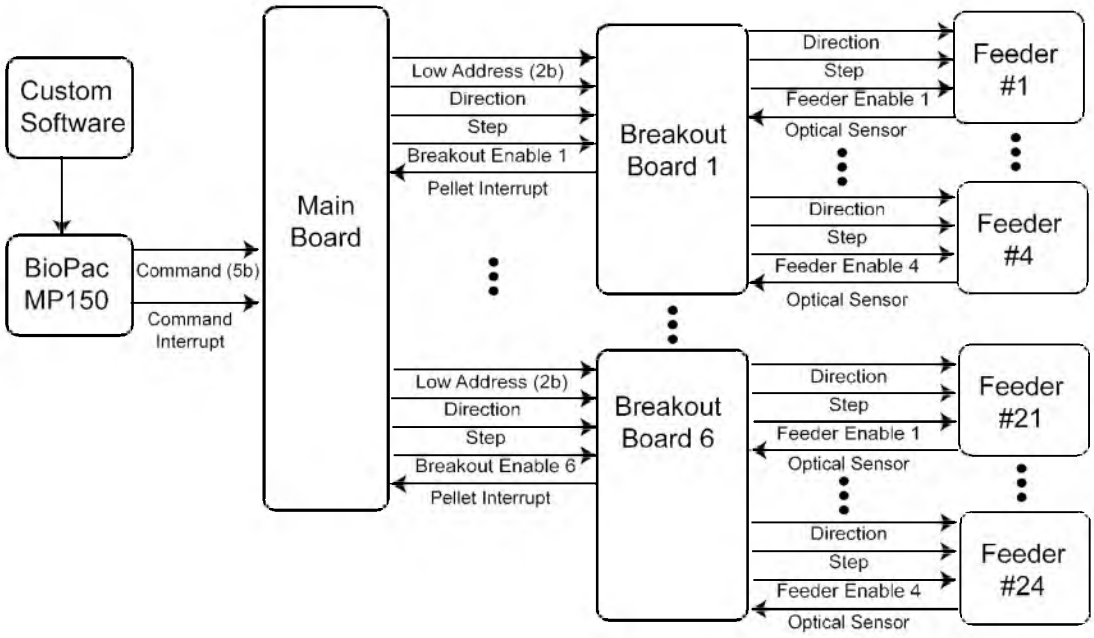

Figure 2.4. Layout of the data flow for feeding control. This setup allows for 24 feeders to be independently controlled with six breakout boards, and one main board.

unsigned five-bit parallel output. An additional single digital output serves as a strobe to signal that a new command is present on the digital output lines. The software sends a single feeding command per data request cycle. The strobe bit from the MP150 is connected to an interrupt pin on the main control board. The schematic for the main board is included in Figure 2.5.

Command Communication

Each unsigned five-bit command consists of either a feeder address, pellet count, or an execute command. To execute a single animal's feeding, the feeding software must sequentially send a feeder address (0 to 23, i.e., 0b00000 to 0b10111) followed by a pellet count (1 to 30, i.e., 0b00001 to 0b11110). If more than 30 pellets are required for a feeding, the software will split the feeding into multiple sets of feeder addresses and pellet count commands. After all commands for all animals have been sent, an execute command (0b11111) is given to inform the feeder system that all commands are delivered. This command tells the Arduino to begin execution of a feeding. The pinout connection for the BioPac MP150/Main board connection are listed in Table 2.1.

Command Execution

At the occurrence of a low-to-high transition on the strobe line, the main control board will read the state of the digital pins connected to the digital port connected to the MP150. These commands are placed into two buffers, one for feeder address, and a second for pellet count. At the presence of an execute command, these buffers will be read and executed in a FILO (first-in-last-out) order. The feeder address is translated into

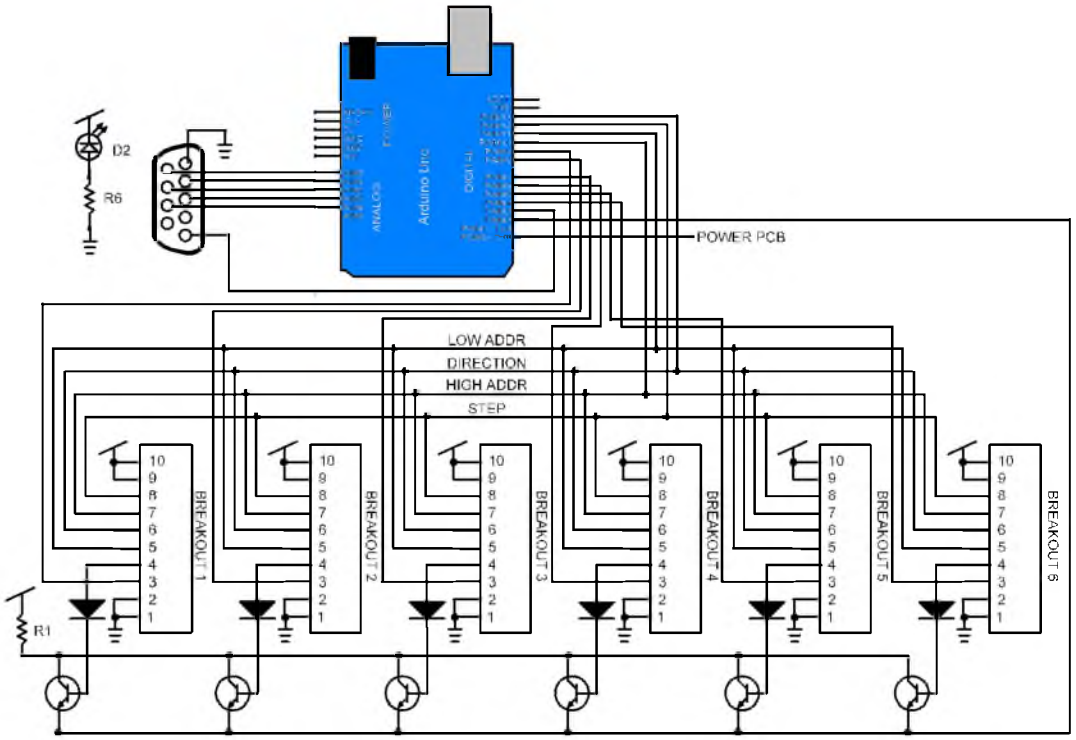

Figure 2.5. Schematic of the main system control board.

Table 2.1. Pinout of the cable that connects the BioPac MP150 to the feeding system

Pin Name	BioPac MP150 DB25	Feeder Main Control Board DB9
GND	5	1
Strobe	17	5
C0	1	6
C1	2	2
C2	3	7
C3	4	3
C4	14	8

hardware by separating the lower two bits from the upper three bits. The lower two bits are sent to each breakout board, while the upper three bits are translated in software to determine which of the six breakout boards to enable.

Feeder Operation

The feeder is attached to the breakout board via a ten-pin ribbon cable, which is separated into a six-pin header, and a custom four-pin connection to the infrared diode/phototransistor pair (Table 2.2). Each feeder receives three inputs; step, direction, and enable. The step and direction pins are concurrently sent to all 24 feeders connected in the system. However, only the feeder that has its enable line set low responds to the step and direction command. The mechanical design of the feeder uses a modified version of a design from a commercially purchased automated pellet dispenser (ENV-203-1000, www.med-associates.com, St. Albans, VT) commonly used for behavioral experiments. This dispenser uses a stepper motor (STP-MTR-23055, www.automationdirect.com, Cumming, GA) to turn a rotary rod, which allows for single 1-gram pellets to be dispensed. Options for the delivery of different size pellets are available from med-associates. The motor is controlled using a "Stepper Motor Driver v3.3" board (www.thingiverse.com/thing:4970/#instructions), an open-source circuit design for stepper motor control. This board allows for TTL level voltages to be used to drive the higher voltage input required to run the stepper motor. To drive pellet delivery, the step pin is driven high for 40ms, then driven low for 40ms, making each step 80ms long. This cycle repeats until the delivery of all pellets has been confirmed.

Table 2.2. Pinouts of the ribbon cables connecting the main board to the breakout board, and the breakout board to the feeders.

	Function:	
Pin Number	Main Control <-> Breakout	Breakout <-> Feeder
1	GND	GND
2	GND	GND
3	ENABLE	STEP
4	PELLET DETECT	DIR
5	FEEDER LOW	ENABLE
6	DIRECTION	N/C
7	FEEDER HIGH	COLLECTOR
8	STEP	EMITTER
9	VDD	ANODE
10	VDD	CATHODE

Feeder Control

An individual feeder can be controlled by individual activation of the enable input on the stepper motor driver controller. The step command is concurrently sent to all feeders, but it is ignored by the feeder so long as the driver's enable line is set to disabled. Each breakout board receives the same lower two bits of the feeder address, i.e., 0b00 to 0b11. Each of these addresses is translated on the breakout board using a SN74HC138N 3-to-8 line decoder (Texas Instruments, www.ti.com). The output from this chip is fed into each enable input for the four attached feeders. The decoder has a separate enable which is sent from the main control board. Thus, one is able to use the same four low bit address lines, coupled with six individual enable lines for the decoders, to independently control all 24 feeders. An outcome of this design is that only one feeder may be active at a time. The schematic for the breakout board is included in Figure 2.6.

Pellet Detection via Infrared Sensor

Positive delivery of a pellet is measured via an infrared sensor/receiver pair positioned across a 1/16" hole in the feeding tube. Pellet detection is done for each feeder individually using an OPB 100 infrared LED and a phototransistor pair (OPTEK TT Electronics). Each pair of transmitter and receiver is mounted perpendicular to the feeding tube, and the phototransistor is normally in the active state. However, when a pellet crosses in front of the LED, the light is blocked, putting the phototransistor in the cut-off state. This transition is long enough to trigger a software interrupt on the Arduino Uno. The data path of this interrupt can be traced in Figure 2.7. Following the interrupt, four step cycles must occur before the next pellet is counted. This serves as a debounce function, to prevent

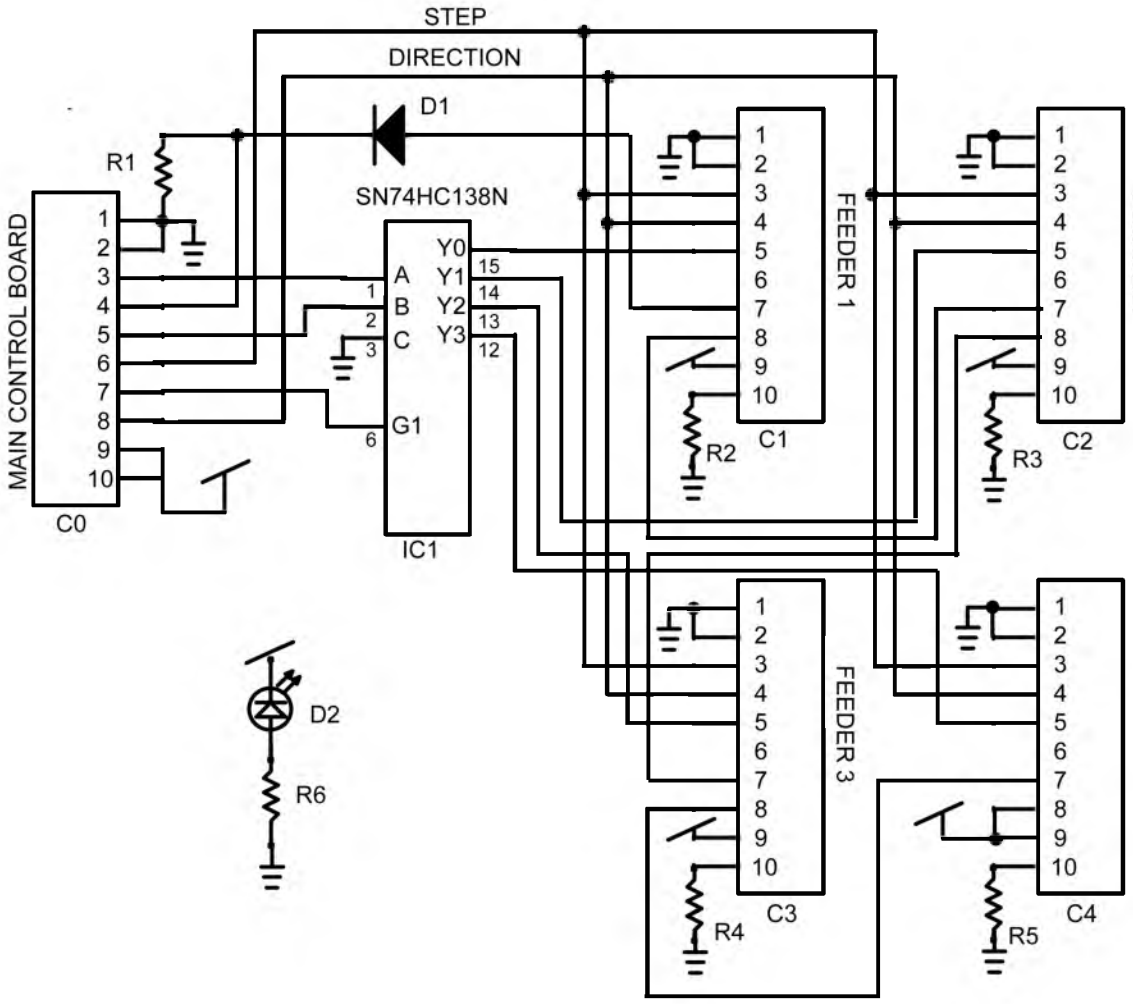

Figure 2.6. Schematic of the breakout board.

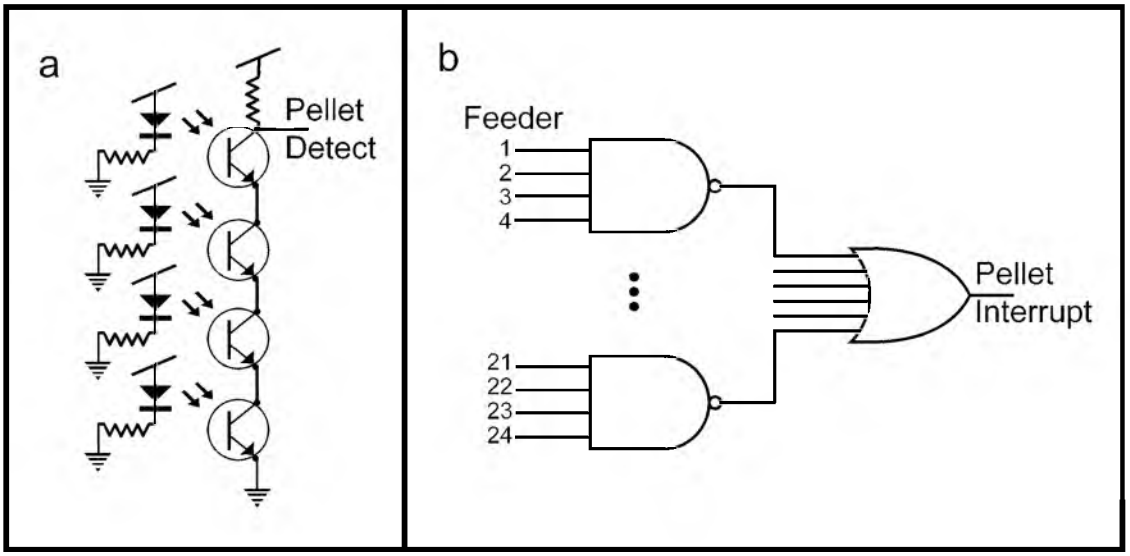

Figure 2.7 Schematic and logic setup for infrared pellet detection system for the breakout boards and main board. (a) Schematic diagram showing the layout of four feeders' infrared sensors attached to a single breakout board. While no sensor is blocked, the signal is pulled low. When a pellet crosses in front of the LED, the pull-up resistor drives the signal high. This layout creates a NAND gate. (b) The NAND structures from each of the six breakout boards are fed into a BJT OR gate on the main board.

a single pellet from being counted more than once.

Each infrared sensor pair on each block of four feeders shares a single output line leaving the breakout board. To allow all four infrared feeders to share the same line, the phototransistors are tied together in serial, creating a NAND logic structure. In other words, while all four transmitters are active, the output line is high. If any transistor is off, (i.e., a pellet crosses in front of the beam) the signal is pulled low. This allows for a single output to monitor the activity of all four feeders (Fig. 2.7A). For successful operation of the feeder system, all four feeders must be attached to a breakout board, or else the sensor logic will be incomplete, rendering pellet detection impossible. The "pellet detect" from each breakout board is fed into an OR gate on the main board (Fig. 2.7B) Thus, a single interrupt pin on the Arduino monitors the state of all feeders currently attached to the system. During pellet delivery, if 125 step cycles occur without the delivery of a pellet, the direction bit is flipped, reversing the direction of rotation, and the feeding continues. Preliminary tests have shown this is sufficient to release jammed feeders during pellet delivery.

Powering the Feeders

The stepper motors require a higher voltage than TTL-level voltage to run. To conserve on power and reduce 60Hz noise, the stepper motor drivers are only supplied 28V power during the execution of a feeding. A separate power PCB was created to control this voltage switching. A power transistor, wired to pin 0 of the Arduino, is driven high at the start of the feeding.

Availability of Software and Designs

All PCBs and software code are open source, and thus are included or freely available on the internet at: https://code.google.com/p/biopacvideo/. Two exceptions exist. The mechanical design of the feeder is based on a commercial design, and thus will require purchase of these to implement the project. Additionally, the software that controls the BioPac MP150 is closed source. Thus, communication with the BioPac MP150 requires the developer dynamic link library, which is available for purchase from BioPac.

Results and Discussion

The result of this work is the design of a flexible system that provides the ability to chronically administer medicated and unmedicated food to 12 animals 24/7 with minimal intervention by staff labor; thereby minimizing handling-induced stress associated with chronic systemic delivery of a drug. The system described herein allows for a large variety of chronic studies to be performed in different disease models.

System Improvements

A consequence of the combination of infrared monitoring is that any malfunction of a single feeder's phototransistor or its connections will cause a failure in the ability to measure all feeders' pellet distribution. Additionally, all four feeders must be connected to a breakout board for the system to be able to record the distribution of pellets. Future iterations of the described system will individually read from each set of feeders based on which breakout board is currently enabled. Also, the feeder system does not skip a permanently jammed or empty feeder. A method in which a feeder is skipped in the queue, and returned to, would allow all other animals to be fed, regardless of feeder faults. This

would require additional circuitry to isolate the infrared sensors on each breakout board. Additionally, it would increase the fidelity of the system if the system had bidirection communication. As it is currently written, errors in the feeding system are not reported or recorded by the system software. The quality of data would increase if the experimenter were able to access data such as the proper completion of a feeding, how long each feeding took, and if any errors occurred during the feeding. Two additional inputs are available on the Arduino and could be utilized for this feedback. Despite these current limitations, a preliminary experiment using this system has shown that the feeders correctly delivered meals greater than 99% of the time over the course of more than 10,000 feedings. The results of this experiment will be published elsewhere.

Other Applications

The current version of this setup relies on the Biopac MP150 to communicate the daily feeding via software control. Other projects may use a different method of supplying the feeding commands, as the MP150 is prohibitively expensive for applications that do not require EEG. This could be achieved without the use of an external source supplying feeding commands; however, an external real-time clock, as well as a reprogrammable source that contains the daily feeding parameters, such as a flash ROM or an SD card, would be required.

Conclusion

This system is the first of its kind, and addresses the goals of bench-to-bedside research. The automated drug-in-pellet deliver system described above permits the delivery of drugs according to a feeding schedule based on the individual pharmacokinetics of a

given drug; i.e., the rate of metabolism and elimination of the drug. Any pharmaceutical intervention that can be integrated into food pellets, and is sufficiently palatable to the animal employed in the study could be used in this model. Thus, the system described herein provides the opportunity to ask pressing questions about the effects of nonadherence or long-term efficacy in chronic animal disease models.

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CHAPTER 3

MODELING THE IMPACT OF ANTISEIZURE DRUG NONADHERENCE IN NEWLY-DIAGNOSED EPILEPTIC RATS USING A NOVEL AUTOMADED DRUG-IN FOOD SYSTEM

Abstract

Nonadherence to prescribed antiseizure drug (ASD) treatment is associated with many negative consequences, including increased seizure burden, morbidity and mortality, and health-care costs. Despite this, 25 to 50% of epilepsy patients have been reported to be nonadherent. Previous nonadherence research has been limited to clinical observations. Here, we present the results from a novel model that permits the study of nonadherence in newly diagnosed epileptic rats

In a study designed to simulate the effect of complete nonadherence (0/%), partial adherence (50%), and full adherence (100%) to the ASD carbamazepine (75 mg/kg, p.o., q.i.d x 6 weeks) on seizure control, newly-diagnosed epileptic rats were randomized to one of three different treatment groups. In a separate study, newly-diagnosed epileptic rats were stabilized on carbamazepine (75 mg/kg, p.o. qid) for two weeks; carbamazepine was then abruptly removed for two weeks and then reinstituted for a final two weeks (six weeks total). This 100%-0%-100% adherence protocol was designed to assess the impact of sudden carbamazepine withdrawal on seizure control and the development of carbamazepine resistance. Carbamazepine was administered using a novel automated

dosing system which was capable of delivering medicated or placebo pellets to simulate taking or missing a dose. Video-EEG (24/7) monitoring was employed to assess the effect of treatment on seizure burden.

Seizure control in rats randomized to the 100% treatment group was significantly better than that of rats assigned to either the placebo control (0%) or nonadherent (50%) treatment schedule. Seizure control of the nonadherent group (50%) was indistinguishable from that observed in the population of epileptic rats assigned to the placebo control (0%) group. For the second study, removal of CBZ treatment resulted in a marked increase in seizure frequency; however, seizure control was regained to a level not significantly different from that obtained with the initial treatment trial with CBZ. Complete seizure control was only obtained in one of 11 rats randomized to the adherent (100%) treatment schedule

This chapter describes results obtained from a novel preclinical adherence study designed to assess the impact of varying levels of drug adherence on seizure control in newly diagnosed epileptic rats using a novel automated drug-in-food delivery system and chronic 24/7 video-EEG recording. The results reported herein demonstrate the utility and practicality of preclinical nonadherence studies to assessing the impact of nonadherence to a widely prescribed ASD on seizure control.

Introduction

Patient nonadherence to doctor-prescribed medication dosing regimens is a widespread, extensive problem across all disease states and is often associated with expensive and fatal consequences. The clinical definition of patient nonadherence is a patient taking his/her medication, as directed by the physician, less than 80% of the time

(Hughes, Bagust et al. 2001). It is estimated that one-third to one-half of patients across many disease categories are classified as nonadherent (Osterberg and Blaschke 2005). Nonadherence is estimated to cost as much $290 billion (Cutler and Everett 2010) and results in approximately 125,000 deaths annually (McCarthy 1998). Surprisingly, even in HIV/AIDs where nonadherence can result in the emergence of treatment-resistant strains of HIV (Ho 1996), 50% to 70% of patients receiving antiretroviral therapy are estimated to be nonadherent (Chesney 2000). In the treatment of diabetes, nonadherence is directly related to a higher number of complications (Stratton, Adler et al. 2000), yet as high as 64% of patients are nonadherent (Cramer 2004). In epilepsy, nonadherence to antiseizure drugs (ASDs) is associated with a number of consequences; some of which can be life threatening; e.g., seizures (Stanaway, Lambie et al. 1985; Manjunath, Davis et al. 2009; Modi, Rausch et al. 2014), depression and other neuropsychiatric disorders, increased mortality as a result of accidents and sudden unexplained death in epilepsy (SUDEP) (Gaitatzis and Sander 2004; Faught, Duh et al. 2008) have all been associated with nonadherence and all are associated with increased health care expenses and hospital visits (Faught, Weiner et al. 2009). Despite these consequences, 25 to 50% of patients with epilepsy do not take their ASDs as prescribed (McAuley, McFadden et al. 2008; Manjunath, Davis et al. 2009; Zeber, Copeland et al. 2010; Modi, Rausch et al. 2011).

Several investigators have evaluated the effects of nonadherence in patients with epilepsy on seizure control (Stanaway, Lambie et al. 1985; Manjunath, Davis et al. 2009; Modi, Rausch et al. 2014); however, quantifying the direct relationship between nonadherence and seizure burden is difficult. Patients that practice nonadherence are also more likely to display similarly poor performance in reporting of seizure events (Buelow

and Smith 2004; Hoppe, Poepel et al. 2007) and often overestimate their level of adherence; effects that lead to a required correction factor (Modi, Guilfoyle et al. 2011). Thus, in order to avoid these confounding factors and directly test the consequences of nonadherence on seizure control and severity, we developed a novel automated drug-delivery system that allows for tightly controlled delivery of medicated food pellets to newly diagnosed epileptic rats to test the impact of experimenter-defined levels of nonadherence on seizure control.

Modeling nonadherence in animals requires multiple daily administrations spread over 24 hours, which requires experimenter-administered dosing both day and night. Manually administering these doses over a long-term, chronic experiment, requires a substantial dedication of research staff, and thus is ultimately infeasible. To be able to address questions about nonadherence on seizure control, we have created a computer-controlled automated dosing system, as outlined in Chapter 2. The present chapter describes the utility of this automated system using a drug-in-food protocol wherein carbamazepine (CBZ), a widely-prescribed antiseizure drug (ASD), is administered to newly-diagnosed epileptic rats. It was previously demonstrated that CBZ-in-food is effective in reducing the frequency of seizures in a fully-epileptic animal (Grabenstatter, Clark et al. 2007; Ali, Dua et al. 2012); i.e., a chronically epileptic rat that displays a stable seizure frequency. The advantage to this approach is that the efficacy of an administered treatment on seizure control can be compared to the pretreatment seizure frequency of each individual rat. However, this approach does not accurately reflect the clinical treatment of epilepsy, wherein treatment is initiated at the time of initial diagnosis. In contrast to previous studies, the model and approach described herein seeks to model clinical reality

wherein treatment is initiated soon after diagnosis is made; i.e., the second confirmed seizure.

In this chapter, we employed a novel computer-controlled device wherein drug-in-food pellets can be delivered to individual newly diagnosed epileptic rats using an experimenter-defined dosing protocol. The results reported herein describe a new animal model of nonadherence that provides a unique opportunity to directly test the consequences of nonadherence in newly-diagnosed patients with epilepsy. In addition to modeling nonadherence on seizure control, we also describe a novel study design that represents a paradigm shift in the conduct of antiseizure drug evaluation; i.e., initiation of chronic antiseizure drug dosing at the time of epilepsy onset.

Methods

Animals and EEG Electrode Implantation

Male Sprague-Dawley rats weighing between 190-230 gm (Charles River Laboratories, Raleigh, NC) were used for this study. Animals were housed in individual cages in a temperature controlled room (70-74°F) with a 12h light/12h dark cycle, and had *ad libitum* access to water for the entire study. All animal procedures were performed inaccordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the University of Utah Institutional Animal Care and Use Committee. The overall experimental design is outlined in Figure 3.1a. A three-wire EEG recording electrode (Plastics One, Roanoke, VA) was stereotaxically implanted onto the skull of rats via sterile technique under ketamine/xylazine anesthesia.

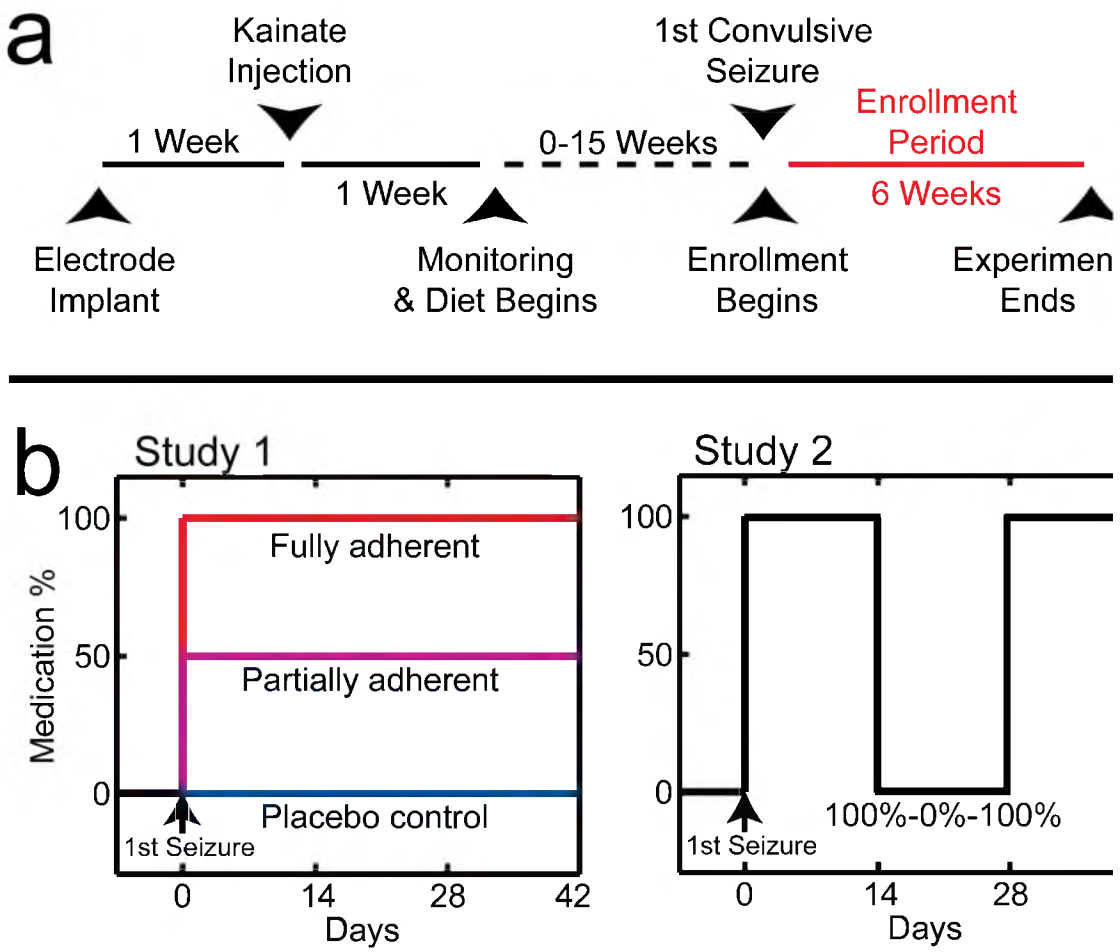

Figure 3.1: Study design for testing nonadherence. (a) Experimental timeline. Data obtained during the enrollment period (shown in red) were subjected to statistical analysis. (b) Time line of enrollment for the first study shows the time course of adherence for both studies.

vEEG Recording

Rats were permitted six days to recover from surgery before being placed into one of two vEEG suites (12 rats per suite), and monitored 24/7 using vEEG for the duration of the experimental trial. Animals were tethered to a rotating commutator (Plastics One, Roanoke, VA) which in-turn was connected to an EEG100C amplifier. EEG was streamed from a BioPac MP150 Recording system (BioPac Systems Inc., Goleta, CA), and video was captured by a pair of DVP 7020BE Capture cards (Advantech, Milpitas, CA). Video and EEG data were synchronized and written to disk using custom software that also controlled the feeding system (See Chapter 2). Video was stored in MPEG4 format, and EEG were stored in a custom file format based on the Acknowledge File Format 3.8 by BioPac (BioPac Systems Inc., Goleta, CA).

Induction of Status Epilepticus

Status epilepticus (SE) was induced using the repeated low-dose systemic kainic acid (KA) (Tocris Bioscience, Bristol, UK) protocol as previously described (Hellier, Patrylo et al. 1998). Rats were allowed access to rat chow (22/5 Rat Diet, Harlan Teklad) food *ad libitum* for the first seven days following KA treatment.

Drug and Placebo Pellet Delivery

Seven days following KA treatment, all rats were placed on a food-restricted diet of placebo food pellets. Rats received four equally spaced daily feedings of 15 g/kg of placebo food pellets; i.e., 60 g/kg of food per day. Pellets were delivered using a novel computer-automated dosing system that has demonstrated reliable delivery on an

experimenter-defined dosing protocol, as outlined in Chapter 2. Each cage was equipped two automated pellet dispensers; one filled with medicated food, and one filled with placebo food. At the time of each scheduled feeding, one of the two pellet dispensers would automatically dispense placebo food pellets or medicated food pellets as per study protocol to each enrolled subject (see Study Design). The number of pellets per feeding was based on the individual weight of the rat, recorded weekly at the time of the cage cleaning. The number of nonconsumed pellets each week was also recorded at the time of cage cleaning.

One-gram chocolate minitreat placebo or medicated food pellets were obtained from BioServ (Frenchtown, NJ). Each 1g medicated pellet contained 5 mg of CBZ (Sigma Aldrich, St. Louis MO). The number of medicated pellets delivered at each of four feedings was computer controlled to deliver a dose of 75 mg/kg CBZ q.i.d,. i.e., 300 mg/kg CBZ daily. This dose was previously shown to be efficacious in reducing the seizure frequency in chronically epileptic rat (Grabenstatter, Clark et al. 2007; Ali, Dua et al. 2012).

EEG Review

The EEG record of the previous 24 hour period (midnight to midnight of the previous day) was reviewed daily by a trained EEG reviewer. The clinical behavior associated with any recorded abnormal EEG event was evaluated by a review of the synchronized video and any observed seizure recorded. Seizure severity, using a modified Racine Scale (Racine 1972; Ben-Ari 1985), was recorded for each confirmed seizure. Any rat that did not develop epilepsy over the course of sixteen weeks following SE, or who lost their EEG implant over the course of the study was removed from the study.

Study Design

In Study 1, upon confirmation of their first convulsive seizure, newly diagnosed epileptic rats were randomly enrolled into one of three treatment arms (see Figure 3.1b); 0% adherent, 50% adherent, 100% adherent. The 0% adherent group received unmedicated pellets for the duration of the six-week study and served as the placebo control. Epileptic rats enrolled into the 50% adherent arm received medicated pellets for 50% of their meals on a computer-generated randomized feeding schedule. Epileptic rats enrolled into the 100% adherent arm received medicated food pellets at each scheduled feeding. Upon randomization into one of the above described treatment arms, rats received their assigned treatment four times daily for six consecutive weeks.

In Study 2, diagnosed epileptic rats were enrolled into the 100% arm; i.e., CBZ-medicated pellets at every meal for two weeks. They were then crossed over to the placebo arm (0% medicated chow) for two weeks and then crossed back to the 100% medicated arm for the remaining two weeks of the study (see Figure 3.1b).

Data Analysis

The results from all review sessions were electronically stored into a single project file that contained the time and medication history of each individual feeding, the time of each seizure event, and the reviewer determined seizure stage. These data were analyzed in MATLAB. For all analysis, Grubbs exclusion outlier analysis was used, and we report the statistical significance for each test without outliers. A significance level of ($p < 0.05$) was used for all tests.

To evaluate the effect of nonadherence on seizure control, we analyzed the

observed seizure frequency and seizure severity for each rat. Statistical difference in seizure frequency between groups was determined using pair-wise student's t-test. To determine the difference in severity, the proportion of Stage 5 seizures was calculated for each individual animal and groups were compared using pair-wise student's t-tests.

Survival curves, i.e., time to nth seizure, were constructed to demonstrate the effect of treatment on the time to the nth seizure (3^{rd} consecutive convulsive seizure). The number of days observed for each rat to reach the 3^{rd} convulsive (Racine Stage 3 or higher) seizure was plotted for each of the three treatment arms of Study 1; i.e., 0%, 50%, or 100% adherent groups. A Kaplan-Meier estimator with a log-rank test was used to determine the significance level; $p<0.05$ was considered statistically significant.

To determine the relationship between the randomized dosing paradigm in the 50% adherent group and the resulting seizures, we performed a seizure-triggered average of the meals 96 hours prior to a seizure. Only meals that occurred after enrollment were included in the analysis. To determine the relationship between meal timing and the occurrence of a seizure, we binned the time at which a seizure occurred into 6 one-hour periods following meals. The goal was to determine if seizures occurred at a specific time in relationship to the most recent meal. The probability of a feeding being more likely to be medicated or unmedicated was tested using a two-tail binomial test.

Results

The results described herein demonstrate the feasibility of using a novel computer-automated pellet delivery system to deliver drug-in-food pellets to newly diagnosed epileptic rats to test the consequences of nonadherence on seizure control. This novel system and approach allows for the conduct of a chronic and controlled evaluation of the

impact of nonadherence on seizure control with a minimal impact on staff resources and minimal stress to the epileptic rat. In addition to being a novel combination of pharmacology, biology, and engineering, this study is particularly unique in that it mirrors the clinical management employed in the treatment of patients with newly diagnosed epilepsy using a commonly employed ASD.

A total of 101 animals were implanted with EEG electrodes and treated with kainic Acid (KA) to induce status epilepticus. Of these, 19 rats died as a result of the KA-induced SE, four rats did not meet enrollment criteria, i.e., first convulsive seizure following SE, and 29 lost their EEG implant before being enrolled. Of the 49 rats that were enrolled, eight did not complete the study due to EEG implant loss; 30 animals completed Study 1, and 11 animals completed Study 2. The breakdown of animals that successfully completed each study per group is summarized in Table 3.1. On average, rats reached enrollment criteria 24·8±20·0 s.d. days after KA-induced SE. The experimental timeline is shown in Figure 3.1a.

The EEG record of each rat was reviewed daily by a trained EEG reviewer. Figure 3.2a shows 24 hours of EEG recording from a single epileptic rat enrolled in the nonadherent (50%) group. Seven Racine Stage 4-5 seizures are embedded in this 24 hour EEG recording. The two hours surrounding a single Stage 4 seizure is shown in Figure 3.2b. Figure 3.2c illustrates the two minutes surrounding the same seizure. As shown in this montage, seizures were identified on the basis of their EEG pattern and confirmed by a review of the corresponding video. Electrographic seizures are characterized by rhythmic spiking lasting a minimum of five seconds and increased amplitude of two-fold or greater over baseline.

Table 3.1: Adherence groups evaluated in these studies. Following the first post-SE observed convulsive seizure, rats were randomly enrolled into one of the four listed experimental groups. Treatment consisted of delivering either all medicated pellets or all unmedicated pellets for a single meal. The name of each adherence group corresponds to the percentage of meals that were medicated over the course of the 6 week treatment trial.

Group	Adherence	N	Treatment
Study 1			
Placebo control	0%	9	No medicated meals, vehicle control group
Nonadherent	50%	10	Randomly administered medicated meals 50% of the time
Adherent	100%	11	Always administered medicated meals
Study 2	100-0-100%	6	100% for 2 weeks, 0% for two weeks, 100% for two weeks

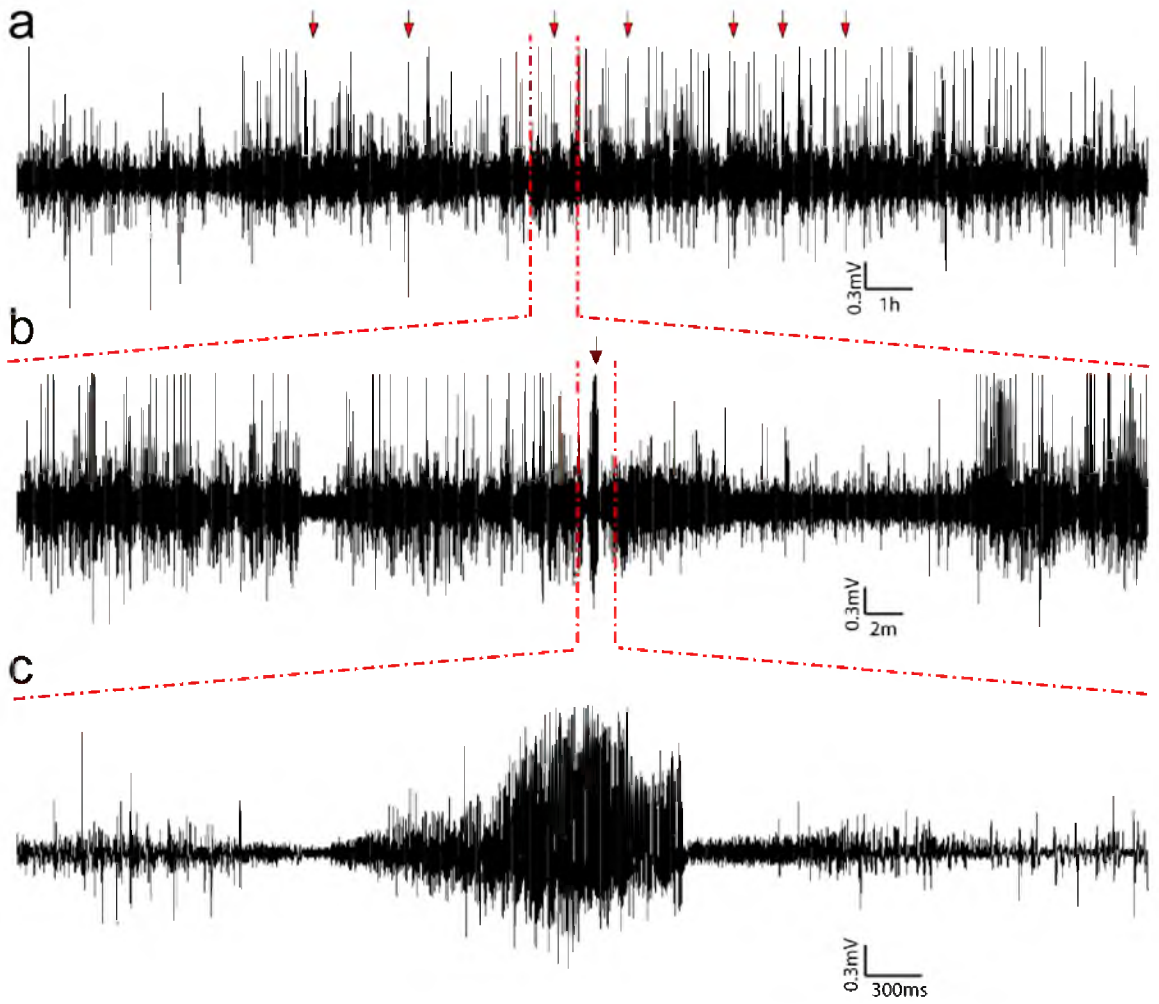

Figure 3.2. EEG data from a single epileptic rat observed to have several Racine Stage 4-5 seizures. (a) Over the course of 24 hours, seven Stage 4-5 seizures, indicated by red arrows, were recorded. Note that seizures are not easily distinguished at this time scale. (b) One hour of EEG centered on a Stage 4 seizure highlighted in (a). (c) Two minutes surrounding the same seizure from (b). A typical seizure characterized by increasing amplitude followed by fast cessation of spiking activity, is viewable at this time scale.

The raw seizure data from 2 rats in each of the treatment arms from Study 1 (0%, 50%, and 100%) and Study 2 (100%-0%-100%) are summarized in Figure 3.3. The relationship of each confirmed seizure to the feeding and dosing schedule is also shown in this figure. A qualitative review of the results plotted in Figure 3.3 illustrates the improved seizure control of epileptic rats in the 100% group compared to that of rats in either the 0% or 50% treatment arms. Note that animal A6 was the only animal in the 100% arm that achieved 100% seizure control; i.e., no further observed convulsive seizures after treatment initiation. It is also interesting to note the relationship of seizure control to dosing in the 100%-0%-100% treatment arm. Clearly, seizure control is improved during the two week period that the epileptic rats were receiving drug-in-food with every meal. Animal A8 is one of two rats from the 100%-0%-100% group that achieved full seizure control while on the medicated arm of their treatment schedule but lost control when removed from treatment. None of the animals in either the placebo arm (0%) or nonadherent (50%) group achieved full seizure control.

Animals that were adherent (100%) demonstrated a significantly lower seizure frequency and seizure severity when compared to rats that were enrolled in the nonadherent (50%) ($p=0.012$), or placebo control (0%) arms ($p=0.002$, Figure 3.4a,b). Interestingly, no detectable difference in seizure frequency or severity between epileptic rats in the placebo (0%) group compared to those in the nonadherent (50%) group was observed ($p=.28$, Figure 3.4a,b).

Survival curves are often used in clinical trials to illustrate the efficacy of a given treatment on outcome. As shown in Figure 3.4c, newly diagnosed epileptic rats enrolled into the adherent (100%) arm of Study 1 were more likely to remain seizure free than those

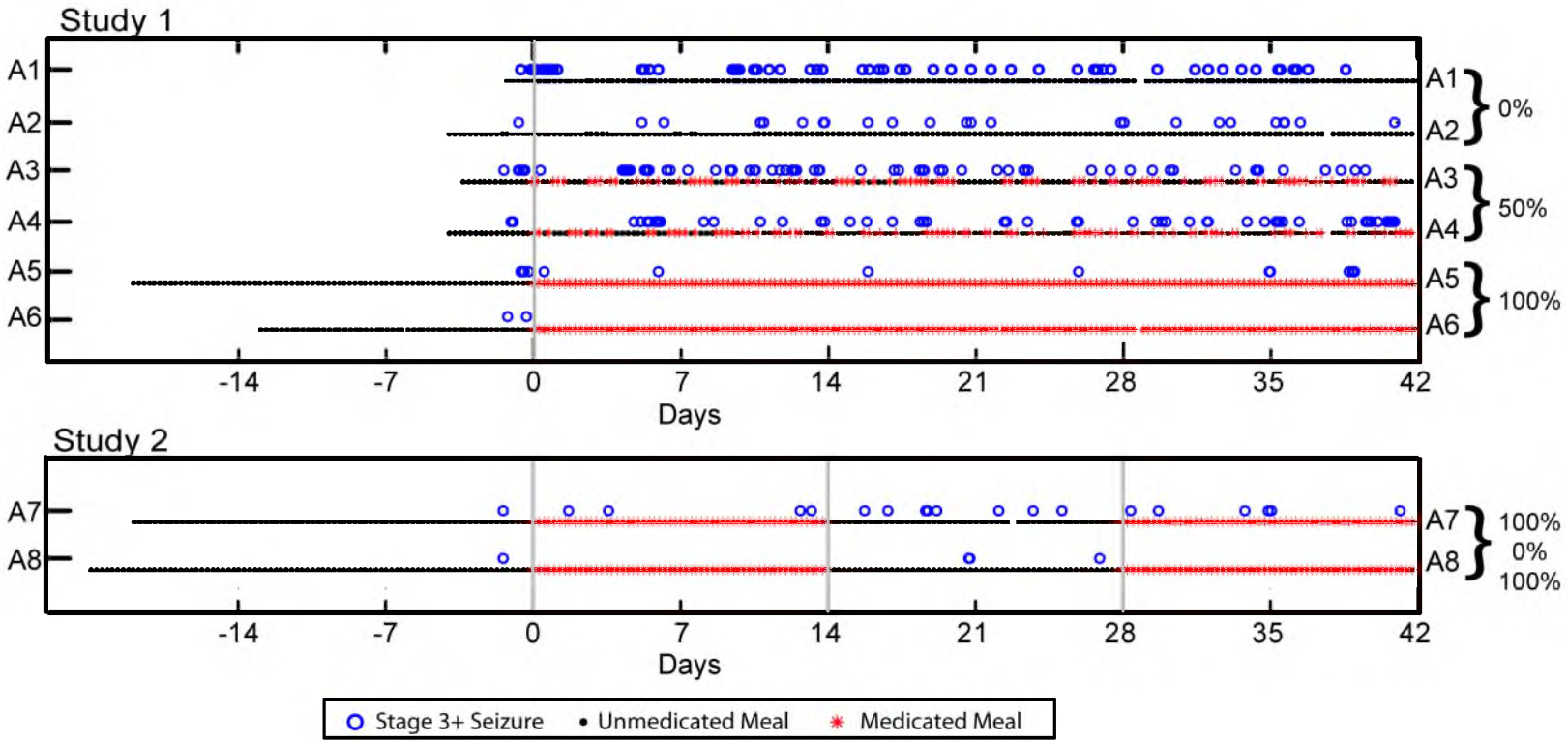

Figure 3.3. Raw seizure data in relationship to feeding schedule observed in eight individual animals, two from each experimental group. Data are aligned from the point of enrollment, negative time is the period following kainate treatment. Of the two animals in the 100% adherent group, one rat (A6) had full seizure control. In contrast, the second animal (A5) experienced frequent breakthrough seizures. Only one of 11 animals enrolled in the 100% group experienced full seizure control. In Study 2, animal A8 is one of two rats to have full seizure control during the periods with CBZ treatment.

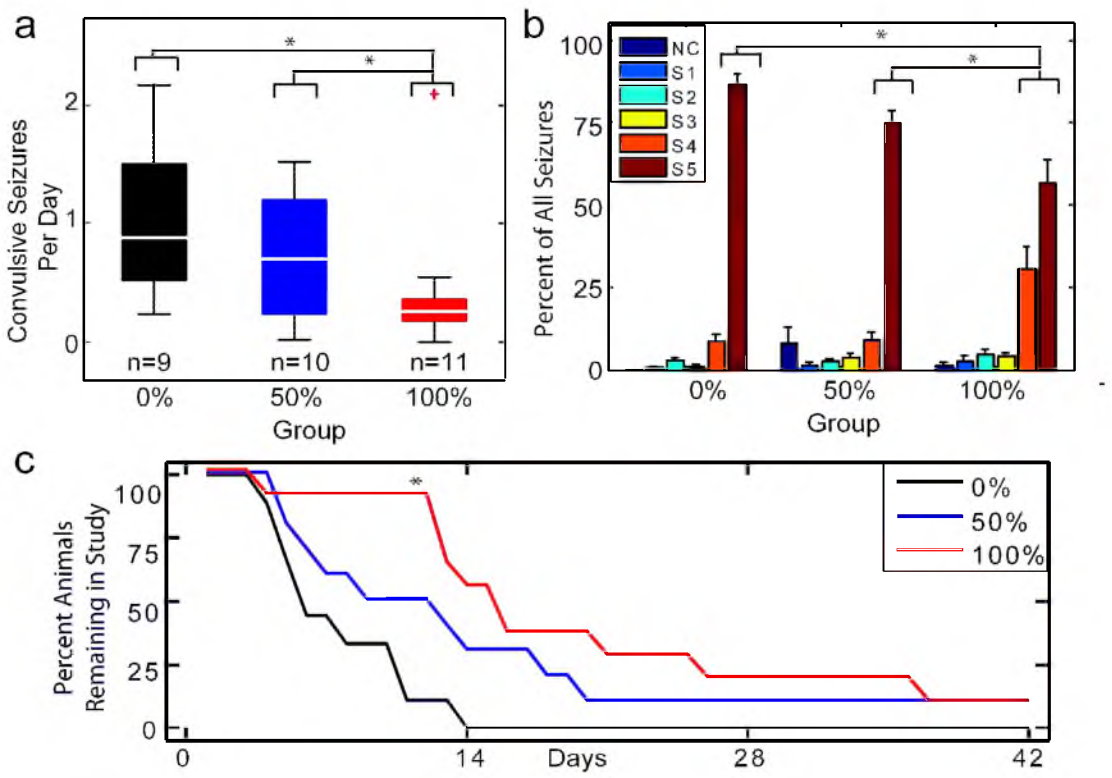

Figure 3.4. Results from the different groups show that newly diagnosed epileptic rats enrolled into the 50% adherent group performed similarly to animals that never received medication. (a) The dispersion of Stage 3-5 seizures per day for each group shows that the seizure frequency of epileptic rats enrolled into the100% group was lower than that of rats enrolled into either the 0% or 50% group (t-test, $p < 0.05$). (b) An analysis of the percentage of seizures by seizure stage reveals that the seizure severity of epileptic rats assigned to the 100% group was less on average than the seizure severity of those epileptic rats enrolled into either the 0% or 50% adherence groups (t-test, $p<0.05$). (c) A survival analysis of seizure freedom shows that animals in the 100% group were more likely to remain seizure free than those enrolled into the 0% or 50% adherence groups (Log rank, *$p<0.05$).

enrolled in the placebo (0%) treatment ($p = 0.008$) and nonadherent (50%) group ($p=0.012$). In contrast, rats enrolled into the nonadherent (50%) group showed no improvement in seizure free survival when compared to those enrolled in the placebo (0%) control ($p=0.91$) (Figure 3.4c).

We tested the hypothesis that the probability of a newly diagnosed epileptic rat experiencing a seizure was related to their medication history by evaluating the seizure history and medication record of each newly diagnosed epileptic rat enrolled into the 50% adherence group. Specifically, we analyzed the timing of the feedings to each observed seizure to determine if a relationship could be established between missed doses, i.e., unmedicated meals, and the occurrence of a seizure. To analyze missed doses and their relation to subsequent seizures, we looked at the 96 hour period prior to each confirmed seizure. The results of this analysis, i.e., the percentage of meals that were medicated prior to all seizures, are summarized in Figure 3.5. A significant probability between a seizure and a missed dose was found when the second ($p<0.001$), fourth ($p=0.006$), fifth ($p=0.0291$), and sixth ($p<0.001$) meal prior to a seizure were not medicated.

In Study 2, we sought to determine whether abrupt withdrawal of treatment with CBZ from epileptic rats in the 100% treatment group would result in an altered pharmacological response upon resuming treatment; i.e., 100%-0%-100%. Results summarized in Figure 3.6a demonstrate that abrupt CBZ withdrawal leads to an increased seizure frequency ($p<0.01$); however, the loss of seizure control was not associated with a worsening of seizure control upon reinitiating CBZ treatment ($p = 0.79$, Fig. 3.6a). Five rats were rejected from the analysis of Study 2 due to improper switching of the medication, however, inclusion of these animals into analysis did not change the significance or

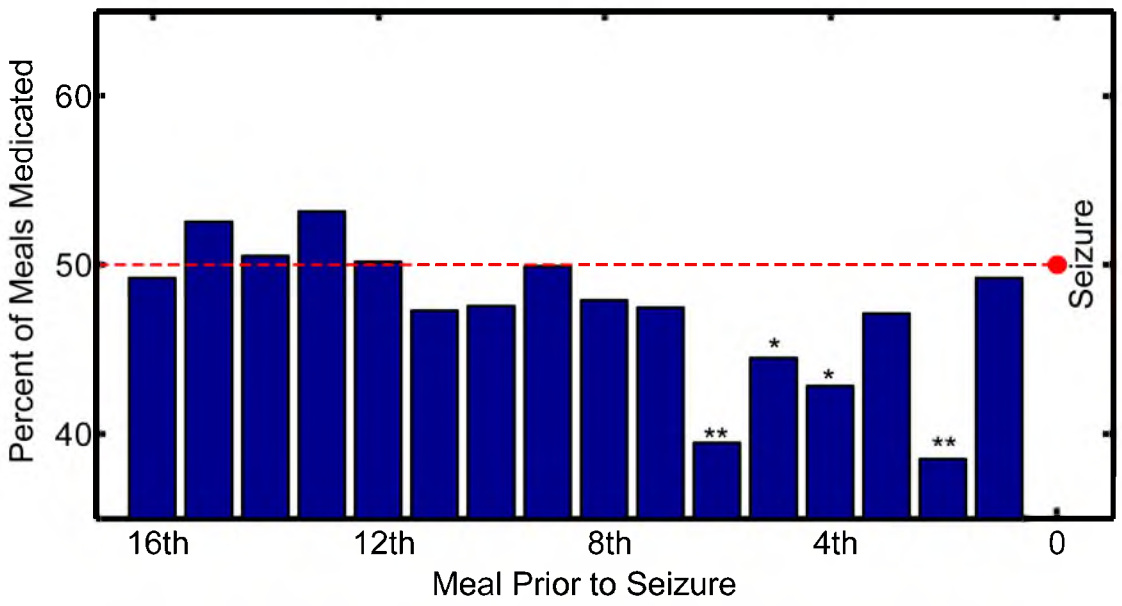

Figure 3.5: Historical relationship between medicated meals and seizures. For the nonadherent (50%) group, we examined what percentages of meals were medicated prior to a seizure. For example, 42% of all second meals prior to seizures were medicated, while approximately 49% of all of the meals directly before all seizures were medicated. A significant relationship was found for the second, fourth, fifth, and sixth meal. (*$p < 0.05$,**$p < 0.01$)

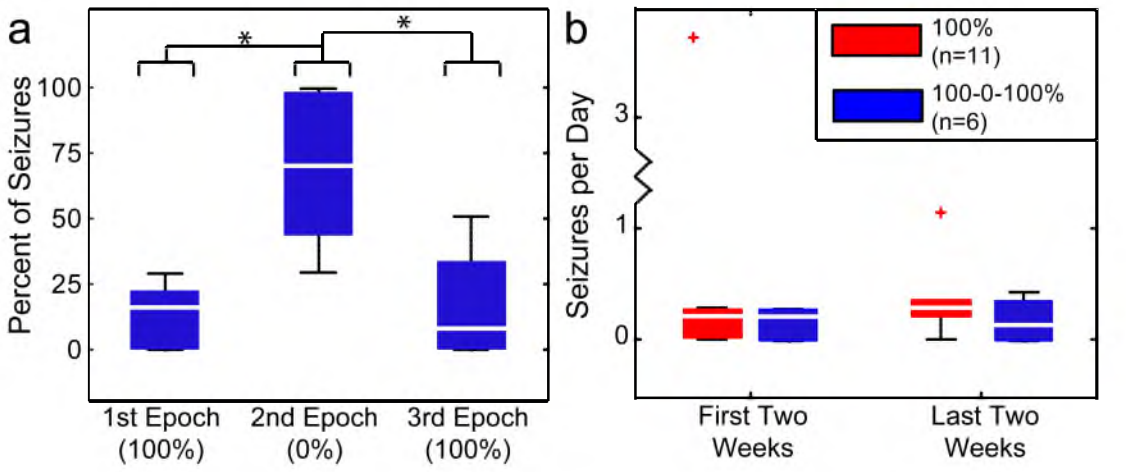

Figure 3.6. Observed seizure frequency of newly diagnosed epileptic rats enrolled into the 100%-0%-100% adherent group. (a) Newly diagnosed epileptic rats displayed significantly more seizures during the period when they were removed from CBZ (2nd epoch). However, despite an increase in seizures during this drug-free period ($p<0.01$), the same degree of seizure control for the population as a whole was regained when CBZ treatment was restored ($p=0.79$). (b) Additionally, animals from Study 2 showed no difference in seizure control during the last two weeks from animals in Study 1 enrolled into the 100% treatment arm ($p=0.42$).

interpretation of the data. Interestingly, the degree of seizure control in the last two week epoch of the 100%-0%-100% study was not significantly different from the last two week epoch of the adherent (100%) animals from Study 1 ($p = 0.42$, Fig. 3.6b).

Discussion

This study is the first preclinical study to model the impact of nonadherence in an animal model of chronic epilepsy. Previously, the study of nonadherence has been limited to clinical observations which are reliant on patient reporting. The results described herein demonstrate the value of directly testing the consequences of nonadherence in newly-diagnosed rats with epilepsy. Further, the present investigation describes the utility of a novel automated feeder system for the chronic delivery of drug-in-food pellets to newly diagnosed epileptic rats.

In an effort to mimic the clinical management of human epilepsy, we designed a novel treatment paradigm to treat newly diagnosed epileptic rats. Previous studies investigating drug response in chronic epileptic animals have traditionally initiated drug treatment immediately after the insult and before diagnosis, or after a fixed period after the animal has become fully epileptic. However, these paradigms do not accurately mimic what takes place in the clinic, where patients typically begin treatment after their second seizure. The approach described herein, in which rats initiate treatment at the time of diagnosis; i.e., the first unprovoked seizure, provides a paradigm shift which closer reaches the goals of bench to bedside translational science.

In all metrics used to analyze seizure outcome, rats enrolled into the nonadherent (50%) group displayed less seizure control than those in the adherent (100%) group.

Although it is not necessarily surprising that nonadherence leads to less seizure control, as this has been shown in numerous clinical studies, the results obtained from this first preclinical epilepsy study of animal adherence, serves as an important validation of the experimental approach using a computer-controlled delivery system that provides tight control over oral medication dosing (Stanaway, Lambie et al. 1985; Manjunath, Davis et al. 2009). Interestingly, the lack of any significant difference between the nonadherent (50%) and the placebo control (0%) group, may suggest that patients practicing nonadherence at a rate of 50% are as likely as fully nonadherent patients to experience poor seizure control. However, in the clinical setting, nonadherence follows more complex trajectories than that modeled in the present investigation (i.e., a fixed rate of 50%). Previous investigations have shown that children with newly-diagnosed epilepsy display adherence patterns that follow one of five patterns over six months, ranging from fast-nonadherence to near-perfect adherence (Modi, Rausch et al. 2011). While our experimental paradigm only tested a single fixed rate, future studies seek to model real-world patterns of nonadherence.

Interestingly, a significant relationship between meal history and seizures was observed. For example, a rat was more likely to have a seizure when the second, fourth, fifth and sixth meal prior to a seizure was unmedicated. Interestingly, the probability of a seizure occurring when the meal prior to a seizure was unmedicated was not different from that when the meal prior to a seizure was medicated. These results suggest that the consequences of a missed dose are not immediate and a seizure may occur up to several days after the missed dose. It is well established that CBZ has a measurable therapeutic effect well beyond its half-life, due to the presence of its active metabolite, carbamazepine-

epoxide (Morselli, Baruzzi et al. 1977). Therefore, the finding that a seizure may be the result of missed dose that occurred well beyond the half-life of CBZ is not necessarily unexpected. However, the results summarized in Fig 2.5 also demonstrate that there are likely to be scenarios in which patients practicing nonadherence would likely experience a seizure even though they took their prescribed ASD immediately prior to a seizure. In such a case, the patient may consider the seizure as a failure of the medication to control their seizures rather than a consequence of their own nonadherence.

Clinical observations have shown that rapid CBZ withdrawal leads to an increased seizure burden (Malow, Blaxton et al. 1993). Thus, it is not surprising that an abrupt worsening of seizure control was recorded after animals stabilized on 100% CBZ were abruptly switched to the placebo food (0%). Interestingly, this abrupt worsening of seizure control did not negatively affect subsequent seizure control when animals were placed back on the 100% CBZ-in food diet; i.e., substantial seizure worsening off of CBZ did not lead to a subsequent CBZ-resistant state. This finding is in agreement with clinical experience which suggests that the majority of patients are fortunately able to regain seizure control if they experience breakthrough seizures as a result medically supervised ASD withdrawal (Schmidt and Loscher 2005). However, clinical observations compiles results from patients who have been prescribed a variety ASDs, and have a complicated medication history. Since navigating these variables adds a layer of complexity, the model and approach to ASD delivery described here provides a mechanism to eliminate the myriad of factors that can lead to seizure breakthrough, thereby leading to a focused approach under tightly controlled conditions.

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CHAPTER 4

A SIMPLE QUANTITATIVE METHOD FOR ANALYZING ELECTROGRAPHIC STATUS EPILEPTICUS IN RATS

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Innovative Methodology

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A Simple Quantitative Method for Analyzing Electrographic Status Epilepticus in Rats

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Lehmkuhle MJ, Thomson KE, Scheerlinck P, Pouliot W, Greger B, Dudek FE. A simple quantitative method for analyzing electrographic status epilepticus in rats. *J Neurophysiol* 101: 1660–1670, 2009. First published January 7, 2009; doi:10.1152/jn.91062.2008. Electrographic status epilepticus (ESE) is a medical emergency consisting of repetitive seizures and may result in death or severe brain damage. Epilepsy can develop following ESE. The properties of ESE (e.g., duration and intensity) are variable, as are the effects of putative therapeutic treatments. Therefore a straightforward method to quantify different components of ESE would be beneficial for both researchers and clinicians. A frequency range close to the gamma band was selected for extraction of seizure-related activity from the EEG. This filtering strategy reduced motion artifacts and other noise sources in the electrophysiological recordings, thus increasing the signal-to-noise ratio of the EEG spike activity. EEG spiking was quantified using an energy operator and modeled by an eighth-order polynomial. In a benzodiazepine-resistant rat model of pilocarpine-induced ESE, the efficacy of various pharmaceutical agents at suppressing ESE was analyzed with this and other methods on data collected for ≤24 h after ESE induction. This approach allows for the objective, quantitative, and rapid assessment of the effects of both short- and long-lasting pharmacological manipulations on ESE and other forms of prolonged repetitive electrical activity.

INTRODUCTION

Electrographic status epilepticus (ESE) is defined clinically as repetitive seizures lasting 30 min or longer, although recent publications have suggested that seizures lasting only 10 min (or even a few repetitive seizures) should be treated as quickly as possible (Bleck 2005; Chen and Wasterlain 2006; Treiman et al. 1998). EEG has been successfully incorporated in the early diagnosis of nonconvulsive ESE (Bautista et al. 2007). ESE often occurs after various forms of brain injury such as stroke (Arboix et al. 1997; Davalos et al. 1988; Young 2007), can arise in patients with epilepsy who are withdrawn from anti-epileptic drug medication (Gayatri and Livingston 2006; Martinez-Cano et al. 1995), and results from exposure to nerve agents (Holstege and Dobmeier 2005). As EEG becomes more widely used for clinical diagnosis, a simple but quantitative method for assessing the properties of ESE is likely to be useful for clinical research concerning the short- and long-term effects of repetitive seizures associated with brain injury.

ESE is also used experimentally as a brain insult to model the development of epileptogenesis in rodents (Ben-Ari 1985; Loscher 2002; Lothman and Collins 1981; Nadler 1981; Turski et al. 1989). It is widely known that chemo-convulsants (such as kainate and pilocarpine) and certain patterns of repetitive electrical stimulation of the amygdala, hippocampus, and other structures can cause prolonged and repetitive seizure activity (i.e., ESE) that persists for hours and can even extend into days. These bouts of ESE are generally followed by the development of chronic epilepsy, which has been widely used as a model for studying the mechanisms of epileptogenesis. Numerous experimental issues in this area of research would benefit from better quantification of the ESE.

The clinical relevance of ESE and the wide use of ESE models in the study of epileptogenesis show the need for methods to quantify ESE activity. The purpose of this study was to develop such a method and compare its performance with other means of scoring ESE activity. Specifically, we determined whether quantifying and modeling the power in the γ-band frequencies (20–70 Hz) in the raw EEG signal would provide a simple quantification of activity representing ESE. The γ-band, an approximate frequency range of field excitatory postsynaptic potentials that would be expected to occur during seizures, was shown to contain dynamic power in the EEG during ESE. We compared the performance of the γ-band power method with human evaluation methods and also with quantification of the number of spike events and the spike frequency. Human evaluation of the various distinct stages of ESE has been extensively characterized (Treiman et al. 1990) and has been modified and adapted to provide for analysis of a drug's effectiveness for the treatment of ESE (Prasad et al. 2002). Groups of benzodiazepine-resistant rats were compared in terms of the duration and severity of the ESE or the effects of pharmacological intervention on ESE. The γ-band power method detected differences between treatments that were not observed with human scoring or spike-based analyses, suggesting that this method may identify prominent temporal features of ESE that are not noticeable with categorical or rate-based observations. This method for assessing the severity of ESE has implications for objectively screening treatment therapies for benzodiazepine-resistant ESE.

METHODS

Electrode implantation

All surgical procedures were performed under protocols approved by the University of Utah Animal Care and Use Committee. Sprague-Dawley rats (180–220 g) were implanted with surface electrodes as follows. The animals were anesthetized with isoflurane (2%) and

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placed in a stereotaxic unit. A bipolar (with ground) electrode (MS333-3-B, Plastics One, Roanoke, VA) was used for surface recordings. Two holes (500 μm) were drilled on the right side of the midline, and one lead was placed into each of the craniotomies to provide differential recordings. A third lead was placed in a third craniotomy left of the midline to be used as the ground electrode. The electrodes were fixed in place with dental cement, and the skin was sutured around the skull. After surgery, the animals were put on a 12-h light/dark cycle and fed standard rat chow ad libitum. After recovery from the surgery (≥7 days), the animals were treated with pilocarpine.

Video and EEG recording

Implanted animals were placed into cages equipped with commutators and cables (Plastics One) and were connected to the cables via their skull caps for EEG recording. Signals were amplified using EEG100C amplifiers (high-pass filter, 1 Hz; low-pass filter, 100 Hz; 5,000× gain), digitized at 500 Hz using an MP150 digital-analog converter, and acquired with AcqKnowledge acquisition software (BioPac Systems, Santa Barbara, CA). While the rats were tethered in these cages, they were continuously video monitored using eight color cameras linked to a multiplexer to allow for eight animals to be recorded on one DVD. Recordings were made for 24 h across three DVD recorders (DMR-ES20, Panasonic), each recording 8-h periods.

Pilocarpine and drug treatment

After recovery from surgery, animals were connected to the video-EEG recording setup and pretreated 18–24 h before pilocarpine administration with LiCl (127 mg/kg, ip). Scopolamine bromide (1 mg/kg, ip) was administered 30 min before the administration of pilocarpine (50 mg/kg, ip). Animals were grouped in four categories: *1*) animals receiving vehicle (saline) injection 60 min after the first convulsive seizure (vehicle), *2*) animals receiving propofol (100 mg/kg) 60 min after the first convulsive seizure (propofol), *3*) animals receiving diazepam (10 mg/kg) 60 min and vehicle 70 min following the first convulsive seizure (diazepam), and *4*) animals receiving diazepam (10 mg/kg) 60 min and a test compound (60 mg/kg) 70 min following the first convulsive seizure (test). The test compound was a coded compound and part of the Antiepileptic Drug Development Program to identify potential new treatments for ESE. A final group of animals, which were not treated with pilocarpine, were injected with either saline (vehicle) or saline plus propofol to assess the effects on EEG power without ESE.

Identifying the stages of ESE

Two different human-based evaluations of ESE were made (Table 1).

TABLE 1. *Human-based evaluation of electrographic status epilepticus*

Five-Point	Six-Point
1) Normal or slow isolated EEG spikes	*1*) Normal or slow isolated EEG spikes
2) Intermittent discrete seizures	*2*) Discrete seizures
3) Continuous seizure activity with fast spiking	*3*) Merging seizures
4) PEDS with intermittent superimposed seizures	*4*) Continuous ictal activity
5) PEDS alone	*5*) Continuous ictal activity with flat periods
	6) PEDS

The EEG records were scored based on these descriptors. PEDS, periodic epileptiform discharges.

These methods were based on previously published scales here termed the "five-point" method (Prasad et al. 2002) and the "six-point" method (Treiman et al. 1990; Walton and Treiman 1988). Each animal's EEG was evaluated at each hour starting at the injection times described above through 10 h. In a 5-min epoch after each hour, the EEG was scored according to the stage of ESE. Kruskal-Wallis one-way ANOVA on ranks for repeated measures was performed to determine if median scores were significantly different at 1, 3, and 10 h after the injection times.

Spikes and spike rate

The number of spike events from each EEG trace was calculated by setting thresholds 3 SD above and below the mean of a baseline period taken before pilocarpine injection. Each threshold crossing (both positive and negative) was counted over time to determine the number of spike events and the spike rate. The mean and 95% CIs of the spike rate were calculated for each group. This process was repeated for EEG data filtered in the γ-band (20–70 Hz, as described above).

Quantitative modeling

The raw EEG data were defined as $x(t)$, $t \in T$, with T being the discrete time interval of the entire experiment, where $t = 0$ was defined as the time of the first pharmacological treatment injection. This aligned all data to the same physiologically relevant time point. First, data were filtered with a fifth-order Butterworth band-pass filter. The Butterworth filter was $H(t)$ selected due its constant magnitude response across frequencies in the pass-band, as well as the quick roll-off in the stop-band. The γ-band filtered EEG signal $f(t)$ was generated by convolution (*Eq. 1*), and the power in the γ-band $e(t)$ was calculated by squaring the value of each sample (*Eq. 2*). The exclusion of frequencies outside the γ-band reduced the contribution of movement artifacts and other noise sources present in the EEG signal during analysis.

Because the analysis aimed to quantify gross seizure activity, the energy was convolved with a 5-min "boxcar" window to smooth the signal (*Eq. 3*). The resulting smoothed data $c(t)$ were downsampled to 1 sample/s, shown by *Eq. 4*, where k is the resulting time index

$$f(t) = x(t) \times H(t) \tag{1}$$

$$e(t) = |f(t)|^2 \,\forall_t \tag{2}$$

$$c(t) = e(t) \times boxcar \tag{3}$$

$$d(k) = c(500k) \forall k \in \frac{T}{500} \tag{4}$$

A "baseline", b, representing seizure-free EEG for each EEG data trace was selected by hand and calculated from 10 min of data from $d(k)$ ~20 min before the pilocarpine injection (*Eq. 5*), where t_b was the selected starting location for calculation

$$b = \frac{1}{600} \sum_{k=t_b}^{t_b+600} d(k) \tag{5}$$

The time epoch used to determine baseline was hand selected to ensure a noise- and event-free signal. The mean of these 10-min baseline intervals was meant to represent the activity generated by normal cortical behavior of the awake but quiescent animal. These baseline calculations were used to adjust the power of the EEG signal to account for amplifier variability. Electrographic seizure activity was modeled with a eighth-order polynomial, $p(k)$, fit to the data, $d(k)$, from $k = 0 - 10$ h. Because the EEG activity and severity of ESE can vary on the time scale of

seconds, the polynomial served to model the trend of the electrographic response and remove intra-animal variability. After the polynomial was fit, the starting point was scaled to be 100%, and the baseline remained as 0%. Statistical analysis was performed on both the spike rate analyses and the model estimations of each treatment group using one-way ANOVA with Tukey's post hoc analysis and Bonferroni correction for repeated measures at 1, 3, and 10 h after the treatment injections. Spectrograms were calculated from 1–100 or 200 Hz in 10-s bins with 5 s of overlap (Chronux Matlab toolbox, http://chronux.org/).

RESULTS

ESE

Video EEG data were acquired from 38 animals across the four treatment groups (vehicle, $n = 11$; propofol, $n = 6$; diazepam, $n = 11$; and test, $n = 10$) for 24 h after Li-pilocarpine injection. The quality of recordings included *1*) artifact-free EEG, characterized by high signal-to-noise ratio and distinct seizure

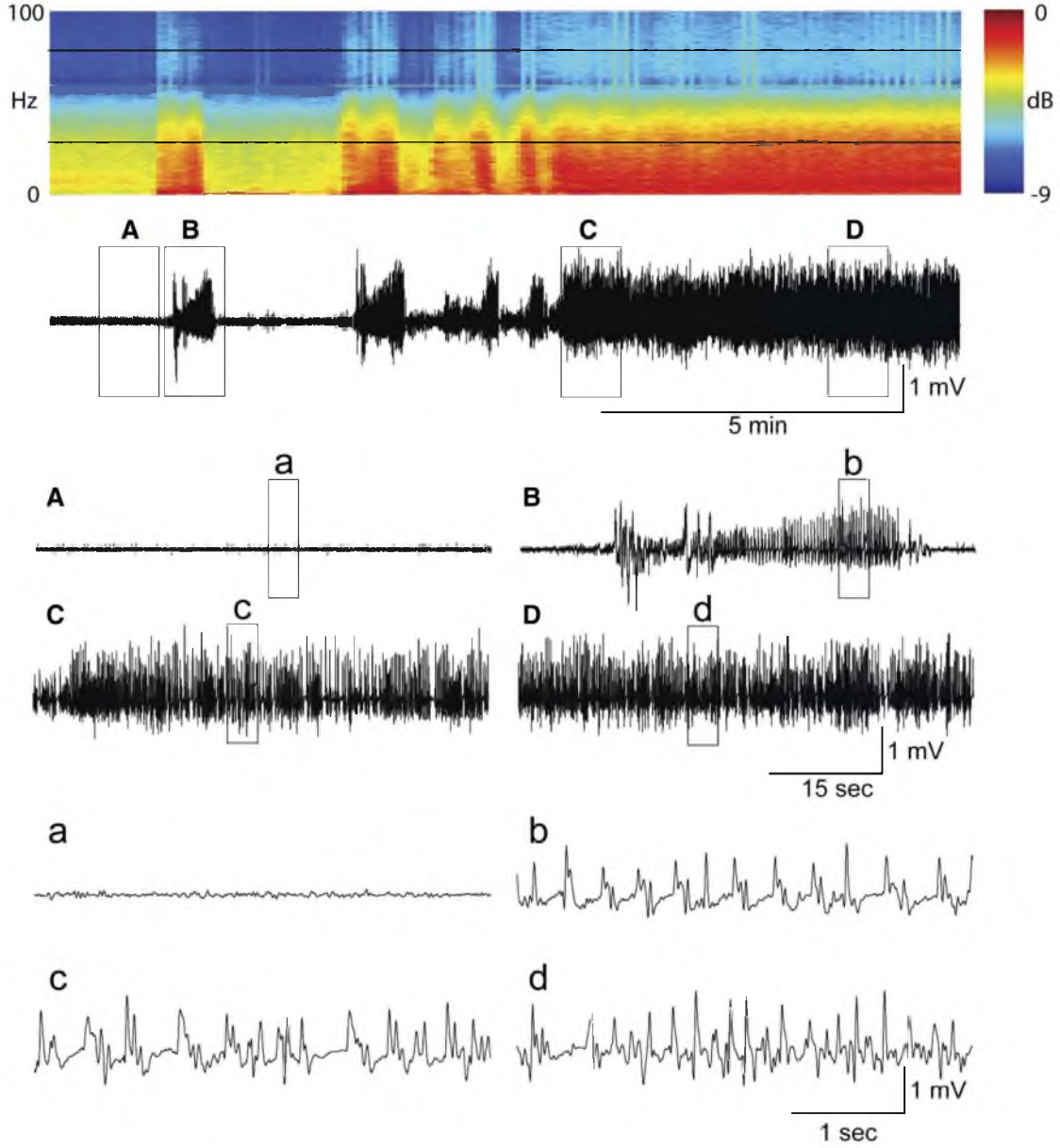

FIG. 1. Artifact-free electrographic status epilepticus (ESE). The raw EEG trace is 15 min long and was taken at the beginning of ESE after Li-pilocarpine treatment. At the *top* of the figure is the spectrogram (i.e., power spectrum over time) of the raw EEG. The light black bars bracket the γ-band frequencies (20–70 Hz) used in this study and show an increase in EEG power during ESE. Each box (A–D) shows 1-min enlargements of the data. These traces are further enlarged to 5-s (a–d). *A*: normal EEG before seizures. *B*: the 1st electrographic seizure. *C*: sample EEG trace during the beginning of ESE. *D*: sample EEG trace ~5 min after the start of ESE. This trace represents raw (unfiltered) EEG that is free of movement and electrical artifacts.

activity, and *2*) artifact-prone EEG that was corrupted with movement-induced artifacts that could be traced to convulsive or other motor activity. An example of a typical artifact-free EEG recording from the diazepam group can be seen in Fig. 1. Particular features of this EEG recording have been enlarged to show temporal detail. Conversely, the data in Fig. 2 are representative of an artifact-prone EEG recording (from the diazepam group) in which high-amplitude, movement-related artifacts are present (marked with arrows in the expanded traces). All recordings showed power in the gamma and other frequencies during status epilepticus, as can be seen in the spectrograms of Figs. 1 and 2, regardless of signal quality. The first steps for analyzing ESE were to benchmark the EEG response in the four treatment groups using *1*) human categorical scoring and *2*) by calculating the number of spike events over time.

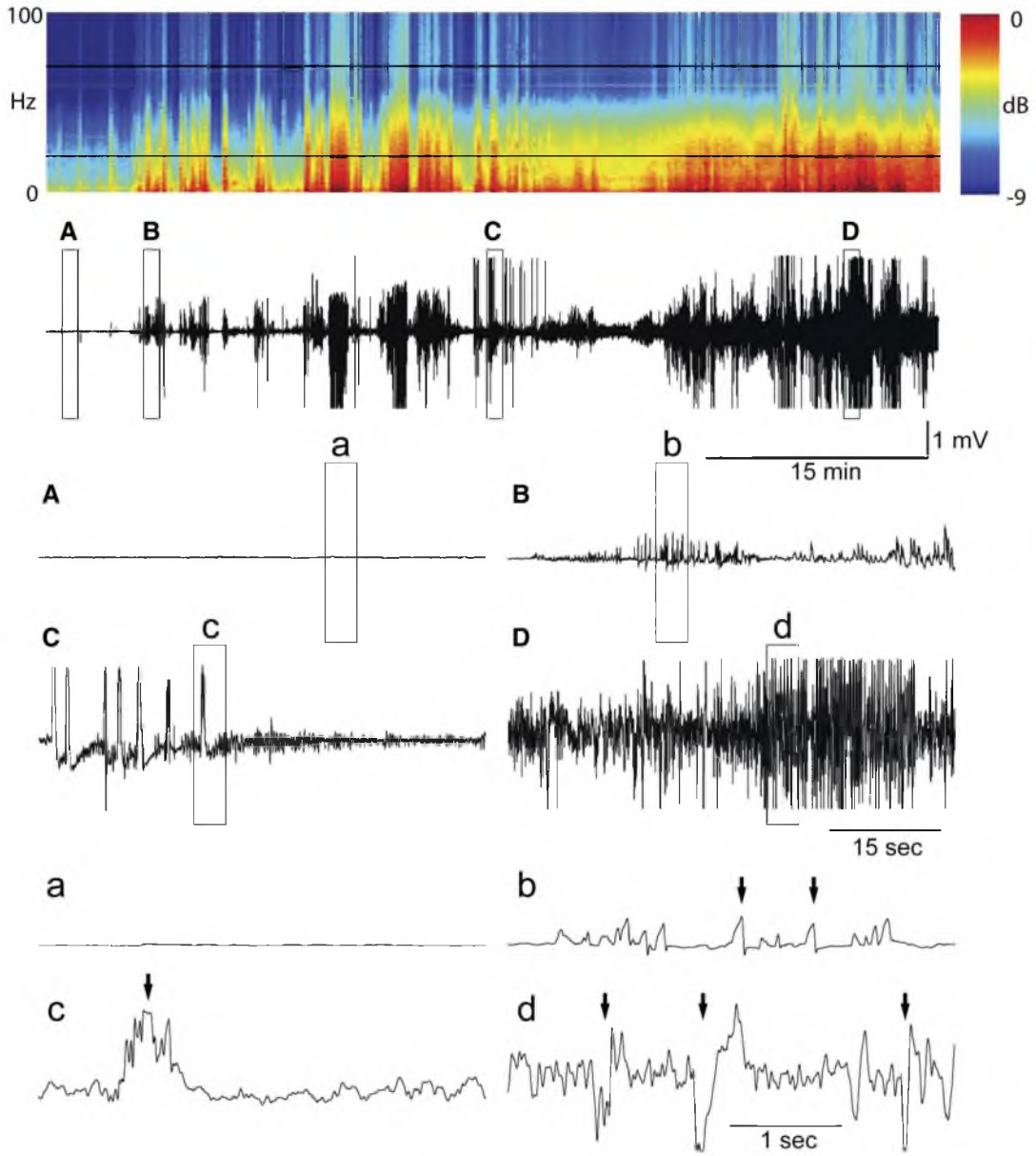

FIG. 2. Artifact-prone ESE. The format is similar to Fig. 1. The raw EEG trace is 60 min long and was taken at the beginning of ESE after Li-pilocarpine treatment from a different animal. This EEG recording contains numerous movement-induced artifacts, suggested by the arrows. *A*: normal EEG before seizures. *B*: the 1st electrographic seizure. *C*: sample EEG trace during seizure. *D*: sample EEG trace after the start of ESE. The voltage scale is conserved across traces.

Human analysis

Human evaluation of every raw EEG recording trace was performed by a single expert in the laboratory with years of experience recording and observing rodent ESE. The human analysis involved identifying particular EEG waveforms in rodent ESE in hourly increments following the initial treatment injection. The EEG's were scored based on the descriptors in Table 1 according to the five-, and six-point methods (see METHODS). The median scores of each treatment group were determined from scored ESE of each animal and Kruskal-Wallis ANOVA were performed at three different time points: 1, 3, and 10 h after the initial treatment injection (Fig. 3). Propofol treatment by 3 h after the first motor seizure significantly changed the ESE score based on the five- and six-point methods (five-point: propofol-vehicle, $P < 0.01$; propofol-diazepam, $P < 0.05$; propofol-test compound, $P < 0.01$; six-point: propofol-vehicle, $P < 0.001$; propofol-diazepam, $P < 0.01$; propofol-test compound, $P < 0.05$). No other treatment groups were significantly different from each other at the times tested. These categorical methods were derived from published methods for comparison between treatment groups enabling us to evaluate the results of the γ-band power algorithm.

Spike rate calculations

An automated routine that measures the number of spike events over time was applied to EEG data to further bench-

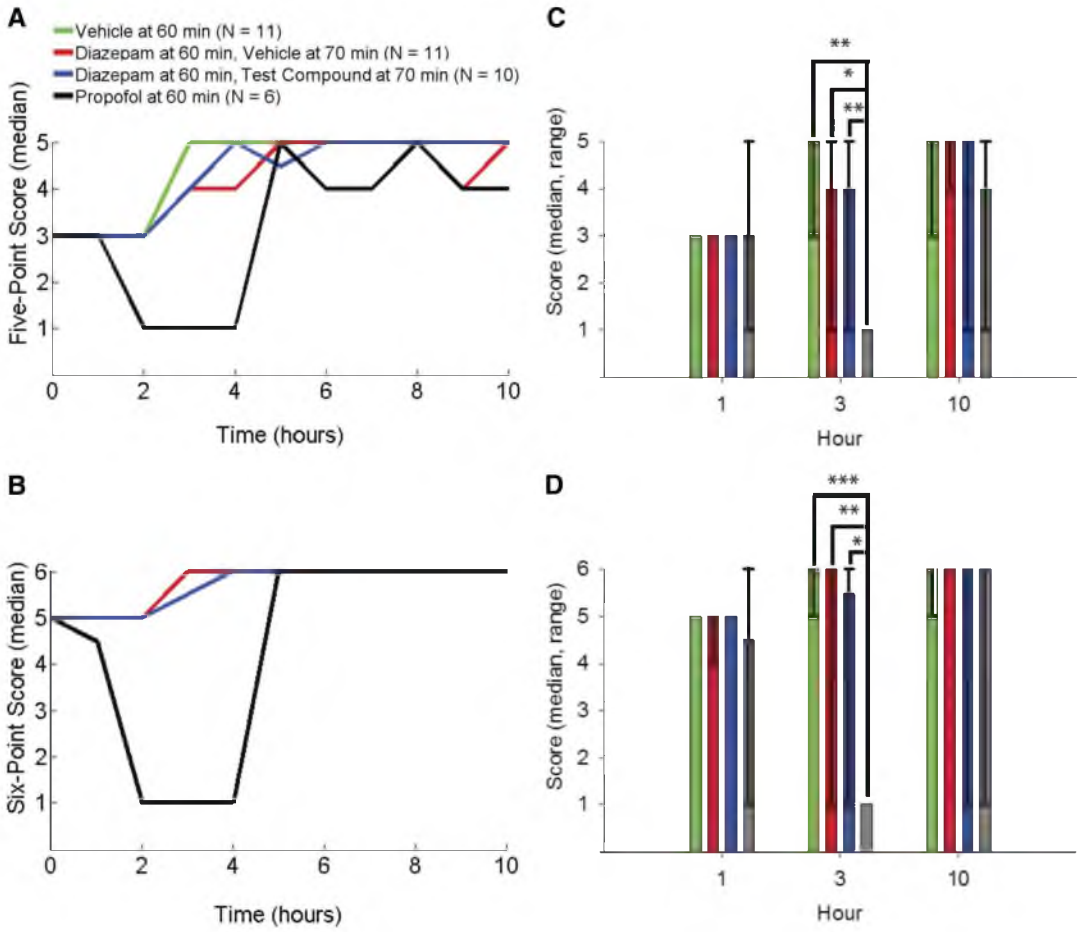

FIG. 3. Human evaluation of ESE. Data are the median of the human-scored ESE starting 1 h after the 1st motor seizure (the initial treatment injection, time = 0 h) in 1-h increments (±5 min) for 10 h. Individual data points are connected with lines for clarity and are not meant to represent a continuous function. *A*: "5-point" method. *B*: "6-point" method. Green trace shows vehicle (saline) injection delivered 60 min after the first motor seizure ($n = 11$). Red trace shows diazepam injection delivered 60 min after the 1st motor seizure followed by vehicle (saline) injection delivered 70 min after the 1st motor seizure ($n = 11$). Blue trace shows diazepam injection delivered 60 min after the 1st motor seizure followed by test compound injection 70 min after the 1st motor seizure ($n = 10$). Black trace shows propofol injection delivered 60 min after the 1st motor seizure ($n = 6$). *C* and *D*: Kruskal-Wallis ANOVA of the data in *A* and *B* at 1, 3, and 10 h after the initial treatment injection (median and range). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Note the vehicle trace (green) in *B* follows and is obscured by the diazepam + vehicle trace (red). Human evaluation of ESE indicated the effectiveness of propofol treatment at reducing ESE to near baseline (determined before inducing ESE) levels. Kruskal-Wallis ANOVA suggested that the 5-point and 6-point methods closely match each other for describing changes in ESE over time.

mark responses to the four treatments. The number of spike events from each EEG trace was calculated by selecting thresholds 3 SD above and below the mean of a baseline period taken before pilocarpine injection. Differing from the format for human evaluation, the mean and 95% CIs of each treatment group were calculated from the spike rate of each animal and one-way ANOVA were made (Fig. 4). This process was repeated for EEG data filtered in the γ-band (20–70 Hz). The raw EEG spike rate indicated that propofol treatment was significantly different only from the diazepam group on the third hour following injection ($P < 0.05$). The filtered EEG spike rate, however, indicated that propofol treatment was significantly different from all other treatments the first hour after injection (propofol-vehicle, $P < 0.01$; propofol-diazepam and propofol-test compound, $P < 0.05$) and from all other treatments except the test compound group treatment on the third hour after treatment injection (propofol-vehicle, $P < 0.05$; propofol-diazepam, $P < 0.01$). These results differed from the human scoring data possibly suggesting that the spike rate method (after band-pass filtering to isolate the γ-band activity) may be more sensitive to changes between treatment groups than human scoring.

Model estimations

Because EEG data show increased activity in the γ-band during ESE, a quantitative method was developed to estimate drug treatment effectiveness on changes in EEG power over the frequency range of 20–70 Hz. The raw EEG was first filtered and energy in the signal calculated. An eighth-order polynomial was fit to the energy data as an estimation of the animals' electrographic response to treatment. An example of this method applied to artifact-free and artifact-prone ESE in response to the diazepam treatment can be seen in Fig. 5 representing the data in Figs. 1 and 2, respectively. Filtering data from 20 to 70 Hz removed harmonics of 60-Hz electrical noise and attenuated much of the movement-related artifacts. This process was repeated for every animal in every treatment group. Examples of the polynomial modeling of ESE energy in each of the treatment groups can be seen in Fig. 6. The mean and 95% CIs of all the γ-band energy and polynomial models in each of the treatment groups (vehicle, diazepam + vehicle, diazepam + test compound, propofol) were calculated (Fig. 7). Model estimations were normalized to the polynomial power estimation at the first treatment injection such that comparisons could be made between the effectiveness of each treatment for

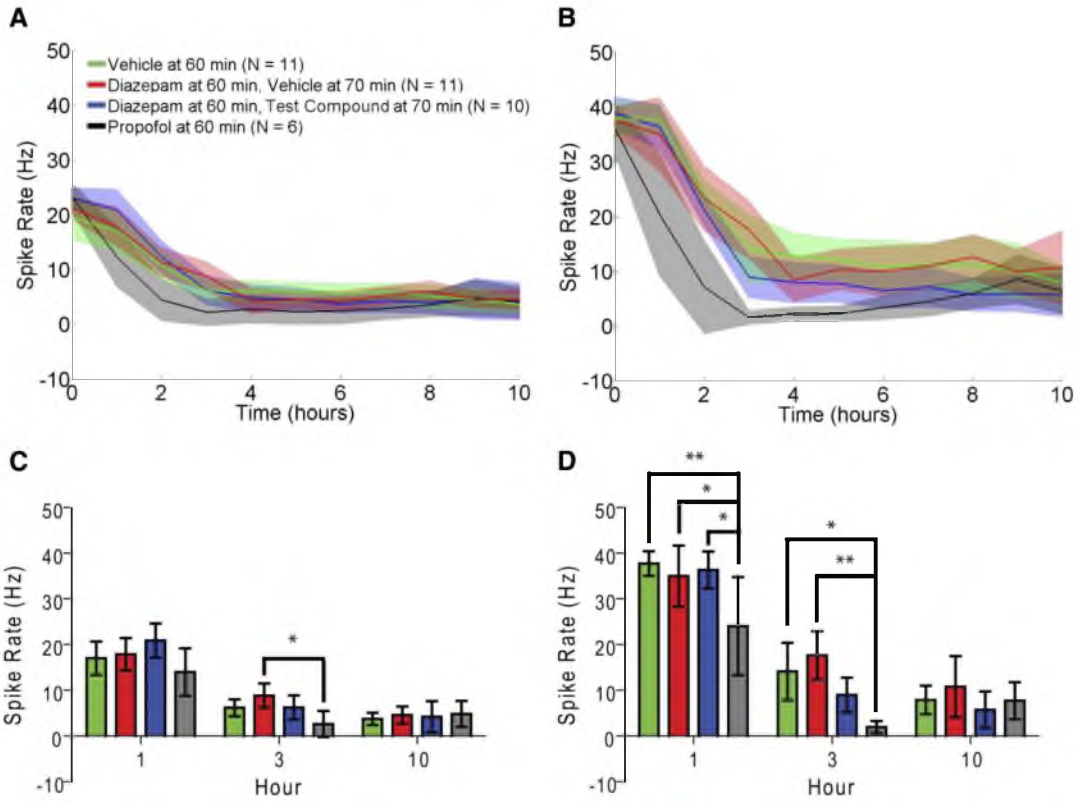

FIG. 4. Spike rate evaluation. Data are the mean and 95% CIs of the spike rate calculated in 1-h increments. *A*: raw EEG. *B*: EEG data filtered in the γ-band (20–70 Hz). Data groups are the same as in Fig. 3. *C*: data selection from *A* at 1, 3, and 10 h. *D*: data selection from *B* at 1, 3, and 10 h. $*P < 0.05$, $**P < 0.01$. Calculating spike rate from the raw EEG did not describe changes in ESE as well as human evaluation. Filtering the EEG in the γ-band improved the divisibility of the treatment groups, but identified differences in the 1st hour after treatment that were not identified by human evaluation. Filtered EEG spike rate did fail to detect a difference between propofol and diazepam plus test compound treatments observed in human evaluation.

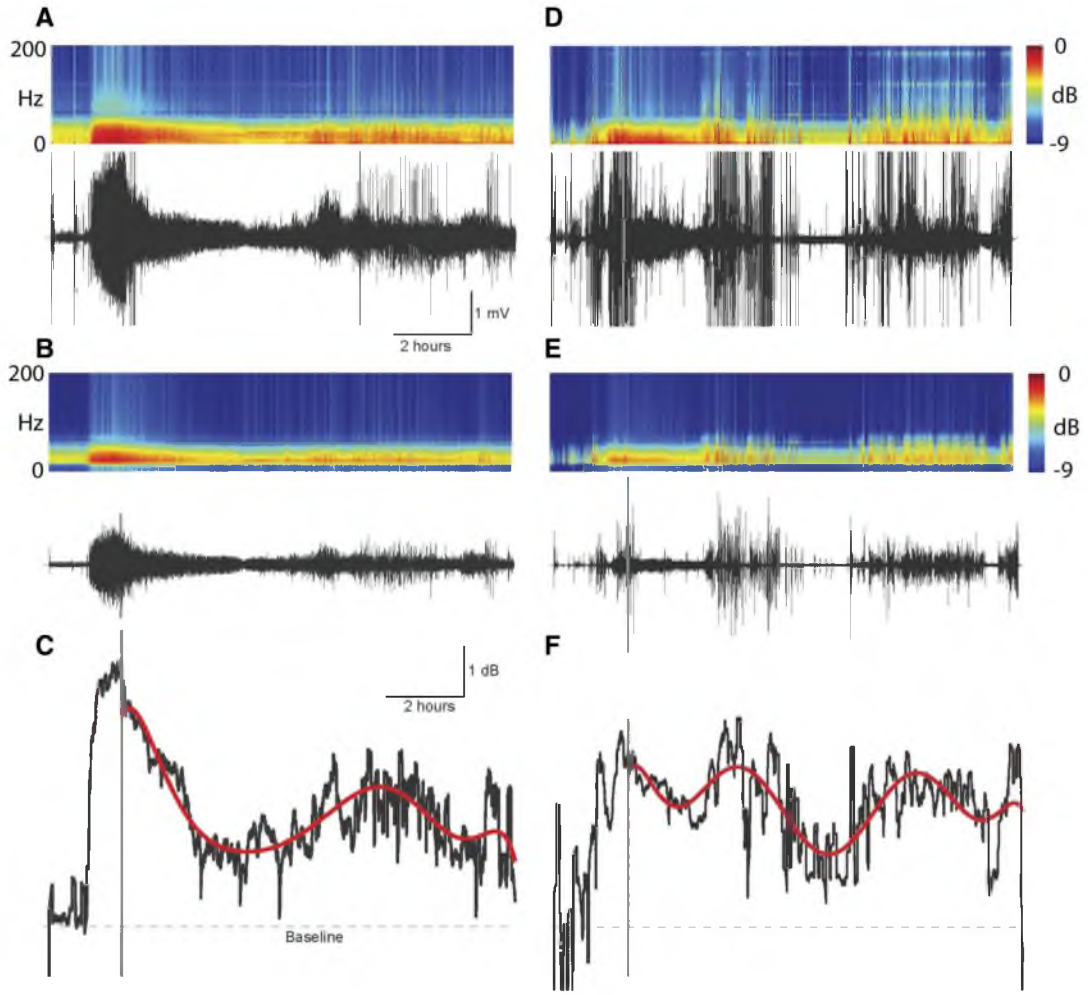

FIG. 5. Estimating artifact-free ESE with the model. *A*: data are the spectrogram (*top*) of an extended raw EEG recording (*bottom*) from Fig. 1. Note the frequency range on the spectrogram is wider than in Fig. 1. *B*: the raw EEG was filtered off-line between 20 and 70 Hz. *C*: data are the $\log_{10}$ power of the filtered EEG trace in *B* slid along in time in 5-min increments and adjusted to the EEG power in a 10-min window before pilocarpine injection (termed "baseline"; not shown). The sharp increase in power was characteristic of ESE. The gray bar indicates 60 min after the 1st motor seizure and was the time point of an injection of diazepam. Overlying the EEG power is an 8th-order polynomial estimation of the power of the EEG signal in response to the diazepam injection, referred to as the quantitative model of ESE (red trace). *D*: data are an extended raw EEG recording from Fig. 2. *E*: the filtered EEG. *F*: power of the filtered EEG trace in *E*. Format is similar to *A*–*C*. Filtering the signal reduced artifacts associated with convulsive movements, animal handling, and electrical artifacts such as harmonics of 60 cycle noise. Modeling of the EEG power with the polynomial reduced short-term fluctuations in EEG power, showing the overall temporal trend in the response to the treatment.

reducing ESE (Fig. 8*A*). Finally, ANOVA were calculated from normalized model estimations (Fig. 8*B*). Quantitative modeling of γ-band power indicated a significant difference between propofol treatment and vehicle and diazepam treatments on the first hour following treatment injection ($P < 0.05$). On the third hour, propofol treatment was only significantly different from vehicle treatment ($P < 0.01$) and test compound treatment was significantly different from vehicle treatment ($P < 0.05$). Both human categorical scoring and spike rate-based analyses failed to detect differences at these time points, suggesting that the quantitative model may detect differences between treatment groups with greater sensitivity than both human and spike rate based estimations of ESE.

As a final control, the quantitative model was also applied to groups of animals given saline (vehicle) or propofol without pilocarpine treatment (Supplementary Fig. S1).[1] These data suggest, in the absence of ESE, that saline injection had no effect on power in the 20–70 Hz band. Propofol injection, however, reliably increased power by ~1 dB, an effect that can be attributed to the biphasic response profile of propofol at intravenous doses of ~1

[1] The online version of this article contains supplemental data.

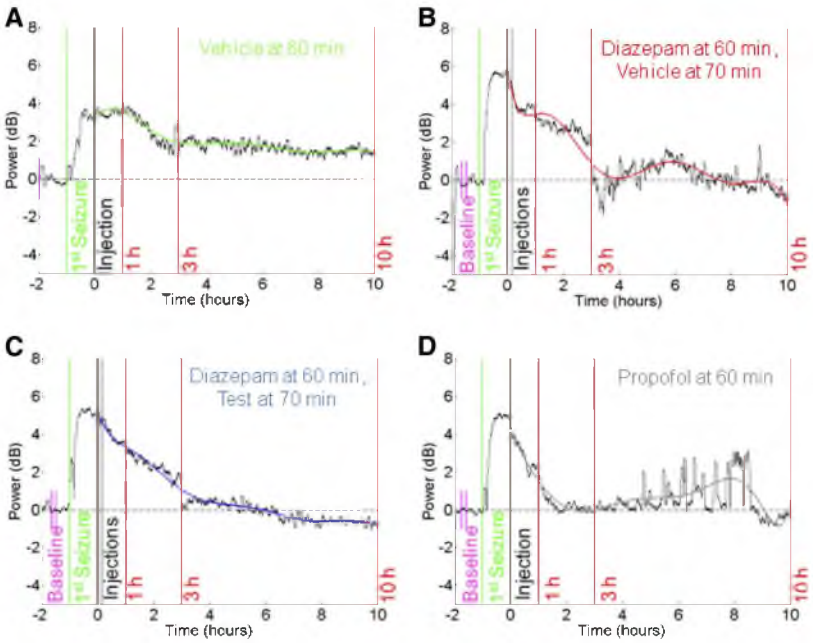

FIG. 6. Quantitative modeling of ESE. Data (black traces) are the $\log_{10}$ power of a single EEG trace adjusted to baseline EEG power before pilocarpine injection (calculated between the short pink vertical bars). The sharp increase in power is indicative of ESE. *Time 0* (black vertical bars) is 60 min after the 1st motor seizure (green vertical bars indicated) and is the time point of the 1st injection of (*A*) vehicle, (*B*) diazepam + vehicle 10 min later (2nd gray vertical bar), (*C*) diazepam + test compound 10 min later (2nd gray vertical bar), or (*D*) propofol. Overlying the EEG power is the quantitative model of ESE described in Fig. 5. Red vertical bars indicate 1, 3, and 10 h after the initial treatment injection (indicated). Data in each figure are representative of a single animal in each of the treatment categories. Note that baseline was calculated earlier than shown in *A*. These data show the varying temporal effects of each treatment on ESE. The polynomial allowed combining data from animals within each treatment group for comparisons across treatment groups.

mg/kg (Dutta et al. 1997). While propofol was delivered at a dose of 100 mg/kg intraperitoneally in the experiments reported here, it is not clear how this dosage and route correlate to intravenous dosing. Despite the uncertainty behind the mechanism of the biphasic response of propofol, there was a clear increase in electrographic activity at this dosage and route that was reflected by the quantitative model. Thus modeling of γ-band energy provides quantitative estimations of ESE and should be useful for estimating the effectiveness of drug treatment for reducing the severity of ESE.

DISCUSSION

Quantifying ESE

Developing quantitative means for detecting repetitive seizure activity from EEG recordings in humans and animals has

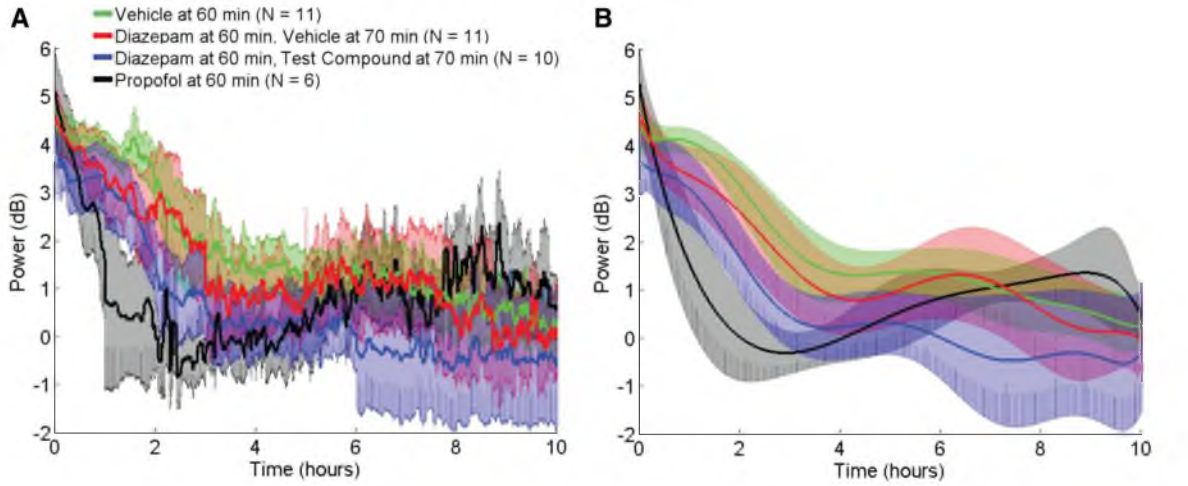

FIG. 7. γ-Band energy and model predictions. *A*: data are the mean (solid traces) and 95% CIs (shaded) of the γ-band energy for each of the treatments after the baseline (prepilocarpine) power has been removed and aligned to the initial treatment injection (time = 0). Format is similar to Figs. 3 and 4. *B*: data are the mean (solid traces) and 95% CIs (shaded) of model predictions for each of the treatments aligned to the initial treatment injection as a function of time. Data groups and format are the same as in *A*. These data show the effectiveness of propofol treatment at reducing ESE to prepilocarpine treatment levels, albeit for short periods.

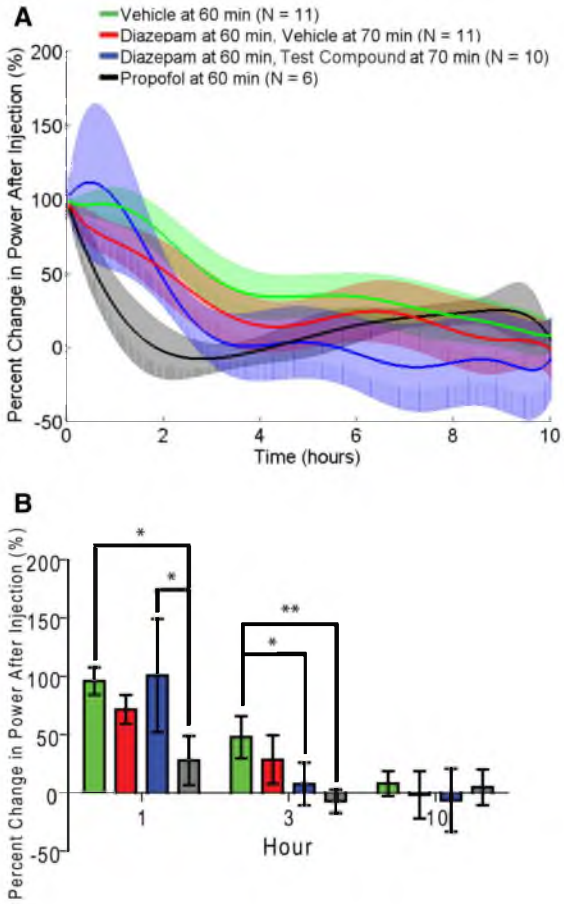

FIG. 8. Percent change in power following the initial injection treatment. *A*: data are the mean (solid traces) and 95% CIs (shaded) of model predictions for each of the treatments normalized to the power at the initial treatment injection. Data groups and format are the same as in Fig. 7. *B*: ANOVA of the data in *A* for all 4 conditions. $*P < 0.05$, $**P < 0.01$. The data show benzodiazepine-resistant ESE as indicated by marginal improvement in ESE power with diazepam treatment (compared with vehicle treatment) similar to human evaluation and filtered spike rate analysis. However, ANOVA suggested no difference between propofol treatment and diazepam treatment, an observation in disagreement with human evaluation and filtered spike rate analysis. Finally the quantitative model identified a significant difference between diazepam with test compound treatment and vehicle treatment. This last difference suggested that this particular test compound was effective with diazepam at reducing ESE, similar to propofol treatment, a potential improvement in benzodiazepine-resistant ESE.

been done using a variety of signal processing techniques with varying success. Repetitive seizures characteristically change in both amplitude and frequency as a function of time (i.e., they are a nonlinear and nonstationary) (Gabor et al. 1996). Techniques that have been used to track the time-varying frequency components of electrographic seizures include chirp detection and match filter construction (Schiff et al. 2000), wavelet (Adeli et al. 2003), wavelet and fractal estimates (Jacquin et al. 2007), and Bayesian probability (Grewal and Gotman 2005; Saab and Gotman 2005) to name a few. However, little has been done to quantify the severity (intensity and duration) of ESE. The goal of this study is to provide a simple, fast, and objective means for measuring the severity of ESE and the pharmacotherapeutic effects of anti-epileptic drugs.

The logic underlying this strategy is that the spike-like events that occur during seizures are very similar in waveform to the events often termed interictal spikes that occur in the EEG between seizures in humans with temporal lobe epilepsy and animal models of this disorder. These interictal spikes have historically been studied in vitro as paroxysmal depolarization shifts that were induced when $GABA_A$ receptors were blocked pharmacologically, thus showing large synchronous excitatory postsynaptic potentials (EPSPs) with superimposed action potentials (Khazipov et al. 2004; Kohling et al. 2000). Because high-frequency action potentials are often filtered out in traditional EEG frequency ranges, the key events in both hippocampal and neocortical seizures are likely to be field EPSPs (Andersen et al. 1966, 1971). The optimum frequency range based on field EPSPs is close to the high β-, low γ-band (20–70 Hz). Likewise, in kainic acid–treated animals, an increase in 20- to 80-Hz power was observed in the neocortex and predominantly in the hippocampus (Medvedev et al. 2000). This led to the selection of those frequencies in the EEG as likely containing the majority of seizure information while excluding or attenuating other EEG signals >70 and <20 Hz and in particular various forms of electrical noise and artifacts. EEG data below 20 Hz also showed increased power during ESE. To focus on electrographic activity, these frequencies were attenuated to reduce artifacts caused by convulsive movements.

Eliminating electrical noise and movement-related artifacts are critical to accurately evaluating ESE. Handling of the animal to administer injections, animal scratching, and convulsive seizures are all sources of EEG contamination. Furthermore, there exists variability within each treatment group that reduces treatment specificity. Three approaches were taken to address these issues: *1*) filtering the EEG reduced many artifacts <20 and >70 Hz, *2*) smoothing the EEG energy reduced variability associated with brief changes in energy, associated with unfiltered artifacts within each treatment group, and *3*) modeling the energy with an eighth-order polynomial estimated the time course of treatment effect over a 10-h period with 10-min resolution, allowing statistical comparisons to be made across treatment groups at selected time points. While the γ-band energy certainly did not encompass all spectral content of ESE, selection of the 20- to 70-Hz frequencies was based on physiological parameters and reliably showed clear differences between treatment groups. The proper selection of frequencies to analyze will likely depend on the signal to be modeled and could vary, even among different rodent models of ESE and other applications. The fully quantitative method described here was able to analyze 24 h of EEG data from 38 animals in four treatment groups on the order of minutes.

Limitations of the model estimations

Modeling EEG power to quantify drug-induced reduction of ESE poses a number of challenges comparing short- and long-term effects. Changes in the EEG on the time scale of minutes, particularly individual seizures, were not well represented by this analysis. However, the model was sensitive to quick-acting treatments such as propofol that tended to abruptly reduce EEG power back to baseline levels. The

modeling estimation in general is dependent on the amount of time and the dynamics of changes in EEG energy that was estimated. However, the dynamics of changes in EEG energy during ESE in the rat was slow (h) and repeatable within treatment groups. Likewise, the effects of any drug that reduce the severity of ESE are time sensitive in that the sooner ESE can be reduced (as judged as a return to baseline EEG power), the better the outcome.

An important issue in making modeling estimations for comparing across treatment groups is knowing the exact time of the first seizure. This dependence may make application of this analysis difficult for data where the time of seizure is unknown. However, this procedure is conducive to quantitatively screening antiepileptic drug compounds under controlled conditions in terms of speed, temporal specificity, and repeatability without the limitations of qualitative judgment. Despite these caveats, the quantitative method described here could be applied to other rodent models of ESE and possibly be used to determine post hoc drug efficacy in human manifestations of status epilepticus.

Comparison of the model with human evaluation and spike rate

ESE proceeds through various distinct stages as it develops and eventually resolves. These electrographic stages have been characterized (Treiman et al. 1990) and they have been modified and adapted to allow for analysis of a drug's effectiveness on treating ESE (Prasad et al. 2002). In an attempt to compare automated ESE quantification with human evaluation, we visually defined the stages of ESE for each animal. A limitation of this human analysis is that it does not lend itself to a description of continuous changes in the severity of ESE, and it only provides a categorical assay of the EEG series. A failure to find a difference between treatments may be caused by human error associated with scoring, a problem that could be compounded by multiple scorers. To address this issue, human scoring was compared with the rate of epileptiform discharges (spikes, spike rate) over a 10-h period following treatment injection (Pitkanen et al. 2005). These rates would ideally make a direct transformation from the stage of ESE to the rate of seizure activity and therefore provide an electrographic measure of the severity of ESE. These rates allow direct comparison between treatment groups similar to the γ-band power method. However, arbitrarily selecting a threshold (in this case 3 SD from mean baseline EEG amplitude) failed to replicate human evaluation results. Additional filtering of the signal in the γ-band improved the divisibility of the treatment groups but not with the statistical power afforded by human evaluation or the specificity afforded by modeling γ-band energy.

Benzodiazepine-resistant ESE

Diazepam, a benzodiazepine, is effective for treating ESE when administered shortly after the onset of seizures (Gastaut et al. 1965; Jones et al. 2002; Lowenstein and Alldredge 1998; Young 2006). However, the effectiveness of diazepam is decreased the longer the time interval between the onset of seizures and administration, known as benzodiazepine resistance (Bleck 2005; Jones et al. 2002; Loscher 2007; Rice and DeLorenzo 1999; Treiman et al. 1998; Walton and Treiman 1988). Benzodiazepine resistance has driven the development of alternative pharmacological treatments for ESE that have the potential to be administered in the field by emergency personnel (Deshpande et al. 2007). There is currently no satisfactory pharmacotherapy screen for animal models of ESE. Quantitative modeling of ESE presents an opportunity to rapidly evaluate new antiepileptic compounds (e.g., the test compound in this study), a valuable preclinical screen. In this study, a coded antiepileptic drug was administered in addition to a typical dose of diazepam. The human categorical scoring did not detect a difference between saline, diazepam at 60 min, and diazepam plus the test compound. The γ-band power method also did not detect any difference between saline and diazepam at 60 min; however, it did detect a significant difference between saline and diazepam plus the test compound treatment 3 h after the treatment injection. Although this effect may or may not be clinically relevant, it suggests that the γ-band power method may be able to extract important features of ESE that human categorical analysis cannot. Additionally, the γ-band power method showed no difference between propofol and diazepam, as well as propofol and test compound treatment groups 3 h after the treatment injection. Despite incremental improvement in γ-band power 3 h after treatment, a new antiepileptic compound would ideally need to work as well as or better than propofol, an anesthetic that is not conducive to administration by first responders within minutes after ESE. The quantitative modeling approach described here could be used as a prescreening tool to reduce the amount of time needed for human evaluation of potential antiepileptic compounds. Although modeling EEG may never be as critical as human diagnoses, collectively, these data suggest that model estimations of γ-band power provide a simple and effective quantitative means for evaluating antiepileptic drug efficacy in the rodent model of benzodiazepine-resistant ESE.

This study provided a rapid and objective method for quantifying the intensity and time course of ESE. This approach has potential for facilitating the screening of possible pharmacological interventions that may suppress convulsive and nonconvulsive seizures during status epilepticus. The analytical method extracts the energy of the EEG γ-band oscillations to estimate the relative response to different treatments and may lead to better understanding of the mechanisms responsible for benzodiazepine resistance. Modeling EEG energy was as effective as human scoring, yet was less subjective, faster, and more sensitive, particularly in the temporal domain. Although changes in EEG power have yet to be correlated to associated brain damage, epileptogenesis, or cognitive impairment after ESE, this method provides a rapid and objective measurement for screening ESE therapies.

GRANTS

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CHAPTER 5

DISCUSSION

Background and Significance

One in 26 people in the United States will develop epilepsy in their lifetime (Hesdorffer, Logroscino et al. 2011; England, Liverman et al. 2012). A diagnosis of epilepsy has a profound negative impact on a person's quality of life, including loss of independence, embarrassment, and social isolation (Fisher, Vickrey et al. 2000; Fisher, Vickrey et al. 2000). Furthermore, approximately one out of every three patients with epilepsy does not have control of their seizures on their current antiseizure drug (ASD) treatment (Mohanraj and Brodie 2005). Despite 20 years of successful ASD development, and 16 new ASDs being made available since 1993 (Shorvon 2009a, Shorvon 2009b), the fraction of patients with uncontrolled epilepsy has remained largely unchanged (Loscher and Schmidt 2011). Thus, there is a significant need for new approaches that include new models to help aid in the search for new therapies, and to address alternate reasons for the lack of seizure control in one-third of the patient population.

In the clinical treatment of epilepsy, it has been widely demonstrated that patients who suffer from epilepsy practice nonadherence at a high rate; approximately 25-53% of patients take their ASD regimen properly less than 80% of the time (McAuley, McFadden et al. 2008; Ettinger, Manjunath et al. 2009; Manjunath, Davis et al. 2009; Zeber, Copeland

et al. 2010; Modi, Rausch et al. 2011; Faught 2012). While clinical observations have demonstrated a link between nonadherence to ASD treatment and an increased risk of seizures (Stanaway, Lambie et al. 1985; Manjunath, Davis et al. 2009), estimates of nonadherence can be problematic, primarily because patients tend to over-report adherence (Buelow and Smith 2004; Modi, Guilfoyle et al. 2011), and can be unaware of seizure events, such as nonconvulsive seizures (Hoppe, Poepel et al. 2007). Nonadherence leads to periods of ASD withdrawal, which can cause status epilepticus (SE), a medical emergency, requiring immediate interventional therapy (Trinka, Hofler et al. 2012). Due to the associated risks and better experimental control, an animal model of nonadherence would be able to address questions that are otherwise infeasible or unethical to test in patients with epilepsy.

The studies conducted over the course of this dissertation describe the creation and characterization of an etiologically relevant model of nonadherence as well as the development of an algorithm for the quantification of electrographic SE (ESE) using an existing animal model of SE. Together, these results aim to aid in the treatment of epilepsy by providing etiologically relevant models and a method for the quantitative analysis of ESE.

Pellet Delivery System

Previous to this work, modeling nonadherence would have required a substantial dedication of staff to deliver medications to animals at all times of day, thus making the study of nonadherence practically infeasible. To address this issue, a novel system for pellet delivery of drug-in-food pellets to chronically epileptic rats was developed. The technical schematics of this unique system are described in Chapter 2. This system is capable of

delivering drug-in-food pellets to food-restricted animals at experimenter-defined levels of adherence 24 hours a day, seven days a week. The creation of this system allows for the study of nonadherence in epileptic animals for the first time, allowing for the investigation of the biological consequences of nonadherence on disease progression and outcome.

In the current form, the system outlined in this dissertation has been integrated with a video+EEG system. However, it would not require substantial effort to re-engineer the system to work independently in a manner that would permit the study of nonadherence in a variety of other animal disease models. As is, the EEG system provides the link between the computer software and the feeder control system, but the computer interface could be replaced by any number of methods, that communicate and record the commands delivered to the feeding system. Thus, the system described herein is highly versatile and could be used in the study of nonadherence across fields, and not necessarily limited to the field of epilepsy. The major requirements of this replacement technology are a real-time clock, which can communicate the date and time to the microcontroller, and a set of feeding commands that tell the system which feeder to run at which times, and how many pellets to deliver per feeding.

Modeling Nonadherence

We sought to characterize our new model of nonadherence, which was enabled by the creation of a novel system for drug-in-food delivery. To do this, we performed an experiment designed to test the difference in seizure control associated with carbamazepine (CBZ) treatment between animals randomly assigned to one of three treatment groups; i.e., placebo (0%), 50% and 100% adherence. The results of this study are described in Chapter 3. In summary, no difference in seizure control was observed between animals in the 50%

and the placebo control groups. In contrast, a significant difference in seizure control was observed between animals assigned to the 50% and 100% groups. This result suggests that taking CBZ at a 50% adherence rate provides little benefit in the way of seizure control. Importantly, a worsening in seizure control was not noted in the 50% adherence group.

The 50% adherence rate was chosen as the halfway point between 0% adherent, i.e., placebo control, and 100% adherent, as it was determined that it would be the best way to perform a first-pass characterization of the model. Other rates of nonadherence could be tested, such as the clinically relevant threshold of 80% used to categorize patients in clinical nonadherence studies (Hughes, Bagust et al. 2001). Whether or not this threshold is biologically relevant is of question, and it may be that a different rate of adherence would serve as a better threshold for studying the impact of nonadherence on seizure control and seizure freedom. Additionally, previous clinical studies have combined results from patients on different ASDs, and tended not to distinguish between monotherapy and polytherapy. As such, the consequences of improper adherence may change as a result of the current ASD treatment regime. By using this novel method, questions regarding adherence based on different ASD treatments can be directly addressed under tight experimenter control.

Further, experiments could be performed using this model which implement more complex, yet more clinically-relevant, trends of nonadherence, such as those observed in newly-diagnosed pediatric patients with epilepsy (Modi, Rausch et al. 2011). When these trends were compared to long-term seizure outcome, it was found that patients that practiced near-perfect adherence had a higher likelihood of having complete seizure control (Modi, Rausch et al. 2014). However, this work cannot answer questions regarding the

worsening of epilepsy as a result of nonadherence; it is unlikely that patients practicing less-than-perfect adherence would later practice perfect adherence. However, an animal model of nonadherence could subject animals to a paradigm of nonadherence, and then place those animals on a paradigm of perfect adherence, and compare the resulting change in seizure control and seizure burden to that obtained in animals that were perfectly adherent for the entire experiment. The results of an experiment such as this would inform clinicians and caregivers of the long-term consequences associated with nonadherence to any given ASD treatment. In short, many future questions concerning the effects of nonadherence in epilepsy can now be asked using a clinically-relevant model of nonadherence.

Initiating Treatment Following the First Seizure

The work performed in this dissertation sought to model the impact of treatment and nonadherence on seizure control in the patient with newly diagnosed epilepsy. In the clinical treatment of epilepsy, patients generally initiate their anti-epileptic therapy after they experience a second seizure (Fisher, Acevedo et al. 2014), as the chance that the patient will have subsequent seizures is high (Hauser, Annegers et al. 1996). We modeled this by considering the SE associated with low-dose KA treatment as the first seizure, and the first observed spontaneous seizure as the animal's second seizure. A rat observed to have this "second" seizure was considered to have epilepsy and thus met the entry criteria for the study and was randomly assigned to one of the treatment groups.

Prior to this work, studies that evaluated the impact of a given ASD therapy on seizure outcome typically began treatment either directly after an insult (Lee, Won et al. 2008; Schauwecker 2010; Bae, Kim et al. 2013), or after the seizure rate had plateaued

(Grabenstatter, Ferraro et al. 2005; Grabenstatter and Dudek 2008). Beginning treatment immediately following the insult is, in reality, treating animals during the latent period; i.e., before the diagnosis of epilepsy (Walker, White et al. 2002). This particular study design is typically employed when attempting to determine whether a given drug might be antiepileptogenic, i.e., prevent the development of epilepsy, or disease modifying, in that it decreases the severity of the seizure disorder or concomitant comorbidities associated with epilepsy.

Beyond the obvious disparity with clinical practice, a disadvantage to treatment during the latent period is that the latent period is associated with distinct morphological processes occurring in the brain that lead to the transformation of a healthy or nonepileptic brain to an epileptic one (Ben-Ari and Cossart 2000; Takahashi, Vargas et al. 2010; Vargas, Takahashi et al. 2013). An additional disadvantage in medicating during the latent period is that a subset of animals never develops epilepsy (Hellier, Patrylo et al. 1998), making statistical inferences more difficult.

Treating animals after the seizure rate has plateaued has advantages; the animals generally have a measurable and more stable seizure frequency at the time treatment is initiated (Grabenstatter, Clark et al. 2007; Williams, White et al. 2009). To this point, an animal's seizure frequency can be used as an inclusion/exclusion criterion in order to have a more homogenous population for therapy testing (Grabenstatter and Dudek 2008) However, in the clinical treatment of epilepsy, it would be horrendously unethical to withhold ASD therapy from patients until their seizure rates stabilize; thus waiting for seizure rates to plateau reduces the clinical relevance of the post-SE animal epilepsy models.

The difficulty in the approach of treating at the initiation of epilepsy lies in the neural processes occurring after the first spontaneous seizure. There are two major contributors to worsening of epilepsy following diagnosis: the underlying process of epileptogenesis, and the seizure events themselves. Epileptogenesis can be occurring in patients or animals who otherwise have their symptoms, i.e., seizures, fully controlled. For example, previous work has shown that CBZ does not prevent the process of epileptogenesis in pilocarpine-treated mice (Capella and Lemos 2002). There is the concept in the field of epilepsy research that each seizure a patient or animal experiences increases the probability of subsequent seizures, i.e., "seizures beget seizures" a phrase coined back in 1881 by William Gowers (Gowers 1881). There is experimental evidence supporting this concept (Berg and Shinnar 1997; Ben-Ari, Crepel et al. 2008), however, it is not universally accepted (Sills 2007). Based on this concept of "seizures beget seizures," it becomes difficult to uncouple the process of epileptogenesis from seizures, i.e., determining if the animal's epilepsy is worsening due to the occurrence of uncontrolled seizures or an underlying biological process, making the approach of treating following the first seizure disadvantageous. Importantly, the advantage of this approach lies in the translatable aspects of this experimental design; i.e., the methodological approach utilized in this dissertation more closely mimics the treatment of newly diagnosed epilepsy in the clinic, something not done previously.

Modeling Pharmacoresistance

The goal of Study 2, as discussed in Chapter 3, was to determine whether or not pharmacoresistance would be induced by removal and restoration of CBZ treatment using a chronic treatment paradigm. The hypothesis of this study was based on the concept that

animals treated with CBZ would exhibit far better seizure control than was actually observed and as previously reported (Grabenstatter, Clark et al. 2007; Ali, Dua et al. 2012). Unexpectedly, CBZ at 75 mg/kg q.i.d. did not provide full seizure control in all of the animals enrolled. In this respect, animals from Study 1 and Study 2 could already be considered to have CBZ-resistant epilepsy, i.e., a group of animals that are refractory to the treatment of CBZ at the dose provided. Thus, a similar group of refractory animals could serve as method of testing investigational compounds, and could be used as a final-stage screening assay for new pharmaceutical treatments for epilepsy. Additionally, results observed following the removal and restoration of CBZ suggests that the sensitivity, or lack thereof, to CBZ is not changed by abrupt discontinuation of CBZ treatment (see Study 2 in Chapter 3 for details).

Often, when patients experience a prolonged period of complete seizure control, they will undergo physician-monitored tapering and discontinuation of their ASD therapy (Hixson 2010). Clinical observations suggest that anywhere from 10 to 70% of these patients will then experience seizures, and need to reinstate their ASD treatment (Berg and Shinnar 1994). When these scenarios occur, a significant but not all-inclusive proportion of patients (84%) are able to regain full-control of their seizures following their return to their previous ASD therapy (Schmidt and Loscher 2005). This result suggests that the majority of patients are not undergoing a change in their pharmacosensitivity to their ASD treatment paradigm, despite both experiencing seizures, and undergoing a period without their ASD treatment. Our model was, however, different from the clinical situation, in that the majority of animals did not exhibit seizure freedom on their initial ASD treatment, and were abruptly removed from their CBZ treatment, thus allowing for the possibility of CBZ-

withdrawal-induced seizures (Malow, Blaxton et al. 1993). Despite the difference from the clinical experience, results obtained from this on-off-on protocol mirrored those from the clinical experience; i.e., we did not observe significant changes in the level of pharmacosensitivity to the previous treatment. In contrast, this result did not match those from the CBZ-resistant kindled rat studies wherein CBZ and another sodium channel blocker, LTG, were found to be effective initially but lost control after a 7-day drug-free interval (Srivastava, Alex et al. 2013). However, the experimental paradigm differs substantially. Even though the kindling model is a model of network hyperexcitability, animals still require focal stimulation for them to express a seizure, i.e., evoked rather than spontaneous seizures. Further, animals in the present study were treated chronically with CBZ, not acutely. These differences may account for differences in efficacy observed between these two animal models.

Quantifying a Model of Electrographic Status Epilepticus

Electrographic status epilepticus (ESE) is a prolonged seizure lasting more than 10 minutes, and is considered a medical emergency (Trinka, Hofler et al. 2012). The currently used pharmacological interventions are inadequate in treating ESE in most scenarios. However, to better characterize the efficacy of current pharmacological interventions and screen putative compounds, a method to quantitate the response of ESE to interventional therapies is described in Chapter 4. The algorithm employs an 8^{th} order polynomial fit of the power of the ESE in the gamma band (20-70Hz). The model performed well when measured against human evaluation methods (Walton and Treiman 1988; Treiman, Walton et al. 1990; Prasad, Williamson et al. 2002), and serves as an unbiased method to quantify the response of ESE to an intervention therapy. For the past five years, this algorithm has

been utilized to quantitate the effect of investigation compounds on ESE as part of the Countermeasures Against Chemical Threats Contract at the University of Utah (Pouliot, Bialer et al. 2013).

Using polynomials to model electrographic status results in a model that follows the time course of power of the EEG. However, this method is subject to the downsides of higher-order polynomial fitting, such that it can result in Runge's phenomenon (Runge 1901), i.e., oscillations present in the model that are a result of the polynomial fitting process, and do not match the underlying signal nor have a biological basis. Thus, selecting a different method of fitting other than a polynomial may result in an improved model. Other possible mathematical methods include exponential fitting or geometric fitting, however, further work would be necessary to test the validity of these attempts to fit the data, and determine whether or not new methods better represent the underlying biological signal that gives rise to the model.

It should be noted that this algorithm was built around pilocarpine-induced ESE in rats, as this model results in a continuous electrographic seizure lasting for five to six hours (Curia, Longo et al. 2008). Different models of ESE may require modification or a complete reworking to properly quantify the severity of the ESE. For example, a new method would be needed for the low-dose KA model of ESE (Hellier, Patrylo et al. 1998), as this model produces multiple punctuated seizure events, rather than a single prolonged seizure (White, Williams et al. 2010). As there may be a link between the severity of the KA-induced ESE and the resulting cortical damage, development of an algorithm that measures the severity of the ESE may produce a quantification method for determining the severity of insult.

Impact and Future Directions

Increasing patient knowledge about the consequence of nonadherence may itself help increase patient adherence, as previous clinical observations have demonstrated a correlation between an increase in education and an increase in medication adherence (Alm-Roijer, Fridlund et al. 2006; Ulrik, Hansen et al. 2010). Additionally, the preclinical model of nonadherence described herein may help characterize the performance of ASDs under conditions wherein a patient practices poor adherence. If certain ASDs perform substantially better or worse in cases of nonadherence, this information may help guide clinicians in choosing an ASD for a nonadherent patient. Further, ASDs could be developed with the concept of nonadherence factored into their performance, such as ASDs displaying a longer half-life, or ASDs that can be formulated into a slow-release pill. While two potential therapies may perform similarly in perfectly adherent patients, an ASD that results in better seizure control when administered in inconsistent dosing regimens may help the patient population that is considered pharmacoresistant due to imperfect adherence (Modi, Rausch et al. 2014).

We also found a long-term correlation between missed doses and seizures (Figure 3.5), suggesting that there may be a critical period following a missed dose in which the probability of a seizure is increased. This information could be used to warn patients of the probability of an increased seizure liability based on their patterns of nonadherence. Another area where the correlation between seizures and missed doses could be of use is in seizure prediction. Seizure prediction utilizes features of neural signals, e.g., EEG and local field potentials, to determine the likelihood of an incoming seizure (Litt and Echauz 2002). This technology has the potential to improve the quality of life for patients with uncontrolled epilepsy, as it may remove the unpredictability of knowing when the next

seizure might occur (Fisher, Vickrey et al. 2000; Snyder, Echauz et al. 2008). The results of the first implanted seizure-prediction device were less than ideal, as patients had a high number of events missed by the device, and an even higher number of false positives (Cook, O'Brien et al. 2013). A possible improvement to this device could come from the study of nonadherence. The technological capability to track patient nonadherence in real-time already exists in the form of the Medication Event Monitoring System (MEMS) cap (Cramer 1995), which could communicate wirelessly to an implantable seizure-prediction device. As previously mentioned, if there is a quantifiable critical period in which seizure probability is increased following a missed dose, that data could be integrated into the feature set of any seizure prediction algorithm. Given this implementation, the knowledge of the relationship between a missed dose and the increase in seizure probability becomes invaluable to the nonadherent patient with uncontrolled epilepsy.

Conclusion

The work in this dissertation covers the creation and characterization of a novel model of drug delivery, used to simulate nonadherence in the newly diagnosed epileptic rat. While this system was developed within the framework of studying nonadherence, chronic drug delivery to epileptic animals has a wide range of possible uses, including testing investigational compounds, testing effects of ASDs on learning and memory, as well as a gamut of behavioral assays. The first "proof of principle" results obtained with this novel system have demonstrated the consequences of nonadherence to ASD treatment in an etiologically relevant rat model of epilepsy. We have also provided a method to analyze the efficacy of investigation compounds to stop ESE, which increases the throughput and quality of this screening test. The hope is that this new model of

nonadherence is used extensively to help neuroscientists and pharmacologists move the field of epilepsy forward, and learn more about the biological underpinnings and clinical consequences associated with ASD nonadherence.

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